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ORIGINAL PAPER

Effect of High-Pressure Processing on Quality and Microbiological Properties of a Fermented Beverage Manufactured from Sweet Whey Throughout Refrigerated Storage

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Abstract
The production of fermented beverages is a promising way to valorize by-products of dairy manufacturing. However, the shelf-life of these products is often limited by the post-acidification process that occurs during storage. In this work, we manufactured a fermented beverage from sweet whey by using the starter lactic acid bacteria (SLAB) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. High-pressure processing (HPP) at 200 MPa for 10 min or 400 MPa for 1 min were applied after manufacturing. The aim of this study was to evaluate the effect of HPP on the quality of the beverage and on the behavior of the SLAB. Both high hydrostatic pressure treatments preserved flavor and texture attributes until 45 days post-HPP, without affecting chromatic parameters. Plate counts for both species were lower in HPP-treated beverages (HB) than in control beverages (CB), although treatment at 200 MPa maintained optimal amounts of total SLAB (6.6–7.9 log CFU/mL). Conversely, quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR) revealed that bacterial DNA or mRNA levels persisted after HPP ($> 1.4 \times 10^5$ genome or cDNA copies/mL), even upon 400-MPa treatments. As a whole, this study indicated that HPP preserved the quality of the beverage until 45 days post-HPP, which is longer than the shelf-life of conventional fermented beverages obtained from milk. Moreover, our results obtained with these SLAB in a fermented dairy beverage upon HPP extend previous findings regarding the limitations of culture-dependent methods to assess microbial viability.

Keywords High-pressure processing · Sweet whey · Shelf-life extension · Sensory analysis · Starter lactic acid bacteria · Culture-independent methods

Abbreviations

BOD	Biochemical oxygen demand
CB	Control beverages
cDNA	Complementary DNA
CFU	Colony forming units
Ct	Threshold cycle
DGGE	Denaturing gradient gel electrophoresis
HB	HPP-treated beverages
HPP	High-pressure processing
ISO	International Organization for Standardization
LOD	Limit of detection
mRNA	Messenger RNA
qPCR	Quantitative PCR
RT-qPCR	Reverse transcription-qPCR
SD	Standard deviation
SLAB	Starter lactic acid bacteria
VBNC	Viable but non-culturable
WI	Whiteness index

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Introduction

The aqueous fluid remaining after milk curdling, commonly known as whey and which is produced mainly during industrial cheesemaking, was considered in the past exclusively as a

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waste product. However, the very high volume of whey produced (160 million tons/year), which is globally increasing by 1–2% every year, in addition to its high biochemical oxygen demand (BOD), makes this by-product an important environmental hazard (Božanić et al. 2014; Prazeres et al. 2012). Therefore, efforts have been made to transform whey or its components into new products, including bioethanol or peptides with potential health benefits (Panesar and Kennedy 2012; Patel 2015). Depending on the type of dairy product used, the resultant whey can be either sweet (pH 5–7) or acidic (pH < 5). The former contains a higher amount of protein and it has more potential to generate further by-products with added value due to its more favorable flavor (Božanić et al. 2014).

High-pressure processing (HPP) is a non-thermal technology which has shown important applications for improving the quality of food products, given its ability to assure food safety and to increase shelf-life by the inactivation of vegetative microorganisms while preserving or improving some quality attributes (Denoya et al. 2016; Wang et al. 2016). HPP treatments affect the functional properties of food proteins through the disruption and formation of hydrogen bonds, thus changing hydrophobic and ion pair interactions of these macromolecules. In the case of the main milk proteins (whey proteins and caseins), HPP induces changes that improve their functionality for some applications. The casein micelles dissociate into smaller subunits with enhanced aggregating properties when subjected to pressures higher than 100 MPa. There is also denaturation and subsequent aggregation of β-lactoglobulin pressurized at the range of 200 to 600 MPa (de Ancos et al. 2000; López-Fandiño 2006).

Because of these physicochemical changes, HPP can improve important quality characteristics associated to fermented beverages and yoghurts such as viscosity and syneresis (separation of the aqueous phase) (revised by Chawla et al. 2011; Trujillo et al. 2002). Furthermore, Krasowska et al. (2005) and Reps et al. (1999) reported that HPP was able to change the metabolism of lactic acid bacteria and to inhibit their acidifying activity. These properties of HPP were used to control excessive acidification during the storage of yoghurt, obtaining a product with improved sensorial attributes (de Ancos et al. 2000; Jankowska et al. 2005).

Previous studies have consistently shown reduced amounts of both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chawla et al. 2011; Trujillo et al. 2002), species commonly used as yoghurt starters (Codex Alimentarius 2003). The loss of culturability reported for these bacteria is dependent on the amount of pressure applied. However, pressures below 300–400 MPa for 10–15 min have shown adequate to maintain the amount of required starter lactic acid bacteria (SLAB) in yoghurt while maintaining or improving rheological aspects (Jankowska et al. 2012; Reps and Wiśniewska 2008; Shah et al. 2008; Walker et al. 2006).

When studying the microbiological aspects of fermented foods, molecular (culture-independent) methods target microbial nucleic acids (DNA, RNA, or both) without the need of selective culture media for their retrospective characterization (Cocolin et al. 2013; Jany and Barbier 2008). In contrast, among other disadvantages, culture-dependent assays are not accurate for the quantification of specific bacterial species during food fermentations, probably due to their inability to detect viable but non-culturable (VBNC) microorganisms (Falentin et al. 2012; Overney et al. 2016; Ruggirello et al. 2016). In this respect, quantitative molecular methods, including quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR), have been successfully used to monitor *S. thermophilus*, *L. delbrueckii*, and other lactic acid bacteria in dairy foods such as yoghurt and cheese (Miller et al. 2012; Pega et al. 2016; Sohier et al. 2014).

In this study, we manufactured a fermented beverage from sweet whey by using the SLAB *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. HPP at 200 MPa for 10 min or 400 MPa for 1 min were applied after manufacturing. Therefore, the aim of this work was to assess the effect of HPP on physicochemical and sensory properties of the fermented beverage, and on the dynamics of the SLAB throughout refrigerated storage. To provide a better understanding of the behavior of these SLAB upon exposure to HPP technology, both culture-dependent and culture-independent (qPCR and RT-qPCR) methods were performed.

Materials and Methods

Elaboration of the Fermented Beverage From Sweet Whey

The fermented beverage was manufactured using the following ingredients: sweet whey (obtained from local dairy industries), ultra-high-temperature-treated milk (3% fat; Mastellone Hnos. S.A., Buenos Aires, Argentina), sucrose (Ledesma S.A.A.I., Jujuy, Argentina), inulin (Beneo GmbH, Mannheim, Germany), guar gum (INS 412) as stabilizer (Biochemical S.A., Buenos Aires, Argentina), and dairy starters (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*; Yogmix, Biochemical S.A., Buenos Aires, Argentina). Sweet whey was filtered through a gauze to remove impurities. Briefly, 75–85% (*w*/*w*) pasteurized (85 °C for 30 min) sweet whey, 8–13% (*w*/*w*) milk, 6–8% (*w*/*w*) sucrose, 1–3% (*w*/*w*) inulin, and 0.1–1% (*w*/*w*) guar gum were mixed gently for 30 min. Next, 1.2 g/L of the starters was added aseptically and the mixture was incubated at 42–43 °C until a pH of 4.7 was obtained. The pH was assessed with a pH meter (6.0226.100 MetroHM AG, Herisau, Switzerland) using a

spearhead electrode for dairy samples. The fermented beverage was rapidly cooled down while shaking it slowly. The final product was divided in high-density polypropylene bottles (250 mL) and stored at 4 °C until HPP.

Compositional Analysis

Table 1 shows the macronutrient composition of the fermented beverage and of the sweet whey used for its formulation. These determinations were performed on fresh samples with an Automatic Julie C8 Milk Scope (Scope Electric Instruments, Regensburg, Germany), according to the manufacturer's instructions.

Sample Preparation and HPP

Each bottle containing the fermented beverage was vacuum-packed in Cryovac BB2800 (Sealed Air, Buenos Aires, Argentina) bags before HPP to prevent potential leakages. Two high hydrostatic pressure treatments were applied in three independent experiments: 200 MPa for 10 min or 400 MPa for 1 min. Bottles without HPP were used as controls. A high hydrostatic pressure equipment with a 2-L capacity (Stansted Fluid Power Ltd., High Pressure Iso-Lab System Model: FPG9400:922, UK) and a maximum working pressure of 900 MPa was used. A mix of distilled water and propylene glycol (70/30) was used as the compression fluid. Pressure was increased at 5 MPa s^{-1}. High-pressure treatments were carried out at an initial temperature of 21–24 °C, which was only modified by adiabatic heating. The temperature of the compression fluid reached a maximum of 30 °C during the 200-MPa treatments and of 35 °C during the 400-MPa treatments. Upon pressure release, these values were reduced to 20 °C. Bottles containing HPP-treated beverages (HB) and control (CB) beverages were kept at 4 °C throughout the complete storage period. Samples from each triplicate HB and from CB were obtained at 1, 30, and 45 days post-HPP for the different analyses (pH determinations, chromatic assessments, sensory evaluations, and culture-dependent and culture-independent experiments).

Table 1 Compositional analysis of fresh sweet whey and fermented beverage

Parameter[a]	Sweet whey	Beverage
Energy (Kcal)	21	53
Carbohydrates (g)	3.6	11
Protein (g)	0.7	1.1
Total fat (g)	0.4	0.7
Saturated fat (g)	0.2	0.4
Trans fat (g)	0	0
Dietary fiber (g)	0	1.0

[a] All data are expressed per 100 mL of sample

Determination of Active Acidity and Chromatic Parameters

Active acidity (pH) was assessed by using a pH meter (MetroHM AG, Herisau, Switzerland). Chromatic parameters of the beverages were determined with a Minolta CR-400 colorimeter provided with a sample holder CR A505 and a Glass Cell 20 mm CM-A99 for measuring liquids (Konica Minolta, Osaka, Japan), using the CIE L^* a^* b^* scale. These values were then used to calculate Whiteness index (WI) = $100 - [(100 - L^*)^2 + a^* + b^{*2}]^{0.5}$ as reported previously (Vargas et al. 2008). The instrument was set up for illuminant D_{65} and 2° observer angle.

Sensory Analyses

A quantitative evaluation of specific sensory attributes was performed according to International Organization for Standardization (ISO) guidelines (ISO 22935–2:2009). A total of ten trained and experienced panelists were selected for the sensory determinations. A scale of 1 to 5 was used and differences were evaluated against fresh control samples. The scale score for each attribute evaluated (flavor, texture) was as follows: 5 (full concordance with established requirement), 4 (slight deviation), 3 (perceived deviation), 2 (marked deviation), and 1 (very marked deviation).

Quantification of SLAB by Traditional Plating (Culture-Dependent Method)

Plate count numbers for *S. thermophilus* and *L. delbrueckii* were carried out using the pour plate method, according to current standard procedures (Ashraf and Shah 2011). Briefly, 1-mL samples of fermented beverage were diluted with 9 mL of sterile peptone water 0.1% (*w*/*v*) (Oxoid, Hampshire, UK) to perform tenfold serial dilutions. *S. thermophilus* was quantified on M-17 agar (Oxoid) supplemented with 1% lactose (Oxoid) after incubations at 37 °C for 48 h in aerobiosis. *L. delbrueckii* subsp. *bulgaricus* was quantified on MRS agar (Oxoid) adjusted to pH 5.4 after incubations at 42 °C for 48 h in anaerobic jars.

Quantification of SLAB by qPCR and RT-qPCR (Culture-Independent Methods)

Isolation of Bacterial Cells

Bacterial cells were concentrated from fermented beverage samples as previously described for milk and cheese samples (Pega et al. 2016), with some modifications. Briefly, 2-mL

samples were centrifuged at 9700×*g* for 15 min at 4 °C and cell pellets were suspended in 20 mL of 2% trisodium citrate-4% polyethylene glycol 8000 (Sigma-Aldrich, MO, USA). The mixtures were centrifuged at 700×*g* for 10 min at 4 °C, and supernatants were transferred to 50-mL tubes. Samples were centrifuged at 9700×*g* for 15 min at 4 °C. The supernatants were discarded and cell pellets were stored at − 80 °C until nucleic acid extraction.

Nucleic Acid Extraction

DNA and RNA from bacterial cells were extracted as reported previously (Pega et al. 2016). Briefly, DNA was extracted by using DNAzol (Invitrogen, MA, USA) and quantified with the Qubit 2.0 Fluorometer (dsDNA Assay BR Kit, Invitrogen). RNA was obtained by using TRIZol Reagent (Invitrogen) and quantified with the Qubit 2.0 Fluorometer (RNA Assay BR Kit, Invitrogen). Then, 1 μg of purified RNA samples was incubated for 15 min with 1 μL (1 U/μL) of DNase I (Invitrogen). Residual DNA was checked by qPCR in controls prepared with non-retrotranscribed RNA.

Primer Design and Specificity Determinations

S. thermophilus specific primers for DNA amplification in dairy samples have been previously described (Pega et al. 2016). The same methodology was used for the design of *L. delbrueckii* subsp. *bulgaricus* specific primers and for assessing in silico specificity. The nucleotide sequence of the primer pair was as follows: 5′-TCA ATC AAG ACC CAC AAA ACT TTC-3′ (f) and 5′-GGA ACC ACC TCT CTC TAG CTG TAG-3′ (r). Cross-amplification experiments to determine primer specificity for *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the fermented beverage were performed with 0.2–30 ng/μL of DNA extracted from *S. thermophilus* (ATCC 19258, Thermo Scientific, MA, USA) or *L. delbrueckii* subsp. *bulgaricus* (ATCC 12315, Thermo Scientific), respectively.

Real-Time qPCR and RT-qPCR Experiments

Experiments were carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA), and the data were analyzed with StepOne software v2.2. The amplification conditions used for *S. thermophilus* have been described previously (Pega et al. 2016). For *L. delbrueckii*, amplifications were performed with 12.5 μL of QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany), 5 μL of DNA or complementary DNA (cDNA), 1.25 μL of each primer (500 nM), and 5 μL of GIBCO ultra-pure water (Invitrogen) (total volume of 25 μL). The thermal cycling conditions were as follows: one uracil-N-glycosylase preincubation step at 50 °C for 2 min, 1 cycle at 95 °C for 15 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min. Reverse transcription was performed with the M-MLV Reverse Transcriptase Kit (Promega, WI, USA), using 5 μL of RNA and following the manufacturer's instructions.

Construction of Standard Curves for qPCR and RT-qPCR

The standard curves used for *S. thermophilus* DNA and cDNA quantifications in dairy matrices have been previously described (Pega et al. 2016). The same protocol was followed to generate standard curves for *S. thermophilus* and *L. delbrueckii* in 2-mL fermented milk samples. Briefly, pasteurized and autoclaved 2-mL fermented beverage samples were spiked with tenfold amounts (0.04 μg–40 mg) of *S. thermophilus* and *L. delbrueckii* (Biochemical 4026) and subjected to DNA and RNA extraction for qPCR or RT-qPCR assays. Standard curves were generated by interpolating threshold cycle (Ct) values (mean of triplicate samples) against the log number of genome or cDNA copies/mL or g of sample. The copy number was calculated as previously described (Pega et al. 2016).

Statistical Analyses

All analyses were carried out with InfoStat software (Di Rienzo et al. 2015). Factorial designs were applied for each determination. One factor was assigned to the type of treatment applied on fermented sweet whey (three levels: CB, 200-, and 400-MPa treated beverages) and the other one to the time point evaluated (three levels: 1, 30, and 45 days post-HPP). Results were analyzed using two-way ANOVA and all-pairwise comparisons were performed by a Tukey HSD test. Differences were considered significant at $p < 0.05$.

Results

Active Acidity Determinations

Active acidity (pH) values for the fermented beverage after HPP during refrigerated storage are shown in Table 2. At 1 day post-HPP, 200-MPa treated beverages and CB showed the same pH values, which were significantly higher ($p < 0.05$) than the ones observed for 400-MPa treated beverages. However, at 30 days post-HPP, 200- and 400-MPa treated beverages showed the same pH values, which were significantly higher ($p < 0.05$) than the ones observed for CB. Accordingly, the results observed at 30 days post-HPP were also recorded at 45 days post-HPP (Table 2).

Table 2 Quality parameters of fermented sweet whey upon HPP during storage at 4 °C

pH	1 day[a]	30 days[a]	45 days[a]
CB	4.6 ± 0.2 b	4.3 ± 0.2 a	4.3 ± 0.1 a
HB: 200 MPa—10 min	4.6 ± 0.1 b	4.6 ± 0.1 b	4.6 ± 0.2 b
HB: 400 MPa—1 min	4.5 ± 0.2 a	4.6 ± 0.2 b	4.6 ± 0.2 b
Flavor	1 day[a]	30 days[a]	45 days[a]
CB	4.5 ± 0.0 a	3.0 ± 0.1 a	1.8 ± 0.6 a
HB: 200 MPa—10 min	4.5 ± 0.2 a	4.1 ± 0.1 b	3.6 ± 0.4 b
HB: 400 MPa—1 min	4.5 ± 0.0 a	4.0 ± 0.1 b	3.3 ± 0.4 b
Texture	1 day[a]	30 days[a]	45 days[a]
CB	4.5 ± 0.0 a	3.0 ± 0.1 a	2.9 ± 0.1 a
HB: 200 MPa—10 min	4.5 ± 0.0 a	4.0 ± 0.1 b	4.0 ± 0.1 b
HB: 400 MPa—1 min	4.5 ± 0.1 a	4.1 ± 0.1 b	3.7 ± 0.4 b
WI	1 day[a]	30 days[a]	45 days[a]
CB	83.1 ± 0.1 a	82.8 ± 0.6 a	83.9 ± 0.1 a
HB: 200 MPa—10 min	83.2 ± 0.1 ab	82.8 ± 0.7 a	83.7 ± 0.4 a
HB: 400 MPa—1 min	83.4 ± 0.1 b	82.7 ± 0.8 a	83.6 ± 0.1 a

Results are expressed as mean values of triplicate samples ± SD. Means from each parameter sharing a common letter in each column are not significantly different ($p > 0.05$)

CB control beverages, *HB* HPP-treated beverages, *WI* Whiteness index

[a] Days post-HPP treatments

Chromatic Parameters

No significant differences ($p < 0.05$) were obtained for the chromatic parameters L^*, a^*, and b^* between HB and CB throughout storage (data not shown). Conversely, in the case of the WI, significant differences ($p < 0.05$) were observed between 400-MPa treated beverages and CB at day 1 post-HPP (Table 2). However, no significant differences ($p > 0.05$) were recorded for this index between HB and CB at 30 and 45 days post-HPP (Table 2).

Sensory Analyses

After 1 day of HPP, the mean scores from the trained panelists revealed no differences in flavor or texture outcomes between HB and CB (Table 2). However, at 30 days post-HPP, HB obtained significantly higher scores for flavor and texture attributes than CB ($p < 0.05$). Moreover, by that time, CB presented a perceived deviation in flavor in relation to HB due to their sour taste and loss of creamy texture. After 45 days of HPP, the deterioration of CB was even more pronounced, with flavor and texture scores significantly lower ($p < 0.05$) than the ones obtained for HB. At this time point, 200-MPa treated beverages obtained slightly higher scores than 400-MPa treated beverages, although these differences were not significant ($p > 0.05$) (Table 2).

Dynamics of SLAB in Fermented Sweet Whey Subjected to HPP: Culture-Dependent Approach

After 1 day of HPP, very similar values were observed for *S. thermophilus* in HB and CB (~8.0 log CFU/mL) (Fig. 1). Conversely, after 30 days of HPP, although similar values were found for this bacterium in 200-MPa treated beverages and CB (~7.9 log CFU/mL), 400-MPa treated beverages showed an ~5 log reduction in *S. thermophilus* plate counts (~3.0 log CFU/mL). Accordingly, a similar scenario was observed after 45 days of HPP, where *S. thermophilus* plate counts in 200-MPa treated beverages and CB showed values of ~7.1 log CFU/mL, whereas 400-MPa treated beverages maintained the levels of this SLAB in 3.0 log CFU/mL. Differences obtained in mean plate counts for *S. thermophilus* between 400- and 200-MPa treated beverages or CB were statistically significant, both at 30 and 45 days post-HPP ($p < 0.05$) (Fig. 1).

Regarding *L. delbrueckii*, very similar values were found between 200-MPa treated beverages and CB (~5.2 log CFU/mL) after 1 day of HPP (Fig. 1). However, 400-MPa treated beverages showed much lower levels (~2.0 log CFU/mL) at this time point, indicating a significant ($p < 0.05$) ~3.2 log reduction for this SLAB in relation to 200-MPa treated beverages and CB. Similarly, after 30 days of HPP, *L. delbrueckii* plate counts in both 200-MPa treated beverages and CB showed values of ~5.3 log CFU/mL, whereas 400-MPa treated beverages showed even lower levels of this bacterium (~0.9 log CFU/mL) than in the previous time point (Fig. 1). This amount corresponded to a significant decrease in *L. delbrueckii* levels of more than 4 log in relation to 200-MPa treated beverages and CB ($p < 0.05$). In accordance with the results obtained after 30 days of HPP, the effect of high pressure on the culturability of *L. delbrueckii* was further evidenced after 45 days post-HPP, where plate counts for this SLAB in CB showed values of ~4.9 log CFU/mL, whereas ~2.6 log and ~0.9 log CFU/mL were recorded in 200- and 400-MPa treated beverages, respectively. At this time point, differences were statistically significant, not only between 400-MPa treated beverages and CB but also between 200-MPa treated beverages and CB ($p < 0.05$) (Fig. 1).

Dynamics of SLAB in Fermented Sweet Whey Subjected to HPP: Culture-Independent Approaches

Primer Specificity

To determine the specificity of *S. thermophilus* primers in the fermented beverage, DNA extracted from *L. delbrueckii* subsp. *bulgaricus* was tested and found to be below the

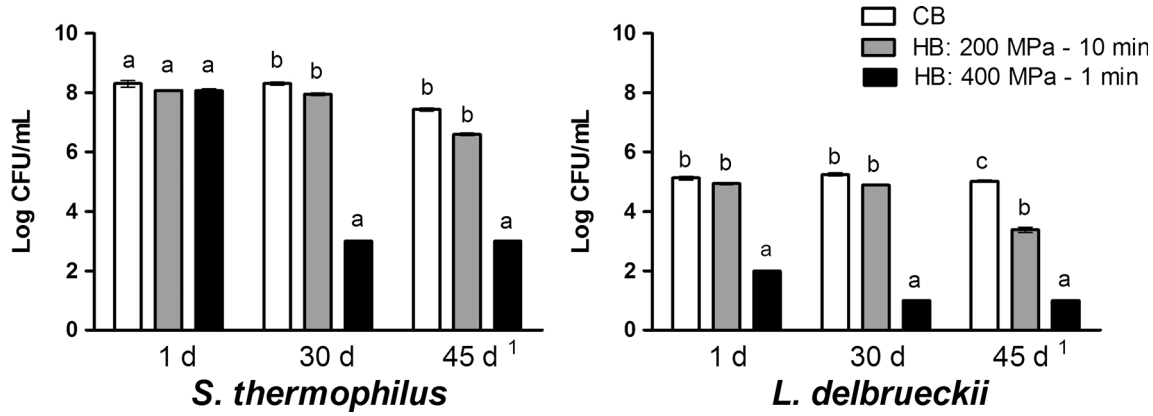

Fig. 1 Effect of HPP treatments on the dynamics of the SLAB used for the fermentation of the beverage manufactured from sweet whey as evaluated by culture-dependent methods. [1]Days post-HPP treatments. *CB* control beverages, *HB* HPP-treated beverages. Results are expressed as the log number of CFU/mL of sample. Each bar represents the mean value of triplicate samples ± SD. Means with the same letter at each time point are not significantly different ($p > 0.05$)

detection threshold of the assay (Ct > 40), even at the lowest dilution of template evaluated. The same results were obtained for cross-reactivity assessments when *L. delbrueckii* subsp. *bulgaricus* primers were tested against the DNA obtained from *S. thermophilus*. Moreover, melting curves generated for these species showed only one peak in every sample, thus confirming the specificity of both primer pairs (data not shown).

Standard Curves

Standard curves generated with negative sweet whey and *E%* values are shown in Fig. 2. An optimum linear correlation between Ct values and copy numbers was recorded for DNA and cDNA standard curves (R^2 values of 0.999) constructed for *S. thermophilus* and *L. delbrueckii*, whereas the detection spectrum was linear across a range of 7 log units. The limit of

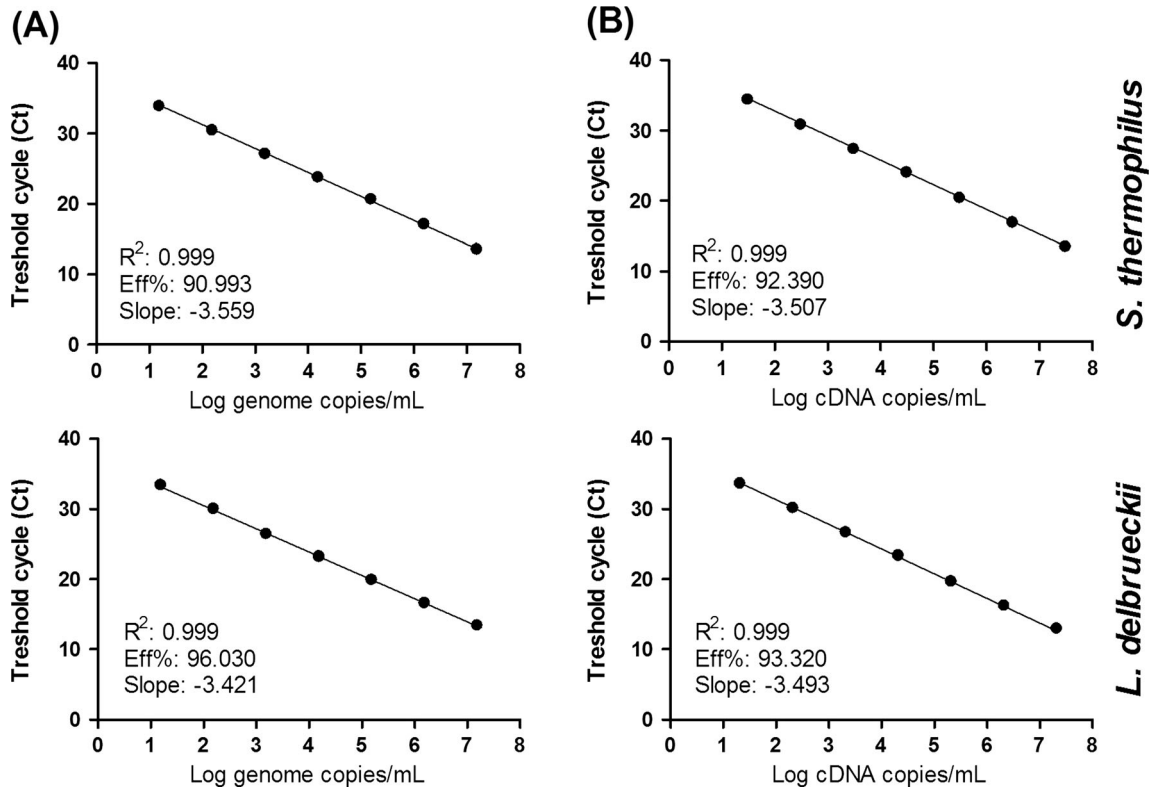

Fig. 2 Standard curves generated for the SLAB used for the fermentation of the beverage manufactured from sweet whey by qPCR (**a**) and RT-qPCR (**b**). Each point represents the mean value of triplicate DNA or RNA extractions ± SD. Standard curves were constructed by plotting Ct values against the log number of genome or cDNA copies/mL of negative samples

detection (LOD) for *S. thermophilus* corresponded to 75 copies of DNA/well and 52 copies of cDNA/well. Similarly, the LOD for *L. delbrueckii* corresponded to 89 copies of DNA/well and 74 copies of cDNA/well.

Quantification of SLAB in Fermented Sweet Whey by qPCR and RT-qPCR

After 1 day of HPP, qPCR experiments revealed similar values for *S. thermophilus* between HB and CB (~2.4×10^5 genome copies/mL). These values were overall maintained in both HB and CB after 30 and 45 days (Fig. 3a). RT-qPCR showed also a similar pattern for *S. thermophilus* cDNA levels, with no significant differences between treatments in any of the time points evaluated (~2.1×10^5 cDNA copies/mL) ($p > 0.05$) (Fig. 3b). Evidently, contrary to the results obtained by traditional plating, the effect of HPP on fermented sweet whey did not influence *S. thermophilus* DNA or messenger RNA (mRNA) levels, even when performing the more drastic treatment (400 MPa for 1 min) and until 45 days post-HPP (Fig. 3a, b).

L. delbrueckii, as evaluated by qPCR, experimented a behavior similar to *S. thermophilus* upon HPP (Fig. 3a). After 1, 30, or 45 days, genome copies remained similar for this SLAB, independently of the treatment evaluated (~2.0×10^5 genome copies/mL). RT-qPCR results also highlighted the presence of cDNA in similar levels among HB and CB (~1.3–~1.7×10^5 genome copies/mL), with no significant differences ($p > 0.05$) (Fig. 3b). The same as with the results obtained for *S. thermophilus*, the effect of HPP on fermented sweet whey did not affect *L. delbrueckii* DNA or mRNA levels throughout refrigerated storage (Fig. 3a, b).

Discussion

The use of HPP to prevent post-acidification in dairy matrices has been reported in previous studies (Chawla et al. 2011; Trujillo et al. 2002). However, in the case of fermented milk treated by this technology, sensory evaluations were not performed after 28 days of refrigerated storage (de Ancos et al. 2000; Jankowska et al. 2005). Therefore, the ability of HPP to extend the shelf-life in this type of food products remains unknown. Moreover, experimental evidence about the use of HPP for preserving organoleptic attributes in beverages produced from whey is currently lacking.

In the present study, we applied HPP (200 MPa for 10 min or 400 MPa for 1 min) to control post-acidification and thus preserve sensory properties in a fermented beverage manufactured from sweet whey. Therefore, our approach transformed an otherwise contaminating by-product (Božanić et al. 2014; Prazeres et al. 2012) into a valuable food product with known potential health benefits such as probiotic bacteria (Guarner et al. 2005) and nutraceutical peptides (Panesar and Kennedy 2012; Patel 2015).

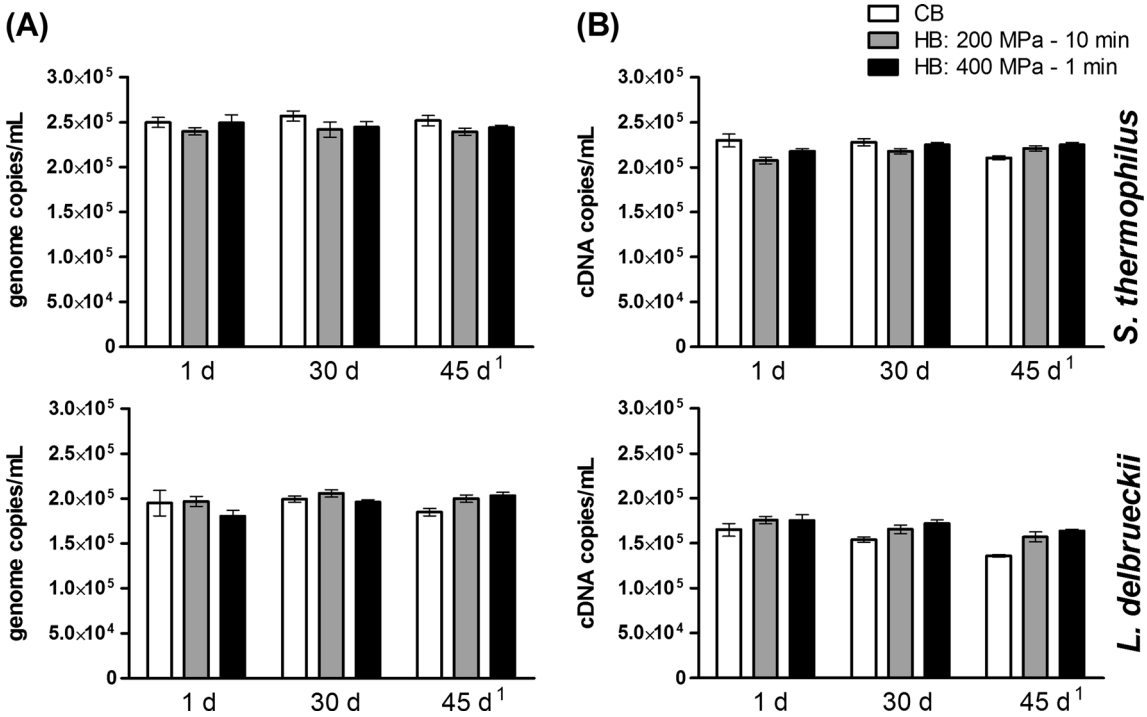

Fig. 3 Effect of HPP treatments on the dynamics of the SLAB used for the fermentation of the beverage manufactured from sweet whey as evaluated by qPCR (**a**) and RT-qPCR (**b**). [1]Days post-HPP treatments. *CB* control beverages, *HB* HHP-treated beverages. Results are expressed as the number of genome or cDNA copies/mL of sample. Each bar represents the mean value of triplicate samples ± SD

The success of HPP to prevent post-acidification in the final product was evidenced both by pH and sensory evaluations. The latter revealed that 200- and 400-MPa treated beverages showed significantly higher scores for flavor and texture attributes than CB, even until 45 days of storage. Furthermore, this storage time is longer than the expiration period of conventional fermented beverages made from milk, which is usually 28–35 days (Codex Alimentarius 2003). Most importantly, to our knowledge, this is the first report showing that HPP extended the shelf-life of a fermented dairy beverage until 45 days of refrigerated storage.

Active acidity values were in line with flavor determinations since CB showed lower acidity than HB throughout refrigerated storage. Only at day 1 post-HPP, 400-MPa treated beverages recorded the lowest pH values among the different treatments.

This early drop in active acidity caused by HPP has also been reported for some liquid foods, including milk and yoghurt, where in situ pH measurements were performed in these matrices after HPP (Samaranayake and Sastry 2013). These events were reported to be reversible, thus explaining the higher pH values obtained at 30 and 45 days post-HPP for 400-MPa treated beverages as compared to CB.

Interestingly, significant differences were also obtained for the WI between 400-MPa treated beverages and CB only at day 1 post-HPP. These differences were not observed at 30 or 45 days post-HPP. Accordingly, it has been shown that chromatic changes in yoghurt made from milk subjected to HPP are also reversible (Harte et al. 2003). Therefore, in the conditions used in the present study, the chromatic parameters of the fermented beverage were not affected by the use of HPP.

As stated above, our results showed that both high-pressure treatments were effective for preventing excessive acidification and improving sensory aspects in fermented sweet whey. However, total SLAB plate counts in 200-MPa treated beverages were 7.9 log CFU/mL at 30 days post-HPP and 6.6 log CFU/mL at 45 days post-HPP. Conversely, in 400-MPa treated beverages, these values were ~4 log CFU/mL, both at 30 and 45 days post-HPP. Therefore, 200-MPa treatments appeared more appropriate than 400-MPa treatments for maintaining the amount of total SLAB considered optimal for standard fermented beverages produced from milk (>6–7 log CFU/mL) (CAA 2012; Codex Alimentarius 2003).

Overall, both high-pressure treatments reduced the culturability of *S. thermophilus* and *L. delbrueckii* during storage at 4 °C. With the exception of *S. thermophilus* plate counts at day 1 post-HPP, where both treatments recorded almost the same values, 400-MPa treated beverages showed lower plate counts for both species than 200-MPa treated samples. Moreover, 400-MPa treated beverages showed significantly lower plate counts than CB for *S. thermophilus* at days 30 and 45 post-HPP, whereas for *L. delbrueckii*, these results were obtained also at day 1 post-HPP. This is in line with the fact that, in the case of *L. delbrueckii*, 200-MPa treated beverages also showed significantly lower plate counts than CB at day 45 post-HPP.

Therefore, the effect of HPP on the loss of culturability appeared earlier (1 day post-HPP) and could be observed with lower pressures (200 MPa) at 45 days post-HPP in *L. delbrueckii*, in comparison to *S. thermophilus.* Since the effect of HPP on the growth of lactic acid bacteria has been shown to be highly strain-dependent (Chawla et al. 2011; Reps et al. 1999), generalizations for defining specific pressure values to obtain specific microbial outcomes, based on different reports, are clearly not reliable. Nevertheless, our findings are consistent with published literature evaluating yoghurt, which has repeatedly shown that higher pressures are more aggressive on microbial cells than lower pressures, with *S. thermophilus* being in general more resistant than *L. delbrueckii* (Jankowska et al. 2012; Trujillo et al. 2002).

Conventional yoghurt produced from milk used in these studies generally contains higher amounts of both protein (~3%) and fat (~2%) (CAA 2012; Codex Alimentarius 2003) than the fermented beverage manufactured from sweet whey evaluated in this study (Table 1). However, despite differences in macronutrient composition, the behavior of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* upon HPP in the present study was similar to the one previously observed in yoghurt samples (de Ancos et al. 2000; Jankowska et al. 2005).

Only a few studies have also investigated the behavior of the microbiota in food matrices upon HPP exposure by culture-independent methods. These studies evaluated microbial diversity by PCR-denaturing gradient gel electrophoresis (DGGE) (Diez et al. 2008; Martínez-Onandi et al. 2017) or pyrosequencing (Pérez Pulido et al. 2017), and they were not focused on quantifying specific bacterial species. Furthermore, the activity of SLAB in food matrices upon HPP has been assessed in the past exclusively by traditional plating methods (Jankowska et al. 2012; Reps and Wiśniewska 2008; Shah et al. 2008; Walker et al. 2006).

In this study, qPCR and RT-qPCR experiments revealed that the effect of HPP on fermented sweet whey did not influence DNA or mRNA levels from any of the two SLAB throughout storage, with values always higher than 1.4×10^5 genome or cDNA copies/mL. Interestingly, these results were observed also in the beverages subjected to the highest pressure (400 MPa) and persisted even until the last time point evaluated (45 days post-HPP).

The effect of HPP on preventing post-acidification in the fermented beverage indicates a decrease in SLAB-mediated lactic acid synthesis. This is consistent with the work of other authors (Chawla et al. 2011). However, SLAB mRNA levels were similar in CB and HB until 45 days post-HPP, suggesting the possibility that other metabolic functions were active in these bacteria. Accordingly, Krasowska et al. (2005) showed

that HPP (300–500 MPa for 15 min) inhibited lactic acid production but maintained peptidolytic activity in lactococci.

The transcript of the *tuf* gene quantified in the present study encodes the GTP binding protein. This gene is considered a housekeeping gene, and although many of its functions remain unknown, it has been used in studies aimed at assessing microbial viability in cheese SLAB *S. thermophilus* (Falentin et al. 2012) and *Lactococcus lactis* (Ruggirello et al. 2016). Therefore, the perceived decrease of lactic acid synthesis correlating with a loss of culturability and, the persistence of mRNA in SLAB throughout refrigerated storage of the fermented beverage, suggests that the *tuf* gene may be also a useful marker to evaluate the effect induced by HPP on cell viability.

We have previously shown under experimental conditions involving the same nucleic acid extraction and qPCR/RT-qPCR protocols, that mRNA may represent an accurate measure of cell integrity, both for *S. thermophilus* in milk, cheese, and yoghurt and for *L. lactis* in milk and cheese matrices (Pega et al. 2017). This suggests that although we cannot conclusively demonstrate the viability of the microbiota detected by RT-qPCR, the mRNA signal detected in this study corresponded only to cells with complete membrane integrity.

As a whole, our results from culture-dependent and -independent experiments suggest that at least some of the bacterial populations which could not be recovered by traditional culturing after HPP were not dead but rather in a VBNC state, and thus, the effect of high pressure on SLAB in fermented sweet whey may have not been completely lethal. This is in line with previous findings regarding the possible induction of VBNC cells by environmental stresses (Overney et al. 2016), high-pressure CO_2 (Zhao et al. 2016), or throughout fermentation processes (Falentin et al. 2012; Ruggirello et al. 2016).

Furthermore, it has been shown by cell viability, permeability, and metabolic activity assays that HPP can induce sublethal injury in some species of lactic acid bacteria such as *L. plantarum*, *L. sanfranciscensis*, and *Lactococcus lactis* subsp. *cremoris* (Ulmer et al. 2000; Vogel and Ehrmann 2008). Therefore, our results extend previous findings indicating that traditional plating assays do not provide complete information regarding bacterial viability when evaluating sublethal stressors such as HPP. However, the functionality and probiotic potential of the VBNC cells, presumably induced by this technology in the fermented beverage, cannot be known based on the present study.

Conclusions

This study shows the usefulness of HPP to preserve the quality of a fermented beverage manufactured from sweet whey until 45 days post-HPP, highlighting the possible use of this technology to extend the shelf-life of fermented food products. Furthermore, treatments at 200 MPa for 10 min maintained optimal amounts of total SLAB (6.6–7.9 log CFU/mL) in the fermented beverage. Thus, a contaminating by-product was transformed into a food product with added value and known potential health benefits.

In addition, the present study describes the effect of HPP on the behavior of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* as evaluated by culture-dependent and quantitative molecular methods, demonstrating marked differences between both approaches. Therefore, although our results do not directly demonstrate the existence of sublethaly injured cells, they provide further evidence for the "VBNC hypothesis" in a dairy beverage fermented with these SLAB.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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