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Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization

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ABSTRACT

The feasibility of high pressure carbon dioxide (HPCD) processing as a non-thermal pasteurization technique for liquid whole egg (LWE) was investigated. First, the influence of process parameters including temperature, pressure, agitation speed, working volume ratio (WVR) and holding time on the reduction of the natural microbial flora of LWE was studied. Temperature, WVR and stirring speed were the most important parameters in HPCD inactivation. HPCD processing at 13.0 MPa, 45 °C, 50% WVR and 400 min^{-1} stirring speed during 10 min proved promising for inactivating the native microorganisms in LWE. Secondly, the effect of HPCD treatment at these "optimal" conditions was evaluated on the microbial quality and pH of LWE under refrigerated storage (4 °C) and compared to stored heat pasteurized samples (69 °C, 3 min). HPCD processing extended the shelf life of LWE up to 5 weeks at 4 °C, which is the current shelf life of heat pasteurized LWE. No pH difference was detected between HPCD and heat treated LWE after 1 week of storage.

1. Introduction

Liquid egg products are widely utilized by the food service industry and commercial food manufacturers because of their convenience and ease in handling and storing as compared to shell eggs. Liquid egg is very sensitive microbiologically, and is therefore heat pasteurized to obtain a microbiologically safe product. Currently, the minimum temperature and time requirements for pasteurization of liquid whole egg (LWE) are 60 °C and 3.5 min in the USA and 64.4 °C and 2.5 min in the UK [1,2]. In most European countries, however, no mandatory process criteria are stipulated for liquid egg processing. Instead, in Europe, egg products have to comply with the European legislation on microbiological criteria for foodstuffs (EU Regulation 2073/2005). This regulation, applicable from January 1, 2006 on, lays down food safety criteria for certain important foodborne bacteria, their toxins and metabolites in specific foodstuffs. These criteria are applicable to products placed on the market during their entire shelf life. In addition, the regulation sets down certain process hygiene criteria to indicate the correct functioning of the production process [3]. In this respect, egg products should be free of *Salmonella* and contain less than 2 log of *Enterobacteriaceae* at the end of the manufacturing process [3].

Although the heat processes used to pasteurize LWE ensure food safety by eliminating *Salmonella*, some heat-resistant microorganisms can survive the pasteurization process and spoil the liquid products even under refrigerated storage [2]. Therefore, the shelf life of LWE is very short, typically not longer than 6 days at 4 °C [4]. To provide a sufficient long shelf life, more intensive heat treatments are needed to reduce the numbers of spoilage microorganisms. Although the conventional heat treatments of LWE are carefully conducted on the critical temperature–time conditions where the egg protein denaturation is minimized, parts of LWEs are frequently overprocessed during thermal treatments, and changes in functional properties due to pasteurization have been reported [5,6]. For instance, egg white began to coagulate at 62 °C and egg yolk at 65 °C [7]. Denaturation of whole egg, indicated by a change in viscosity, occurred in the temperature range of 56–66 °C [5] and can cause important practical problems in the processing of the product.

To minimize the disadvantages of thermal processing of LWE and to extend the refrigerated shelf life of LWE, alternative pasteurization techniques, such as ultrapasteurization combined with aseptic packaging [8], short wave ultraviolet light radiation [9], the use of nisin as a bacteriostatic agent [10], pulsed electric

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fields [11], high hydrostatic pressure [2] and ultrahigh pressure homogenization [12], have been explored. The last two decades, the use of high pressure carbon dioxide (HPCD) has emerged as a promising technology for pasteurization of foods [13]. Processing with HPCD involves contacting foods with either sub- or supercritical (i.e., pressurized) CO_2 during a certain amount of time in a batch, semi-batch or continuous manner. This process effectively inactivates vegetative microorganisms, and because a low temperature can be applied, HPCD processing can produce high-quality, pasteurized food products, meeting the consumer's demand [13]. The bacterial inactivation mechanism of HPCD is, however, not yet fully elucidated, although several theories have been put forward in recent years [13–15]. The different steps in the hypothetical inactivation mechanism can be summarized as follows: (1) solubilization of pressurized CO_2 in the external liquid phase decreasing the extracellular pH, (2) diffusion of CO_2 into the cellular membrane modifying the cell membrane, (3) cellular penetration of CO_2 decreasing the intracellular pH, (4) key enzyme inactivation/cellular metabolism inhibition due to intracellular pH lowering, (5) direct (inhibitory) effect of molecular CO_2 and HCO_3^- on cell metabolism, (6) precipitation of CO_3^{2-} with inorganic electrolytes and Ca^{2+}-binding proteins disordering the intracellular electrolyte balance, and (7) removal of vital constituents from cells and cell membranes [13].

HPCD pasteurization has mostly been applied with promising results on liquid foods, including several fruit juices [16–28], tomato paste [28], milk [18,29,30] and beer [31]. Until now, the feasibility of HPCD processing as an alternative to heat pasteurization for the preservation of LWE has not received much attention. Lehmann and Juchem [32] described a process for enhancing the shelf life of water-containing foods (in particular LWE) using high pressure CO_2 or other inert gases. The described process is, however, not sufficiently performing to be economically viable, in particular because no high reactor fill percentages can be treated, and the treatment time and/or the applied pressure is too high. Wei et al. [17] studied the bactericidal effect of high pressure CO_2 in egg yolk, egg white and whole egg spiked with *Listeria monocytogenes* or *Salmonella typhimurium*. These authors reported that HPCD processing at 13.7 MPa and 35 °C for 2 h completely inactivated *Salmonella* cells in egg yolk and egg white, while *Listeria* cells were reduced by 3 log. The same treatment was, however, less effective in reducing *S. typhimurium* in whole egg and even caused a(n) (unexplainable) twofold *increase* in bacterial numbers of *Listeria*-spiked whole egg samples. Van Ginneken et al. [33] filed a patent for a batch method to inactivate biological contaminants in liquid high fat and/or protein containing food or feed products, such as liquid eggs. Their method comprises the subsequent steps of (i) introducing pressurized CO_2 (e.g., up to 6.0 MPa) in a reactor vessel at a predetermined temperature, (ii) introducing the liquid food in this reactor while stirring, (iii) introducing an additional amount of CO_2 to attain a final CO_2 pressure (e.g., 10.0 MPa) at a predetermined temperature (e.g., 40 °C), (iv) holding the final CO_2 temperature and pressure inside the vessel constant for a certain amount of time while stirring the mixture, and (v) releasing CO_2 pressure and collecting the treated liquid food. A common feature in these three reported studies is that microbial inactivation in the treated liquid egg products was only evaluated immediately after HPCD processing, and not during their entire shelf life. Information on the microbial stability on HPCD treated foods during refrigerated storage is, however, from utmost importance because in order to replace the existing pasteurization methods, non-thermal processing techniques also have to promote an equivalent or enhancement of shelf life [34].

The objective of this study was to examine the feasibility of HPCD processing as an alternative pasteurization technique for LWE. For this purpose, the influence of various process parameters that affect microbial inactivation by HPCD in LWE was studied and the most optimal treatment conditions were identified. Secondly, the microbiological changes and pH of LWE, treated at the most adequate HPCD processing conditions, were evaluated to determine the microbial shelf life under refrigerated storage (4 °C) during 5 weeks, and compared to stored heat pasteurized LWE samples.

2. Materials and methods

2.1. Liquid whole egg (LWE)

Bulk raw as well as heat pasteurized (69 °C, 3 min) LWE samples were provided by a commercial producer (Lodewijckx N.V., Belgium). The LWE samples were transported to the laboratory in insulated polystyrene boxes on ice and then immediately stored at 4 °C. All HPCD treatments and microbial analyses were performed within 24 h after the collection of samples.

2.2. High pressure carbon dioxide processing

The HPCD equipment used in this study has previously been described [35]. To perform an experimental run, the high pressure vessel was loaded with LWE sample. The vessel was tightly closed and immersed in a water bath at a constant temperature. Then, the inlet and outlet tubing were connected to the vessel. When the selected temperature was reached (standard after 15 min), commercially available CO_2 (cooled at 4 °C) was injected into the 100-ml vessel during 1 min to reach the desired pressure. The temperature in the vessel was monitored continuously using a temperature probe and no temperature increase was observed with pressure build-up. Then, the cells were exposed under constant stirring to pressurized CO_2 for a designated time. Afterwards, the pressure was released slowly with the aid of a decompression chamber over a period of 2 min. The suspension was immediately removed aseptically from the vessel, and analyzed microbiologically.

2.3. Experimental design for studying the influence of process parameters

First, a study was carried out to evaluate the effect of pressure, temperature, holding time, working volume ratio (WVR, the ratio of the LWE sample volume and the pressure vessel volume), and agitation (stirring) speed. For this purpose, LWE was subjected to five series of HPCD treatments wherein one process parameter at the time was varied while the other parameters were kept constant. An overview of the treatment conditions can be found in Table 1. Because different batches of bulk raw LWE were used in this study, the initial native microbial flora differed in each of these batches of LWE, as such hampering the comparison of the different experimental series. For this reason, a "reference" experiment at 13.0 MPa, 35 °C, 20 min, 50% working volume ratio and 400 min^{-1} stirring speed was performed in each set of experiments. Samples were taken before and after each treatment in order to determine the microbial counts according to the protocol discussed in Section 2.5. In addition, the reductions of the different populations for every set of HPCD treatments were compared to heat pasteurized samples. Each HPCD and heat treatment were performed in triplicate.

2.4. Shelf life studies

Two replicate shelf life studies were performed to evaluate the effect of HPCD processing on microbial growth during refrigerated storage, taking heat treated LWE samples as a reference. Bulk raw LWE was treated at 13.0 MPa, 45 °C, 50% WVR and 400 min^{-1} stirring speed during 10 min. Immediately after processing, the

Table 1
Summary of the treatment conditions used in order to investigate the influence of the different process parameters on LWE HPCD pasteurization.

Influence	Pressure (MPa)	Temperature (°C)	Holding time (min)	WVR (%)	Stirring speed (min^{-1})
Pressure (MPa)	8.5–13.0–21.0	35	20	50	400
Temperature (°C)	13.0	35–40–45	20	50	400
Holding time (min)	13.0	35	10–20–30	50	400
WVR (%)[a]	13.0	35	20	30–50–70	400
Agitation speed (min^{-1})	13.0	35	20	50	100–200–400

[a] Working volume ratio.

samples were aseptically transferred in sterile flasks and aerobically stored at 4 °C. Heat pasteurized LWE samples (69 °C, 3 min) were stored under identical conditions and used as a reference. Each HPCD and heat treatment were performed in triplicate. On weekly basis, both HPCD and heat treated samples were aseptically withdrawn from the stored samples for microbial enumeration (see Section 2.5). In addition, the pH of all the samples was followed during the storage period with a pH electrode (Hamilton, Switzerland).

The shelf life studies were conducted over a storage period of 5 weeks, which is the current applied shelf life of heat pasteurized LWE. Up to date, the European Regulation 2073/2005 addresses a limited number of food safety and process hygiene criteria for egg products [3]. In this study, however, next to the legal criteria, additional microbial guidelines, recommended by the Laboratory of Food Microbiology and Food Preservation (LFMFP-UGent) at Ghent University [36], were used to evaluate the microbiological safety and quality of LWE. In Table 2, an overview of the legal criteria and microbiological guidelines is given.

2.5. Analysis of the microbiological quality

Tenfold dilution series were made in peptone water (PPS) for plating. PPS consisted of 0.85% NaCl (Merck, Germany) and 0.1% neutralized bacteriological peptone (Oxoid, England). The different populations which were analyzed in this study are total aerobic mesophilic (TAM) count, total anaerobic mesophilic (TAnM) count, total aerobic psychrotrophic (TAP) count, total anaerobic psychrotrophic (TAnP) count, total aerobic spore (TAS) count, total anaerobic spore (TAnS) count, lactic acid bacteria (LAB), yeasts, moulds, *Pseudomonas* spp., *Enterobacteriaceae* and *Salmonella* spp. In Table 3, an overview of these populations is given, together with their growth medium, plating technique and incubation conditions. After incubation, the colony forming units (CFU) were determined. The detection limit was 10 CFU/g. All reported counts were expressed as $\log_{10}$ CFU/g.

2.6. Gram staining

Gram stains were produced with a Gram staining kit (Oxoid, England). The sample was spread in a thin film over a microscope glass slide and dried in the air. Subsequently, the slide was passed through a flame to fix the cells. Once cooled, the slide was flooded with crystal violet for 1 min. The sample was then rinsed with water and treated with iodine for 30 s. Rinsing was followed by treatment with ethanol for 30 s and subsequent washing with water. The slide was then flooded with safranin solution for 30 s, followed by a final rinse with water and drying. The slides were analyzed using an Olympus BX50 microscope (Olympus Optical Co., Ltd., Japan).

2.7. Statistical evaluation

The data presented are means of three replicate experiments ± standard deviation, unless otherwise stated. Analysis of variance (one-way ANOVA) was performed to compare treatment mean values using the Tukey's test. Significance was based on $P < 0.05$. The data were processed using the statistical software package SAS version 9.2.

3. Results and discussion

3.1. Influence of process parameters

In the first part of this study, the parameters influencing the antimicrobial activity of CO_2 in LWE were studied. In addition, the optimum set of operating conditions of HPCD for the storage study was determined. It should be noted that untreated, HPCD treated and heat pasteurized LWE samples were all free from *Salmonella* spp., spores (TAS and TAnS) and moulds (as verified by microbiological analysis after all experiments had been performed), and hence these microbiological parameters are not reported any further.

3.1.1. Influence of temperature

LWE was subjected to HPCD treatments at different temperatures (35, 40 and 45 °C) at 13.0 MPa, 400 min^{-1} stirring speed and 50% WVR for 20 min (Fig. 1a). The results show that the survivors of the HPCD processed samples at 35 °C mainly consisted of LAB (as indicated by the similar counts of TA(n)M, TA(n)P and LAB). Increasing the temperature to 40 °C did not significantly change the inactivation level. However, processing at 45 °C made LAB more

Table 2
Legal criteria and microbiological guidelines for determining the expiry date (shelf life) and microbiological safety of LWE.

Microbiological parameters	Target[a] ($\log_{10}$ CFU/g)	Tolerance[a] ($\log_{10}$ CFU/g)	End of shelf life ($\log_{10}$ CFU/g)
Total aerobic psychrotrophic (TAP) count[c]	3	4	6[b]
Lactic acid bacteria (LAB)[c]	2	3	7
Yeasts[c]	2	3	5
Moulds[c]	2	3	5
Enterobacteriaceae [d]	1	2	Not applicable
Salmonella spp.[e]	Absent in 25 g	Absent in 25 g	Absent in 25 g

[a] Target and tolerance are the guide values that are relevant immediately after production. 'Target' represents the guide value that should be reached in good hygienic conditions. In such cases 'tolerance' represents the upper limit.

[b] When the TAP at the end of the shelf life exceeds the guide value of 10^6 CFU/g the products can only be rejected when it has been shown that the bacteria concerned are different from (homo-fermentative) LAB.

[c] According to the microbiological guidelines of Laboratory of Food Microbiology and Food Preservation (LFMFP-UGent) at Ghent University for pasteurized products susceptible to post-contamination [36].

[d] According to the EU Regulation 2073/2005, process hygiene criteria category 2.3.1 [3].

[e] According to the EU Regulation 2073/2005, food safety criteria category 1.14 [3].

Table 3
Summary of the analyzed populations, together with their growth medium, plating technique and incubation conditions.

Populations	Growth medium[a]	Technique	Incubation conditions
Total aerobic mesophilic (TAM) count	PCA	Pour plate	30 °C, 3 days, aerobic
Total anaerobic mesophilic (TAnM) count	RCA	Pour plate[b]	30 °C, 3 days, anaerobic
Total aerobic psychrotrophic (TAP) count	PCA	Pour plate	22 °C, 5 days, aerobic
Total anaerobic psychrotrophic (TAnP) count	RCA	Pour plate[b]	22 °C, 5 days, anaerobic
Total aerobic spore (TAS) count[c]	PCA	Pour plate	30 °C, 3 days, aerobic
Total anaerobic spore (TAnS) count[c]	RCA	Pour plate[b]	22 °C, 3 days, anaerobic
Lactic acid bacteria (LAB)	MRS	Pour plate[b]	30 °C, 3 days, anaerobic
Yeasts/moulds	YGC	Spread plate	30 °C, 3/5 days, aerobic
Pseudomonas spp.	PAB-CFC	Spread plate	30 °C, 2 days, aerobic
Enterobacteriaceae	VRBG	Spread plate	37 °C, 1 day, aerobic
Salmonella spp.	BSA	Spread plate	37 °C, 1 day, aerobic

[a] PCA, plate count agar, Oxoid, Basingstoke, England; RCA, reinforced clostridial agar, Oxoid; MRS, deMann–Rogosa–Sharpe agar, Oxoid; YGC, yeast glucose chloramphenicol agar, Bio-Rad, Marnes-La-Coquette, France; PAB-CFC, pseudomonas agar base supplemented with pseudomonas CFC, Oxoid; VRBG, violet red bile glucose agar, Oxoid; BSA, brilliance salmonella agar, Oxoid.
[b] Pour plate overlaid with top layer of the same medium.
[c] Tenfold diluted samples were first pasteurized at 80 °C for 10 min to inactivate the vegetative cells.

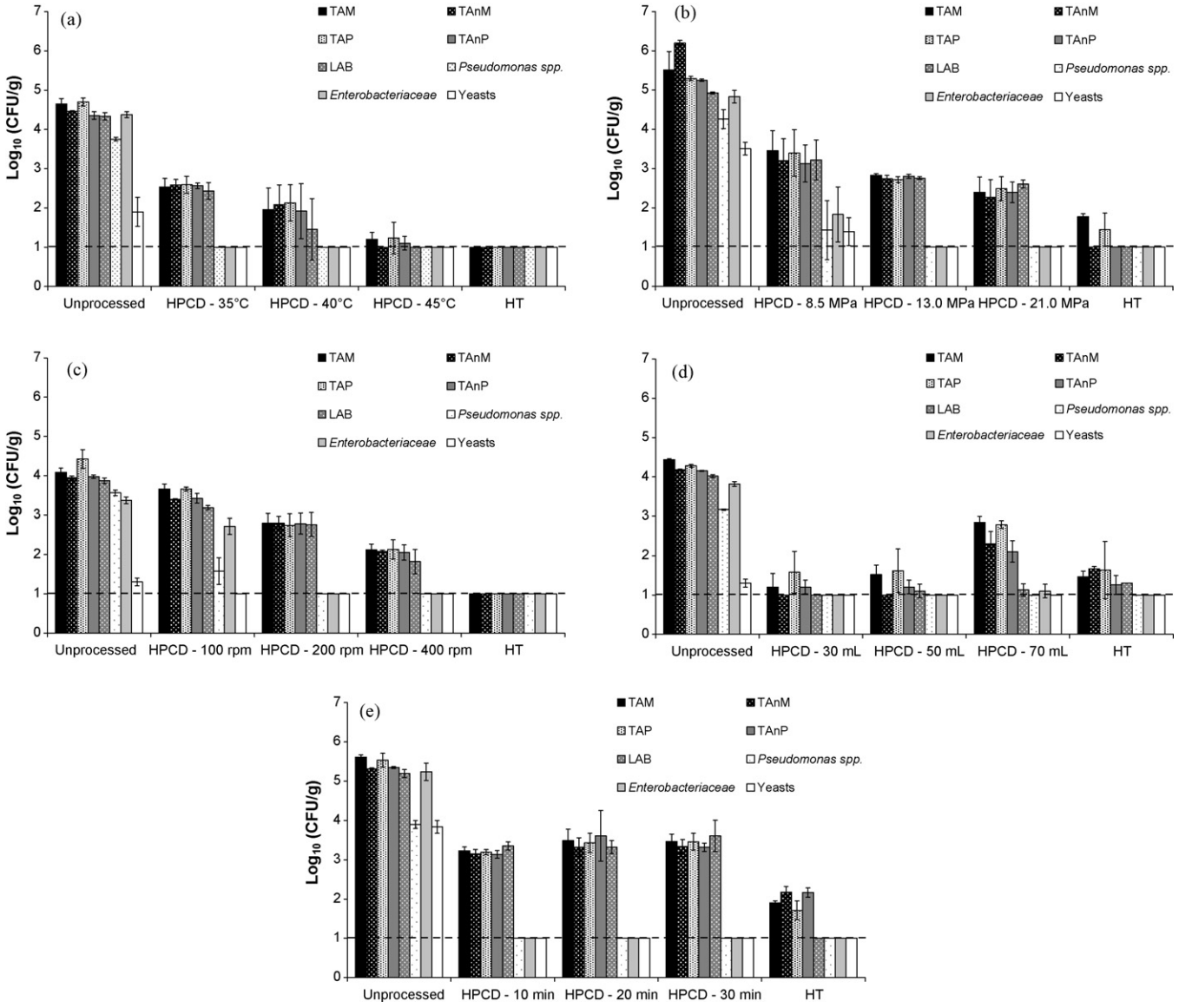

Fig. 1. Effect of process parameters on the microbial inactivation of LWE: (a) effect of temperature at 13.0 MPa, 400 min^{-1}, 50% WVR, 20 min; (b) effect of pressure at 35 °C, 400 min^{-1}, 50% WVR, 20 min; (c) effect of agitation speed at 13.0 MPa, 35 °C, 50% WVR, 20 min; (d) effect of working volume ratio at 13.0 MPa, 35 °C, 400 min^{-1}, 20 min; and (e) effect of holding time at 13.0 MPa, 35 °C, 400 min^{-1}, 50% WVR. Presented data are the mean values of three independent experiments ± standard deviation. Detection limit is indicated by dashed line.

sensitive to pressurized CO_2 (as compared to processing at 35 °C) and significantly increased the inactivation efficacy by approximately 1.2-D ($P < 0.05$) for this group of microorganisms. As for *Pseudomonas* spp., *Enterobacteriaceae* and yeasts, HPCD processing at 35 °C was sufficient to reduce the viability to undetectable levels. Overall, HPCD processing at 45 °C was as effective as heat pasteurization in reducing the naturally occurring microorganisms ($P > 0.05$) in LWE.

The stimulating effect of temperature on the microbial inactivation of HPCD has been frequently reported, as reviewed in [13]. An increase in temperature may stimulate the diffusivity of CO_2, acting on the integrity of the cellular membrane and increasing its fluidity [37–39]. The stimulating effect of temperature can in part be counteracted by its inhibiting effect on CO_2 solubility. However, in our results, treatment at 45 °C significantly increased the antimicrobial efficacy of HPCD by reducing the more resistant LAB population. For that reason, 45 °C was chosen as an optimal temperature condition for the subsequent shelf life study.

3.1.2. Influence of pressure

The effect of pressure was investigated at three pressure conditions (8.5, 13.0 and 21.0 MPa) at 35 °C, 50% WVR, 400 min^{-1} stirring speed during 20 min of treatment (Fig. 1b). In general, an increase in pressure from 8.5 to 13.0 MPa or from 13.0 to 21.0 MPa did not significantly enhance the inactivation efficacy. However, increasing the pressure from 8.5 to 21.0 MPa enhanced the efficacy of the HPCD treatment: the inactivation level of TA(n)M, TA(n)P and LAB significantly increased by 0.9 log cycles ($P < 0.05$) for a treatment at 21.0 MPa, while it reduced the viable cells of *Pseudomonas* spp., *Enterobacteriaceae* and yeasts to undetectable levels. The results indicate that LAB (forming the most abundant group of TA(n)M and TA(n)P in LWE) were more resistant to pressurized CO_2 as these cells survived HPCD treatment. In general, thermal processing appeared to be more effective in reducing microbial populations than HPCD treatment at the tested conditions.

The enhanced antimicrobial activity of pressurized CO_2 with increasing pressure has previously been observed (as reviewed in [13]) and can be attributed to the increased CO_2 solubility in the extracellular medium, facilitating its contact with and penetration into the cells [37–39]. In addition, CO_2 at higher pressures in general exhibits a higher solvating power. The stimulating effect of CO_2 pressure, however, does not go on indefinitely and is limited by the saturation solubility of CO_2 in the suspending medium [40]. This remark was reflected in our results which showed that the HPCD inactivation efficacy was not significantly enhanced when the pressure increased from 8.5 to 13.0 MPa or 13.0 to 21.0 MPa. However, a treatment at 21.0 MPa improved the effectiveness of HPCD as compared to processing at 8.5 MPa. Nevertheless, from an economical point of view, higher pressure significantly increases both operating and capital costs [41] and therefore the optimum pressure condition was set at 13.0 MPa.

3.1.3. Influence of agitation

The influence of agitation was studied by varying the stirring speed of the stirrer in the pressure vessel (100, 200 and 400 min^{-1}) at 35 °C, 13.0 MPa, 50% WVR during 20 min of treatment (Fig. 1c). In general, a higher stirring speed accelerated the inactivation: increasing the stirring speed from 100 to 200 and 400 min^{-1} significantly increased the reduction rate of TA(n)M, TA(n)P and LAB from 0.6-D to 1.3-D and 2.0-D, respectively ($P < 0.05$). The surviving fraction after treatment at 200 and 400 min^{-1} consisted mainly of LAB (as indicated by the similar counts of TA(n)M, TA(n)P and LAB). With regard to *Pseudomonas* spp. and *Enterobacteriaceae*, these cells were reduced to, respectively, 2.0-D and 0.7-D after treatment at 100 min^{-1}, while treatment at 200 and 400 min^{-1} completely inactivated the cells ($P < 0.05$). The yeast population was reduced to undetectable levels, irrespective of the stirring speed. Heat pasteurization, on the other hand, led to a complete inactivation of the naturally occurring microbial population, indicating that this treatment was more efficient than HPCD processing at the investigated process conditions.

The importance of agitation was previously reported by Hong et al. [38] and Lin et al. [42]. In a stirred vessel, the transfer rate of CO_2 is determined by the gas flow rate, the stirring rate and geometrical aspects (such as WVR) [43]. Increasing the stirring speed in our experiments accelerated the process of cell inactivation by increasing the mass transfer of CO_2 in the LWE sample, enhancing the contact between CO_2 and microbial cells, and improving its diffusion across the cells [39,42]. Stirring at 400 min^{-1} significantly improved the HPCD inactivation efficacy and was therefore chosen as an optimum condition for the subsequent shelf life study.

3.1.4. Influence of working volume ratio

The effect of WVR was investigated at three sample sizes (30, 50 and 70% WVR) at 13.0 MPa, 35 °C, 400 min^{-1} stirring speed for a 20 min treatment (Fig. 1d). The inactivation degree of the TA(n)M and TA(n)P significantly decreased by 1.2-D after treatment at 70% WVR as compared to the treatments at 30 and 50% WVR ($P < 0.05$). Regarding the inactivation of LAB, *Pseudomonas* spp., *Enterobacteriaceae* and yeasts, these cells were inactivated below (or at, for LAB) their detection limit, independent of the WVR ($P > 0.05$). No significant difference in microbial inactivation was observed between a HPCD treatment at 30 and 50% WVR on one hand, and heat treatment on the other hand.

The influence of WVR was also related to the mass transfer rate of CO_2, as discussed in Section 3.1.3. Using a smaller LWE sample size, the CO_2 mass transfer is expected to increase, as such accelerating microbial inactivation. In our study, decreasing the WVR from 70 to 50% indeed enhanced the inactivation efficacy, suggesting that the mass transfer was rate limiting at 70% WVR under the selected HPCD conditions. However, further reduction of the WVR to 30% did not further boost the microbial inactivation, indicating that after 20 min of treatment the mass transfer rate was not rate limiting anymore even at 50% WVR in our system under the investigated HPCD conditions. A WVR of 50% was chosen as an optimal condition in the shelf life study because a higher processing capacity is more interesting from a commercial point of view.

3.1.5. Influence of holding time

To study the influence of residence (holding) time, experiments were conducted at 13.0 MPa, 35 °C, 400 min^{-1} stirring speed and 50% WVR during 10, 20 and 30 min of treatment (Fig. 1e). Increasing the holding time did not significantly enhance the inactivation efficacy of HPCD ($P > 0.05$). Furthermore, our results show that the surviving population of HPCD processed samples mainly contained LAB (as indicated by the similar counts of TA(n)M, TA(n)P and LAB). These microorganisms were reduced by 2.0 log, while *Pseudomonas* spp., *Enterobacteriaceae* and yeasts were inactivated to undetectable levels for the three different holding times investigated. Thermal processing, however, appeared to be more effective than HPCD processing at the investigated HPCD conditions.

In our study, the holding time did not influence the antimicrobial power of pressurized CO_2, indicating that after 10 min of treatment the tailing (residual level) of the survival curve was reached. The tailing region mainly consisted of LAB, suggesting that these cells show higher resistance towards a HPCD treatment.

3.2. Shelf life study of LWE under refrigerated storage

In the second part of this research, the effect of HPCD processing at "optimal" conditions (selected on the basis of the results obtained

Table 4
Microbial counts ($\log_{10}$ CFU/g ± SD) of bulk raw LWE.

Populations	Batch 1	Batch 2
Total aerobic mesophilic (TAM) count	4.1 ± 0.2	4.3 ± 0.3
Total anaerobic mesophilic (TAnM) count	4.1 ± 0.2	5.3 ± 0.2
Total aerobic psychrotrophic (TAP) count	4.1 ± 0.1	4.2 ± 0.1
Total anaerobic psychrotrophic (TAnP) count	3.9 ± 0.1	4.2 ± 0.3
Total aerobic spore (TAS) count	<D.L.[a]	<D.L.
Total anaerobic spore (TAnS) count	<D.L.	<D.L.
Lactic acid bacteria (LAB)	4.0 ± 0.2	4.1 ± 0.1
Yeasts	2.6 ± 0.1	<D.L.
Moulds	<D.L.	<D.L.
Pseudomonas spp.	3.0 ± 0.1	3.3 ± 0.1
Enterobacteriaceae	3.5 ± 0.2	4.2 ± 0.1
Salmonella spp.	<D.L.	<D.L.

[a] D.L., detection limit.

in the first part of our research) on the microbial quality of LWE was evaluated under refrigerated storage (4 °C) for up to 5 weeks with conventional heat pasteurized samples (69 °C, 3 min) as a reference. Hereto, two different batches of LWE were subjected to pressurized CO_2 at 13.0 MPa, 45 °C, 50% WVR and 400 min^{-1} stirring speed for 10 min.

The initial microbial loads of unprocessed LWE for the two batches are shown in Table 4. The changes in microorganisms in the HPCD and heat processed LWE samples during storage at 4 °C are presented in Fig. 2. In these figures, the data of the heat pasteurized LWE samples are presented as the means of three independent experiments ± standard deviation, whereas the three replicates of the HPCD treated samples are depicted separately because the samples showed larger variability during the storage period.

In the first batch of LWE, HPCD processing was less effective in decreasing the number of TAM and TAP compared to heat treatment (Fig. 2a, at 0 day). The initial TAM and TAP count of the HPCD treated samples equaled (on average) 2.6 log CFU/g, whereas heat pasteurization reduced the number of TAM and TAP to 1.9 and 1.7 log CFU/g, respectively. Both bacterial groups remained fairly stable during the first 3 weeks of storage at 4 °C. However, as storage continued, significant changes were observed: the TAM and TAP counts continuously increased in two HPCD processed samples, whereas no changes were observed for the third HPCD processed and the heat treated samples. At the end of the shelf life study, the number of TAM and TAP in the HPCD treated samples reached 4.7, 5.1 and 2.3 log CFU/g and 4.2, 5.1 and 2.4 log CFU/g, respectively. After 5 weeks of storage, the TAM and TAP count in the heat pasteurized LWE products was 1.9 log CFU/g. Gram staining of the HPCD treated samples revealed that the population mainly consisted of Gram negative bacteria. In contrast to the aerobic populations, both preservation techniques reduced the anaerobic bacteria below the detection limit and did not allow for their development throughout the duration of the study. Also the number of LAB, *Pseudomonas* spp., *Enterobacteriaceae* and yeasts were inactivated to undetectable levels after treatment and remained below the detection level during the entire storage period. All the LWE samples were free of *Salmonella* spp., spores and moulds.

Similar observations could be made for the storage study of the second batch of LWE (Fig. 2b). Throughout the storage period, variability in microbial quality between the HPCD processed samples was detected. For two replicates, HPCD treatment was as effective as heat pasteurization because both treatments achieved comparable initial inactivation degrees and assured microbial stability during the entire storage study. However, the third HPCD processed sample exhibited a lower initial inactivation efficacy. In this sample, the LAB survived HPCD treatment (as indicated by the similar counts of TA(n)M, TA(n)P and LAB) and considerably started to grow after 3 weeks of storage. At the end of the storage period, the TAM and TAP count in this sample was 6.1 log CFU/g and the number of TAnM, TAnP and LAB equaled 5.3 log CFU/g, indicating that the sample not only contained LAB, but also other spoilage microorganisms were present. Regarding *Pseudomonas* spp. and *Enterobacteriaceae*, no culturable cells were detected during the entire storage period for all the three HPCD processed samples, as well as for the heat pasteurized sample. All the LWE samples of the second batch were free of *Salmonella* spp., spores, yeasts and moulds.

The effect of HPCD treatment on the pH of treated LWE was also studied during the storage period because CO_2 is known for its acidifying properties. During HPCD treatment, CO_2 (present in the reactor headspace) will dissolve in the water-phase of the food to form carbonic acid. This weak acid will dissociate and hydrogen ions will be liberated, decreasing the external pH. Values of pH for HPCD and heat treated LWE samples were similar for each batch and therefore, only the pH evolution of the first batch is shown in Fig. 3. The pH value of unprocessed LWE was 7.4. As expected, immediately after treatment (and after CO_2 pressure release), the pH of HPCD processed LWE was lowered to pH 6.3 (due to residual dissolved CO_2), whereas the pH of heat pasteurized samples was not affected. However, after 1 week of storage, the pH of the LWE samples subjected to HPCD treatment completely returned to its original value as CO_2 returned to its vapor phase and no pH difference was observed between the HPCD and heat processed samples.

In general, treatment at 13.0 MPa, 45 °C, 50% WVR and a 400 min^{-1} stirring speed during 10 min of treatment extended the shelf life of stored (4 °C) LWE with at least 4 weeks. Beyond this period, the psychrotrophic bacterial limit was exceeded only in one out of six samples processed by HPCD. In the first batch, the HPCD treated samples (two out of three replicates) showed an increase in Gram negative bacteria during refrigerated storage. However, the psychrotrophic bacterial limit was not exceeded at the end of the storage study (<6 log CFU/g) and thus acceptable for consumption even after 5 weeks of storage (which is comparable to the shelf life of conventional heat pasteurized LWE, stored at 4 °C). In the second batch, the psychrotrophic bacterial limit did exceed the guide value of 6 log CFU/g at the end of storage study for only one out of three replicates. In this "deteriorated" sample, not only LAB but also other microorganisms were responsible for spoilage, and hence this sample was not in accordance with the microbiological guidelines after 5 weeks of cold storage (4 °C). The difference in microbial growth of the HPCD treated samples in both batches could be attributed to an initial variability in microbial inactivation and should be looked more into detail in order to process homogenous samples. In contrast with HPCD processing, all the heat treated LWE samples were shelf stable throughout the shelf life study.

The (long-term) microbial stability on HPCD treated foods is hardly investigated during its storage. According to the author's knowledge, only Park et al. [19], Kincal et al. [21], Del Pozo-Insfran et al. [22] and Damar et al. [25] performed a shelf life study to evaluate the effect of HPCD treatment on microbial growth during refrigerated storage for other foodstuffs. Park et al. [19] evaluated the effect of a consecutive HPCD (0.1–4.90 MPa, 5 min, 5 °C) and HHP (400 MPa, 5 min, 25 °C) treatment on microbial quality of carrot juice during 4 weeks of storage at 4 °C. During the storage period, no reactivation of aerobes was observed in the pressure treated carrot juice, while the untreated sample showed 8.4 log CFU/mL after 4 weeks. Kincal et al. [21] observed an increase in the bacterial number during storage at 1.7 °C of orange juice treated with continuous high pressure CO_2 at 107 MPa and a CO_2/juice ratio of 1.03 during 10 min, while immediately after HPCD treatment no culturable organisms were present in the juice. By 6 weeks of storage, the microbial counts in the HPCD treated juice reached 6 log CFU/mL, considered unacceptable for juice quality. Del Pozo-Insfran et al. [22] noted comparable microbial counts between HPCD (34.5 MPa, 8 and 16% CO_2) and thermally pasteurized (75 °C,

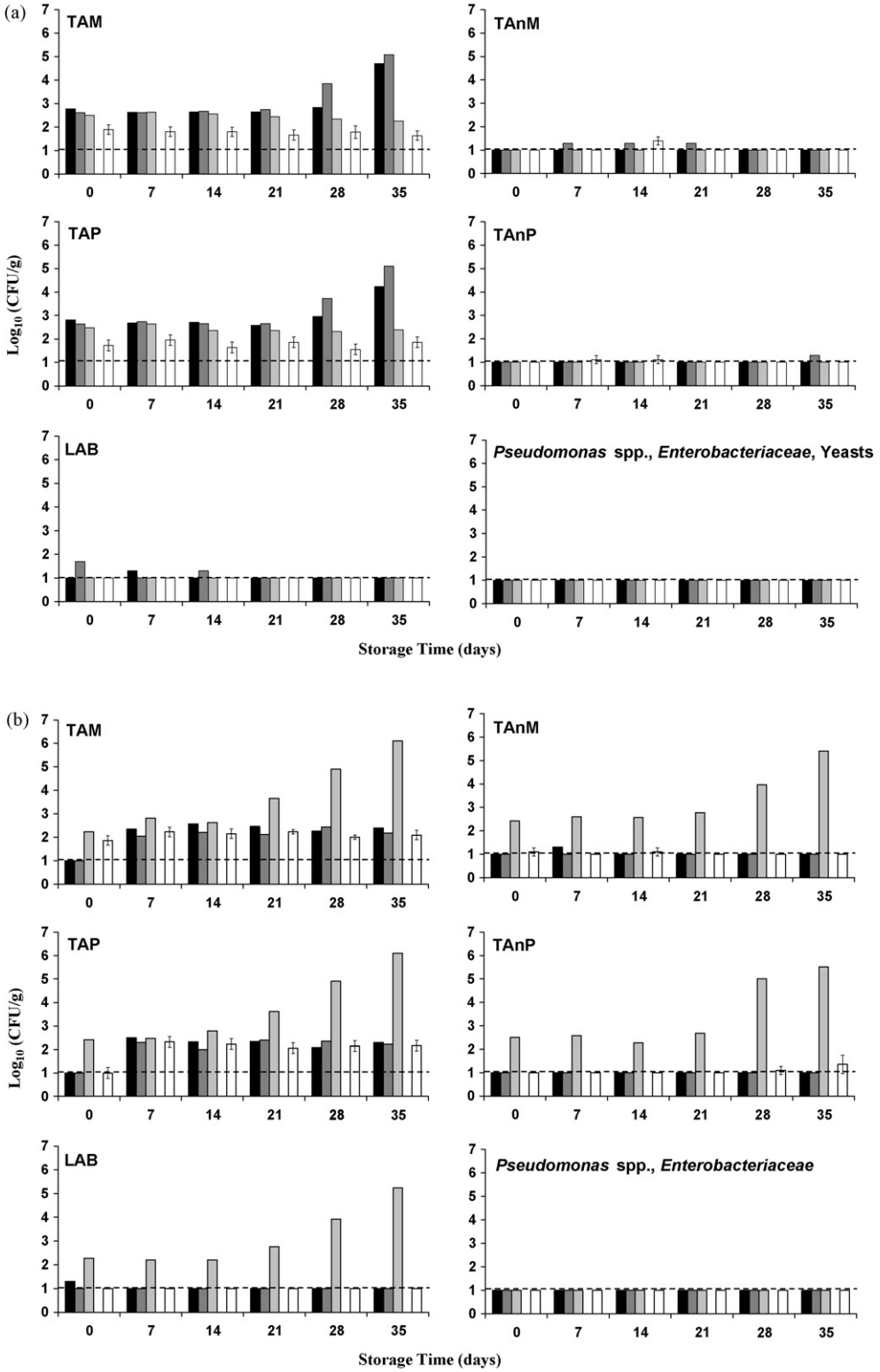

Fig. 2. Changes in microorganisms of HPCD and heat pasteurized LWE in batch 1 (a) and batch 2 (b) during storage at 4 °C. HPCD treatment conditions are 13.0 MPa, 45 °C, 50% WVR, 400 min^{-1}, and 10 min. The data of the heat pasteurized LWE samples are presented as the means of three independent experiments ± standard deviation (indicated by white bars), whereas the three replicates of the HPCD treated samples are depicted separately (indicated by filled bars) due to their larger variability during the storage period. The dashed line represents the detection limit.

15 s) muscadine grape juice during the first 5 weeks of storage at 4 °C. However, yeast/mould counts for HPCD treated juices continuously increased throughout subsequent storage, whereas no changes were observed for heat pasteurized juices. The number of total aerobic microorganisms did not change during storage for both pasteurization techniques. Recently, Damar et al. [25] evaluated microbial growth of HPCD treated (34.5 MPa, 25 °C, 13% CO_2, 6 min), heat treated (74 °C, 15 s) and untreated carbonated coconut water during 9 weeks of refrigerated storage at 4 °C. There was no detectable mould growth in any sample. Yeast counts were only

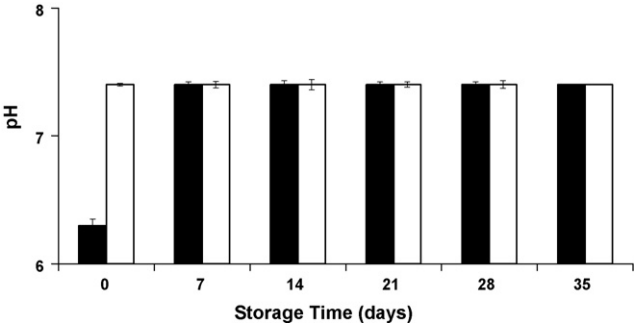

Fig. 3. Effect of HPCD (filled bars) and heat treatment (white bars) on the pH of LWE during storage at 4 °C. HPCD treatment conditions are 13.0 MPa, 45 °C, 50% WVR, 400 min^{-1} and 10 min.

around 1 log initially and decreased to no growth at the end of storage. The total aerobic plate count in untreated samples started to increase after week 6, whereas the total aerobic plate count in the treated samples decreased for an unknown reason by 1–2 log at the end of week 9. HPCD and heat treatment extended the shelf life of the beverage for more than 9 weeks at 4 °C, while the untreated samples reached >5 log CFU/mL at the end of 9 weeks, indicating the end of their shelf life.

4. Conclusions

The natural microbial flora of LWE was decreased by treatment with a batch HPCD treatment system. Various log decreases for total counts of different groups of microorganisms were observed depending on the original starting population and on the pre-set HPCD process conditions. Temperature, working volume ratio and stirring rate were found to have the most profound effect on microbial inactivation of the native flora in LWE. HPCD processing at 13.0 MPa, 45 °C, 50% working volume ratio and 400 min^{-1} stirring speed, during 10 min proved particularly promising for the inactivation of native microorganisms in LWE.

The shelf life of LWE, treated with HPCD under the most preferable process conditions, was in general prolonged with 5 weeks during storage at 4 °C. This shelf life extension is comparable to the current shelf life of heat processed LWE, stored at 4 °C. Only for one out of six samples, a shelf life between 4 and 5 weeks was obtained. No pH difference was detected between HPCD and heat treated LWE after 1 week of storage. Further research is recommended to minimize the observed variation in microbial reduction between HPCD treatments. Furthermore, more research is needed to demonstrate and explain the effect of a HPCD treatment not only on the microbial but also on the sensory and nutritional quality of LWE throughout storage. Also an in-depth economic analysis of the HPCD process has to be assessed in order to offer a viable economic alternative to heat pasteurization of LWE.

With further process optimization and proper scale-up, HPCD processing of LWE can be a promising non-thermal alternative to heat pasteurization.

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