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Abstract: Objectives: The objectives of this work were to enumerate and characterize the pathogenic potential of the Bacillus population that may survive Holder pasteurization of human milk and to evaluate the nutritional damage of this treatment using the furosine and lactulose indexes.
Materials and Methods: Milk samples from 21 donors were heated at 62.5°C for 30 minutes. Bacterial counts, lactose, glucose, myo-inositol, lactulose and furosine were determined before and after the heat treatment. Some Bacillus cereus isolates that survived after pasteurization were evaluated for toxigenic potential.
Results: Non-pasteurized milk samples showed bacterial growth in most of the agar media tested. Bacterial survival after pasteurization was only observed in three samples and, in these cases, the microorganisms isolated belonged to the species B. cereus. Furosine could not be detected in any of the samples while changes in lactose, glucose, and myo-inositol concentrations after Holder pasteurization were not relevant. Lactulose was below the detection limit of the analytical method in non pasteurized samples while it was found at low levels in 62% of the samples after Holder pasteurization. The lactation period influenced myo-inositol content since its concentration in transition milk was significantly higher than in mature or late lactation milk samples.
Conclusions: Holder pasteurization led to the destruction of bacteria present initially in donor milk samples, except some B. cereus that did not display a high virulence potential, and did not modify significantly the concentration of the compounds analyzed in this work.

Heating-induced bacteriological and biochemical modifications in human donor milk after Holder pasteurization

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ABSTRACT

Objectives: The objectives of this work were to enumerate and characterize the pathogenic potential of the *Bacillus* population that may survive Holder pasteurization of human milk and to evaluate the nutritional damage of this treatment using the furosine and lactulose indexes.

Materials and Methods: Milk samples from 21 donors were heated at 62.5°C for 30 minutes. Bacterial counts, lactose, glucose, *myo*-inositol, lactulose and furosine were determined before and after the heat treatment. Some *Bacillus cereus* isolates that survived after pasteurization were evaluated for toxigenic potential.

Results: Non-pasteurized milk samples showed bacterial growth in most of the agar media tested. Bacterial survival after pasteurization was only observed in three samples and, in these cases, the microorganisms isolated belonged to the species *B. cereus*. Furosine could not be detected in any of the samples while changes in lactose, glucose, and *myo*-inositol concentrations after Holder pasteurization were not relevant. Lactulose was below the detection limit of the analytical method in non pasteurized samples while it was found at low levels in 62% of the samples after Holder pasteurization. The lactation period influenced *myo*-inositol content since its concentration in transition milk was significantly higher than in mature or late lactation milk samples.

Conclusions: Holder pasteurization led to the destruction of bacteria present initially in donor milk samples, except some *B. cereus* that did not display a high virulence potential, and did not modify significantly the concentration of the compounds analyzed in this work.

Key Words: human milk, milk bank, pasteurization, *Bacillus cereus*, furosine, lactulose, *myo*-inositol

INTRODUCTION

Human milk is widely recognized as the optimal feeding option for human term and preterm infants because of the wide spectrum of short-, medium- and long-term potential benefits that it provides (1). Unfortunately, there are cases where mother's own milk is not available or enough to cover the requirements of the newborn. Therefore, there is a worldwide increasing demand for donor breast milk, particularly for preterm infants and older infants suffering from diverse medical problems (2). In such situations, clinicians value the importance of banked human milk, not only as a nutritional option, but also as a potentially life-saving therapy.

Up to date, there are not worldwide uniform guidelines for the screening, processing, storage and handling of donor milk among Milk Banks and, in fact, protocols may vary even in banks operating in the same country. However, the potential mother-to-child transmission of certain viruses, such as human immunodeficiency virus (HIV), human T-lymphoma virus (HTLV) or cytomegalovirus, through breastfeeding, together with the difficulties in an exhaustive surveillance of donors' health (including repetitive serum screening), has led to the systematic pasteurization of donor milk in the vastly majority of Human Milk Banks. Human pasteurized milk is considered as the best alternative to non-heated frozen or fresh milk, and has been shown to reduce the incidence of necrotizing enterocolitis, sepsis, and other infections in premature and high risk infants, resulting in shorter hospital stays (3,4,5).

Although some nutrients and bioactive compounds present in fresh human milk remain active after such heat treatment, the biological activity of others compounds is affected at a variable degree (6,7,8,9,10). As a consequence, questions arise concerning the effects of heat processing on some of the unique components of human milk.

Because of its content in lactose and proteins, heating of human milk can induce chemical changes of important nutrients, leading to adverse nutritional effects (11). The damage extent produced by heating can be measured through the use of chemical indexes, such as the furosine (2-furoylmethyl-lysine) and lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) levels. Furosine is used as an indirect measurement of Amadori compounds formed in the early stages of Maillard reaction between proteins (ε-amino group of protein-bound lysine) and sugar components (carbonyl group of reducing sugar as lactose) during processing (12). Lactulose is a synthetic sugar, which does not occur naturally and it is produced from lactose by isomerization in basic media. This disaccharide is absent in raw milk but the dissolved salt system of milk is a buffered solvent favourable to the formation of lactulose from lactose during heat treatment of milk (13). Both, furosine and lactulose are useful markers for evaluating the extent of heat damage in milk and infant formulas (14,15).

In addition, breast milk is a source of commensal and potentially probiotic bacteria (16,17), which seems to play an important role in gut colonization of the healthy infant (17,18). Such bacteria are killed by the pasteurization process. It is important to note that spore-forming bacteria that may survive the heating process, such as *Bacillus cereus*, or microorganisms that could contaminate milk after pasteurization can grow faster than in raw milk because of the heat damage to the milk bacteriostatic systems, including the absence of natural competitors (6).

In this context, the objectives of this work were, on one hand, to enumerate and characterize the pathogenic potential of the *Bacillus* population that may survive Holder pasteurization and, on the other hand, to evaluate the potential nutritional damage of this thermal treatment using the furosine and lactulose indexes.

MATERIAL AND METHODS

Breast milk samples

Breast milk samples (8 mL) were obtained from the Human Milk Bank located at the Hospital Universitario 12 de Octubre (Madrid, Spain). Milk collection was performed following a specific protocol for donor mothers approved by the local ethical committee. The samples were obtained from 21 donors that fulfilled the requirements of the Bank and informed consent was obtained from each donor. Milk was collected at home using electric (Lactaline; Ameda, Lincolnshire, USA) or manual (Harmony or Lactaset models; Medela, Baar, Switzerland) pumps. An aliquot from each milk sample was separated before pasteurization while the rest of the sample was pasteurized by heating it at 62.5°C for 30 minutes; then, it was cooled in a shaking water bath (Lab Companion, Seoul, Korea) filled with ice-cold water and provided of temperature control. Once the temperature reached 4ºC (always within the first 15 minutes of cooling), it was stored at -20ºC until its analysis. A thermometer, coupled to an external sensor of temperature (DT 132, Fourier, Fairfield, USA), was introduced in a control bottle (cow's milk), and used as a probe to monitor the temperature of the milk batch during the whole heating/cooling process.

Bacterial cultures and identification of isolates

Proper peptone water dilutions of 21 pairs of milk samples (21 before and 21 after pasteurization) were plated onto Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK; a general-purpose medium suitable for the cultivation of non-fastidious bacteria, yeasts and moulds), Columbia Nadilixic Acid Agar (CNA, BioMerieux; a highly nutritious, general-purpose medium for the isolation and cultivation of fastidious

125 microorganisms), Baird Parker (BP, BioMerieux; a selective medium for the isolation of
126 staphylococci), MacConkey (MCK, BioMerieux; a selective medium for the isolation of
127 enterobacteria), Polymyxin-Pyruvate-Egg Yolk-Mannitol with Bromothymol Blue
128 (PEMBA, Oxoid; a selective medium for the isolation of *Bacillus*), and de Man,
129 Rogosa, and Sharpe (MRS, Oxoid; a medium for the isolation of lactic acid bacteria)
130 agar plates, which were aerobically incubated at 37°C for 24-48 hours. Parallel, the
131 samples were also cultured on Wilkins Chalgren (WCh, Oxoid; a general medium for
132 isolating anaerobic bacteria), which were incubated anaerobically (85% nitrogen, 10%
133 hydrogen, 5% carbon dioxide) in an anaerobic workstation (MINI-MACS, DW
134 Scientific, Shipley, UK) at 37ºC for 48 hours. Colonies, from the plates where bacterial
135 growth was detected, were isolated and stored at -20°C in the presence of glycerol
136 (20%, v/v).

137 Identification of the isolates was performed by PCR sequencing of a 470 pb
138 fragment of the 16S rRNA gene as described previously (19). The amplicons were
139 purified using the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and
140 sequenced at the Genomics Unit of the Complutense University of Madrid, Spain. The
141 resulting sequences were used to search sequences deposited in the EMBL database
142 using BLAST algorithm and the identity of the isolates was determined on the basis of
143 the highest scores (>98%).

144 Since the genomes of the *B. cereus* group of species, including *B. cereus* and *B.*
145 *anthracis*, are closely related both in gene content and synteny (20) and their 16S rRNA
146 gene sequences share greater than 99% similarity (21), those isolates identified as *B.*
147 *cereus* were submitted to a repetitive element polymorphism PCR (rep-PCR) assay to
148 assure that they did not belong to the *B. anthracis* species. For this purpose, the BOX-

149 A1R primer, which originates a 390 bp fragment if *B. anthracis* DNA is present in the
150 sample, was used as described previously (22).
151
152 **Genetic profiling of the *B. cereus* isolates and evaluation of their toxigenic potential**
153 A collection of 49 *B. cereus* isolates, including those obtained in this work and
154 those obtained from other samples of pasteurized donor milk that were previously
155 rejected by the same Milk Bank because of the presence of this bacterial species, were
156 typed by random amplification polymorphic DNA (RAPD), using primer OPL5 (5'-
157 ACG CAG GCA C-3') as described previously (23).
158 Then, presence of genes involved in the biosynthesis of the main *B. cereus*
159 toxins was evaluated. In relation to the toxins responsible for food poisoning by *B.*
160 *cereus*, cereulide is associated to the emetic symptoms and is encoded by cereulide
161 synthetase (*ces*) gene cluster while three pore-forming toxins appear to be responsible
162 for the diarrhoeal symptoms: hemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe),
163 and cytotoxin K (CytK-1 or CytK-2) (24). Hbl consists of the three proteins L_2, L_1 and
164 B, encoded by the genes *hblC*, *hblD* and *hblA*, respectively; Nhe is composed of the
165 proteins NheA, NET and NheC, encoded by the *nheABC* operon; finally, CytK-1 or -2
166 are single-component toxins. In order to detect the presence of toxin gene determinants,
167 total genomic DNA from each *B. cereus* strain was extracted by disrupting colonies in
168 deionized water and in a (chloroform:isoamylic alcohol):water (1:1, v:v) solution. Then,
169 three multiplex PCR assays were used for the detection of the *hblCDA* and *nheABC*
170 operons and the *ces* gene cluster using primer sets and PCR conditions previously
171 described (25). Parallel, a duplex PCR assay was performed to detect genes encoding
172 CytK-1 and CytK-2 (26). Presence of toxins Hbl and Nhe in culture supernatants of
173 same *B. cereus* isolates was also analyzed with the Gold Labelled ImmunoSorbent

174 Assay (GLISA)-Rapid Test for the qualitative detection of *B. cereus* enterotoxins
175 (Merck, Darmstadt, Germany) following the instructions of the manufacturer.
176
177 **Determination of furosine**
178 Determination of furosine in the 21 pairs of milks samples was performed by
179 ion-pair RP-HPLC following the method of Resmini et al. (27). Before analysis, milk
180 samples (2 mL) were hydrolyzed with 6 mL of 10.6 N HCl under inert conditions at
181 110ºC for 24 h in a Pyrex screw-cap vial with PTFE-faced septa. The hydrolysate was
182 filtered through Whatman Nº 40 filter paper, and, 0.5 mL of filtrate were applied to a
183 previously activated Sep-Pak C_{18} cartridge (Millipore). Furosine was eluted with 3 mL
184 of 3N HCl and 20 μL was injected into the chromatograph.
185 RP-HPLC analysis of furosine was carried out in a C_8 column (250 mm × 4.6
186 mm, 5 μm) (Alltech furosine-dedicated) maintained at 35°C using a linear binary
187 gradient at a flow rate of 1.2 mL/min. Mobile phase was constituted by solvent A, 0.4%
188 acetic acid, and solvent B, 0.3% KCl in phase A. Detection was performed using a
189 variable wavelength UV detector at 280 nm (LDC Analytical, SM 4000). Acquisition
190 and processing of data were achieved with a HPChem Station (Hewlett-Packard).
191 Calibration was performed by external standard method using commercial standard of
192 pure furosine (Neosystem Laboratories, Strasbourg, France). The detection limit (LOD)
193 of RP-HPLC method was 1.16 mg/100 mg of protein.
194 The determination of protein concentration was done following the Bradford
195 procedure (BioRad) using albumin as external standard.
196
197 **GC Analysis of Carbohydrates**

198 Lactose, glucose, lactulose and *myo*-inositol were also determined, by GC, in the
199 21 pairs of milk samples, following the method of Montilla et al. (28). For this purpose,
200 0.2 mL of sample was made up to 2 mL with methanol in a volumetric flask to remove
201 proteins and fat. Mixtures were vigorously stirred, followed by standing for at least 1 h.
202 The supernatant was used for carbohydrate analysis and a solution of 0.1% (w:v)
203 phenyl-β-D-glucoside in methanol/water (70:30, v/v) was added as internal standard.
204 Before derivatization, equal volumes (0.5 mL) of supernatant and internal
205 standard solution were mixed and dried at 38–40ºC in a rotary evaporator. The dried
206 mixtures were treated with 100 µL N,N-dimethylformamide and held at 70ºC for 1 h to
207 obtain a constant anomeric composition. Then, 100 µL of N-trimethylsilylimidazole
208 were added to silylate the carbohydrates and the reaction was completed in 30 min at
209 70ºC. Silylated carbohydrates were extracted with 0.1 mL of hexane and 0.2 mL of
210 water. Volumes in the range of 0.2–1 µl of the organic phase containing silyl-
211 derivatives were injected into the column.
212 The trimethylsilyl ethers of carbohydrates were analyzed in an Agilent
213 Technologies 7890A gas chromatograph equipped with a commercial 30 m × 0.32 mm
214 inside diameter, 0.5 µm film fused silica capillary column SPBTM–17, bonded,
215 crosslinked phase (50% diphenyl/50% dimethylsiloxane) (Supelco, 595 North Harrison
216 Road, Bellefonte, PA, USA). Separation was performed at 235ºC for 9 min, followed by
217 an increase up to 270ºC at rate of 15ºC/min and keeping this temperature for 15 min.
218 Temperatures of injector and flame ionisation detector were 300ºC during the analysis.
219 Injections were carried out in split mode 1:30, using 1 mL/min of nitrogen as carrier
220 gas. Data acquisition and integration were performed using Agilent Chem-Station Rev.
221 B.03.01 software (Wilmington, DE). To study the response factor relative to the internal
222 standard, solutions containing lactose, lactulose, glucose and *myo*-inositol were

prepared over the expected concentration range in milk samples. The identity of carbohydrates present in milk samples were confirmed by comparison with relative retention times of standard samples.

Statistical Analysis

Microbiological data, recorded as colony forming units (CFU) per mL of milk, were transformed to logarithmic values before statistical analysis. Quantitative biochemical data were expressed as mean ± standard deviation (SD) and 95% confidence interval (CI) of the mean. Values were tested for normality of distribution. Correlations between lactose, glucose, and *myo*-inositol concentration and lactation time were determined by the Spearman method. The effect of Holder pasteurization on sugar concentrations was evaluated with paired Student's *t*-tests. The influence of the lactation period (transition milk, mature milk, and late lactation milk) in lactose, glucose, and *myo*-inositol concentration was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests. Statistical tests were considered significant at $P < 0.05$. The SAS system (Statistical Analysis Systems Institute Inc., Cary, NC, USA) was used to perform these analyses.

RESULTS

Bacterial counts in the milk samples and identification of the isolates

To evaluate the effect of Holder pasteurization on the viability of the milk bacteria, 21 samples of donor milk were cultured before and after heating at 62.5°C for 30 minutes. In all cases, inoculation of non-pasteurized milk samples in BHI, CNA, BP,

MRS, WCh and PEMBA agar plates led to bacterial growth (Table 1). In contrast, bacteria only could be isolated from 13 (62%) of the same samples when inoculated on MCK plates (Table 1). Globally, the bacterial counts in non-pasteurized samples oscillated between 2.60 and 5.22 $\log_{10}$ CFU/mL in BHI medium, with a mean (SD; 95% CI) value of 3.93 (0.85, 3.54 – 4.31) $\log_{10}$ CFU/mL. The lowest mean bacterial counts of donor milk samples were found in MRS medium, and were 0.88 $\log_{10}$ CFU/mL lower than in BHI agar plates (Table 1). In the samples in which growth was observed on MCK agar plates (n=13), the counts oscillated between 1.70 and 4.92 $\log_{10}$ CFU/mL.

Most of the bacteria isolated from the raw milk samples, both qualitatively and quantitatively, belonged to the genera *Staphylococcus* (BHI, CNA, MRS, WCh agar plates), *Streptococcus* (CNA, MRS, WCh agar plates), and *Bacillus* (PEMBA), or to the *Lactobacillus* group (MRS). When growth was observed on MCK plates, all the isolates belonged to the coliform group and, most of them, to the species *Escherichia coli* or to the genus *Enterobacter*.

Pasteurization had a radical effect on the bacterial population of the samples (Table 1); in fact, bacterial growth could not be detected from most pasteurized samples after culturing onto BHI, MCK, CNA, BP, MRS or WCh agar plates. Bacterial survival after pasteurization was observed in one sample when cultured on BHI (1.7 $\log_{10}$ CFU/mL) and PEMBA (3.44 $\log_{10}$ CFU/mL) agar plates, and in two additional samples when cultured on PEMBA agar plates (both at a concentration of ~2.0 $\log_{10}$ CFU/mL) (Table 1). In all these cases, the microorganisms isolated belonged to the species *B. cereus*. A very low number of colonies (n=1–3; dilution 0) of staphylococci or propionibacteria were observed in two samples but their presence seemed to be due to post-processing contamination since these isolates did not survive Holder pasteurization

when they were inoculated in sterile milk at an initial concentration of ~4.0 $\log_{10}$ CFU/mL (results not shown).

Genetic profiling of the *B. cereus* isolates and evaluation of their toxigenic potential

RAPD profiling of the *B. cereus* isolates showed the presence of six different band patterns. Interestingly, each RAPD profile was coincident with one of the six toxin gene profiles observed among the 49 isolates (Table 2).

None of the isolates harboured neither the *ces* gene, associated to the biosynthesis of the emetic toxin, nor *cytK1* while all carried those required for Nhe production. The *cytK2* gene was present in approximately 50% of the isolates. Finally, only one strain (~2%) harboured the complete *hblCDA* operon. The results obtained with the GLISA immunoassays (detection of toxins in culture) were in agreement with the presence of the genes as determined by multiplex PCR. Nhe toxin could be detected in cultures of the *nheABC*-positive strains while Hbl toxin could only be detected in the strain that harboured the complete *hblCDA* operon (Table 2).

Effect of pasteurization and lactation period on the concentrations of furosine and carbohydrates

In this study, no peak of furosine was detected in any of the samples, neither before nor after the pasteurization process; therefore, holder pasteurization did not favour Maillard reaction.

Lactose, glucose, *myo*-inositol, and lactulose concentrations in donor milk samples are presented in Table 3. Mean (SD; 95% CI) concentration of lactose in non-pasteurized milk samples was 64.08 (6.14; 61.28 – 66.88) g/L. Glucose and *myo*-inositol were found in all samples at approximately three orders of magnitude lower

297 concentration than lactose and showed considerable sample-to-sample variation, i.e.
298 206.45 (78.79; 170.59 – 242.32) mg/L for glucose and 196.45 (104.40; 148.93 –
299 243.97) mg/L for *myo*-inositol. However, glucose and *myo*-inositol concentrations were
300 not related in each individual milk sample; in fact the ratio [glucose]/[*myo*-inositol]
301 varied from 0.33 to 3.05, and only 5 samples showed similar amounts of both
302 compounds (glucose/*myo*-inositol = 0.91 – 1.17). There was no correlation between
303 lactose, glucose, and *myo*-inositol concentrations in milk samples (data not shown).

304 Changes in lactose, glucose, and *myo*-inositol concentrations in milk samples
305 after Holder pasteurization were not relevant, although mean lactose concentration
306 (expressed as mean ± SD) increased by 1.42 ± 2.89 g/L (paired Student's *t*-test, *P* =
307 0.036) and mean glucose and *myo-ino*sitol concentrations decreased by 6.82 ± 14.75
308 mg/L and 1.61 ± 19.95 mg/L, respectively (paired Student's *t*-test, *P* = 0.047 and *P* =
309 0.716, respectively). Lactulose was below the detection limit of the analytical method
310 (10 mg/L) in non pasteurized milk samples, and it was found in 62% of the samples
311 after Holder pasteurization with a mean concentration of 18.96 ± 6.14 mg/L (Table 3).

312 Since milk samples were donated by women at different lactation periods,
313 between 6 days and 1.9 years, Spearman's correlation coefficients were calculated in
314 order to compare the relationship between the concentration of lactose, glucose and
315 *myo*-inositol in human milk samples and the lactation time (in days). Lactose and
316 glucose concentration had poor correlation with the length of lactation (Spearman *r* = -
317 0.302, *P* = 0.1836 for lactose and *r* = 0.155, *P* = 0.503 for glucose) but there was a
318 strong negative and significant correlation between *myo*-inositol concentration and the
319 lactation time (Spearman *r* = -0.752, *P* = <0.0001).

320 Lactose, glucose and *myo*-inositol concentration in human milk samples as a
321 function of three different lactation periods is shown in Figure 1. Lactose and glucose

concentrations were slightly higher in mature milk (15-180 days) samples than in transition milk (< 15 days) and in late lactation milk (180-250 days of lactation), but these differences were not statistically significant. In contrast, the lactation period had a significant effect on *myo*-inositol concentration (one-way ANOVA, *F*-value = 11.65, *P*-value = 0.0006). The concentration of *myo*-inositol in transition milk (341.35 ± 95.22 mg/L, n = 4) was significantly higher than in mature (198.04 ± 80.14 mg/L, n = 8, $P < 0.05$) and late lactation (130.64 ± 53.44 mg/L, n = 9, $P < 0.001$) milk samples (Figure 1).

DISCUSSION

In this study, bacteria could be isolated from non-pasteurized human milk in different culture media. This finding is not strange since fresh human milk contains a number (<3 $\log_{10}$ CFU/mL) of viable bacteria and a wide range of free bacterial DNA signatures which may program the neonatal immune system (29). In fact, breast milk has been shown to be a continuous source of commensal and potentially probiotic bacteria to the infant gut, including staphylococci, streptococci, bifidobacteria and lactic acid bacteria (16,17,30).

The fact that, in this study, donors extracted the milk using pumps may explain why many samples had counts higher than 3 $\log_{10}$ CFU/mL and why growth was observed in 62% of samples when cultured on MCK agar plates. It has been shown that the use of milk pumps to collect the samples is associated to a higher level of bacteria, and particularly enterobacteria, which are not related to the usual breast milk microbiota (31). Contamination of milk during pumping has been reported previously and seems to be of particular concern for premature infants or ill infants in neonatal intensive care units (32,33). Many milk pumps and/or their accessories can not be properly sanitized

and/or sterilized and bacteria usually persist after application of current cleaning protocols. Therefore, the design of new pumping devices that can be sterilized and subjected to more efficient cleaning and disinfection procedures is highly desirable.

Holder pasteurization of the milk samples led to the destruction of the bacteria present in the initial fresh samples with the exception of three samples in which *B. cereus* could be isolated. Similarly, a recent study revealed that 93% of milk samples submitted to Holder pasteurization showed no bacterial growth on cultures and that *Bacillus* sp. was the predominant contaminant in those that were positive after pasteurization (34). *B. cereus* is described as being of ubiquitous presence in nature; in addition to a full life cycle in soil, where it is richly present, it is also adapted to human hosts, either as a pathogen or, more frequently, as a part of the intestinal microbiota of a healthy host (24). Additionally, it has been found in breast milk of healthy rhesus monkeys (35) and in the udder of cows (36). The possible adaptation of *B. cereus* to the environment of the animal gut could be the basis of their proposed probiotic effect. In fact, certain strains producing negligible amounts of toxin at 37ºC have been approved for probiotic use by the European Food Safety Authority (EFSA) (24). However, as the level of virulence is highly variable among different strains, caution is strongly required when dealing with this species.

In contrast to vegetative cells, spores of *B. cereus* can survive different heat treatments, including Holder pasteurization. As a consequence, this species is a common inhabitant of milk (36,37), and it can cause a defect known as sweet curdling in dairy products. Considering the non fastidious nature of this microorganism, no type of food with pH < 4.8 can be excluded as a risk of food spoilage or foodborne disease (38). Failure to follow basic food preparation rules, such as slow or inadequate cooling, storage at ambient temperature or prolonged heat-keeping at approximately 60ºC, may

allow growth of *B. cereus*. Therefore, these hygienic rules are critical in a Milk Bank providing milk to preterm neonates. It should be had in account that a negative result for *Bacillus* in a post-pasteurization culture does not mean that this microorganism is absent; it only means that this species is under the detection limit of the technique (for example, 100 CFU/mL if 10 μl of milk were cultured).

Two distinct foodborne disease types, emetic and diarrhoeal, are associated with *B. cereus*. For the both types, 3-8 $\log_{10}$ CFU cells or spores have been indicated as the infective dose (24,38). The count of *B. cereus* in a confirmed foodborne outbreak in Norway was as low as 2 $\log_{10}$ CFU/g of food (39) although further research showed that the actual number was closer to 4 $\log_{10}$ CFU/g, and that the underestimation was due because the bacilli were being present as aggregated spores (24). While the role of cereulide in causing the emetic syndrome of *B. cereus* is well established, that of the cytotoxins as etiological agents of diarrhoeal disease is not so clear. Strong evidences indicate that Hbl, Nhe and CytK cytotoxins are virulence factors usually involved in *B. cereus* foodborne diarrhoeal disease but there are difficulties in establishing a single factor as the etiological agent of gastroenteritis due to this species; this fact reflects that, most probable, the disease is multifactorial and that a number of additional virulence factors may contribute to the overall cellular damage, possibly in a strain-dependent manner. In this study, no strain harboured genes responsible for the biosynthesis of the emetic toxin while all carried those required for Nhe production. However, genes encoding Nhe are now thought to be present in all known *B. cereus* group strains (24). In relation to *cytK* genes, *cytK1* could not be detected in any of the 49 strains but *cytK2* was present in approximately 50% of them. Finally, only one strain (2%) harboured the complete *hblCDA* operon. Hbl and CytK-related genes are present in less than 50% of randomly sampled strains (40,41,42). Hbl is a three-component toxin complex and all

three components are necessary for maximal biological activity (43,44). In conclusion, *B. cereus* strains isolated from pasteurized milk in this study do not seem to possess a high virulence potential.

Holder pasteurization did not significantly modify the concentration of any of the biochemical parameters analyzed in this work. Furosine and lactulose values are used to determine the effects of thermal treatment applied to milk or the addition of reconstituted milk powder to raw, pasteurized or UHT milk. In this work, furosine could not be detected in any of the samples, in contrast with the levels found in Holder and high-temperature (72ºC, 15 s) pasteurized milks, 6.9 - 10.0 and 6.7 - 20.3 mg/100g protein, respectively (45). This could be due to the low protein concentration present in human milk compared to cow' milk. Furosine determination has gained broad attention by food chemists and biomedical researchers, as its formation upon heat treatment is well characterised. Moreover, it represents the Amadori products from early Maillard reactions in which amino acids react with reducing carbohydrates, resulting in a loss of their bioavailability. This is of importance for the essential amino acid lysine, which is also the limiting amino acid in many proteins.

In the pasteurized samples where lactulose was detected, its content was higher than that found in Spanish pasteurized milks (45), a fact that may be attributed to the high content of lactose present in human milk. The lactulose concentrations were well below the limits considered acceptable for infant formulas. A correlation between lactulose and furosine exists (46,47), since both parameters are influenced by the intensity of the heating process and also by the storage conditions (48). However, the concentrations found here do not seem enough to negatively affect protein quality.

No differences were found in the concentrations of lactose and glucose when the fresh samples of donor milk were divided in three groups on the basis of the duration of

lactation (transition milk, mature milk, late lactation milk). Interestingly, there was a statistically significant decrease of the concentration of *myo*-inositol in the samples as the lactation period increased. This finding is relevant since administration of *myo*-inositol to premature infants with respiratory distress syndrome who are receiving parenteral nutrition is associated with increased survival without bronchopulmonary dysplasia or neural developmental handicap and with a decreased incidence of retinopathy (49). Serum *myo*-inositol concentration increases after birth in premature breastfed infants, while it tends to fall in those receiving parenteral nutrition (49). This reflects the fact that concentrations of *myo*-inositol are significantly higher in human milk than in infant formulas or parenteral nutrition solutions (50). Although the observation of a higher myo-inositol concentration in early milk is interesting, the comparison of the three lactation periods has limited value due to the reduced number of samples within each group. Therefore, more work is required in order to establish firm conclusions regarding its influence on preterm health.

Globally, the results of this study showed that Holder pasteurization led to the destruction of bacteria present initially in donor milk samples, with the exception of some *B. cereus* strains that did not display a high virulence potential; in addition, the thermal treatment did not modify significantly the concentration of furosine and lactulose, two compounds that are used as markers for evaluating the extent of heat damage in cow's milk and infant formulas.

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572 **Legend to figures**

573

574 **FIGURE 1.** Lactose, glucose, and *myo*-inositol concentration in transition (TM, <15
575 days, n = 4), mature (MM, 15-180 days, n = 8) and late lactation (LLM, >180 days, n =
576 9) raw donor milk. The results of a one-way ANOVA and Bonferroni's post hoc tests
577 are shown by asterisks.
578 * = $P < 0.05$; *** = $P < 0.001$.

579

580

Figure

FIGURE 1

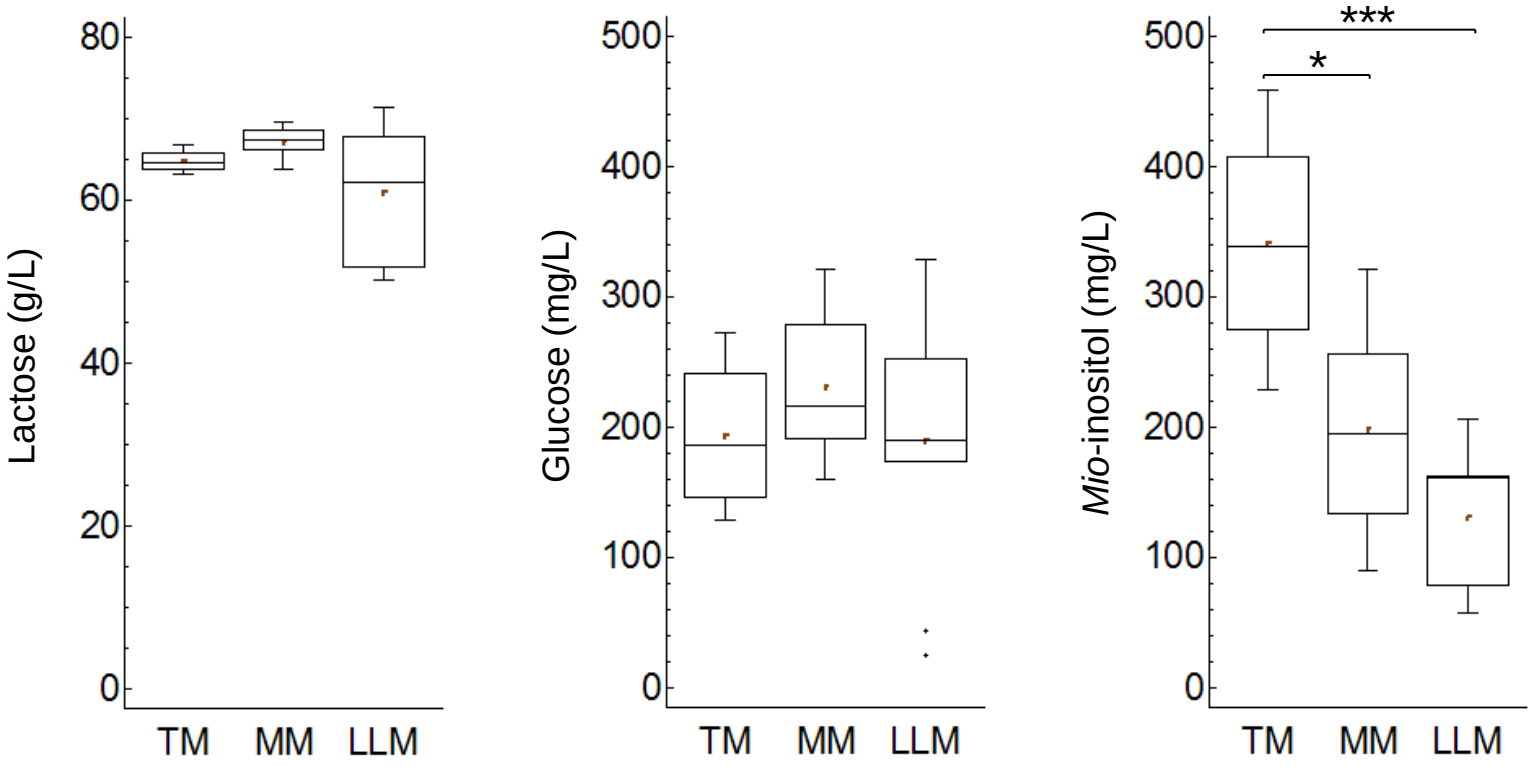

TABLE 1. Bacterial counts in donor milk samples before and after Holder pasteurization

Medium	Heat treatment	Number of samples positive/total	Mean	SD	95% CI	Range	
						Minimum value	Maximum value
BHI	NP	21/21	3.93	0.85	3.54 - 4.31	2.60	5.22
	P	1/21	1.70*				
CNA	NP	21/21	3.65	0.82	3.27 - 4.02	1.70	5.11
	P	0/21	nd				
MCK	NP	13/21	3.34	1.17	2.63 - 4.05	1.70	4.92
	P	0/21	nd				
MRS	NP	21/21	3.05	0.90	2.65 - 3.46	1.70	4.97
	P	0/21	nd				
BP	NP	21/21	3.28	0.92	2.86 -3.70	1.70	5.18
	P	0/21	nd				
WCh	NP	21/21	3.67	0.76	3.32 - 4.01	2.48	5.21
	P	0/21	nd				
PEMBA	NP	21/21	3.29	0.64	3.00 - 3.58	1.70	4.46
	P	3/21	2.48†			2.00	3.44

Values of mean, SD, 95% CI and range are expressed as $\log_{10}$ CFU/mL; BHI = Brain Heart Infusion; BP = Baird Parker; CI = confidence interval of the mean; CNA = Columbia Nadilixic Acid Agar; MCK = MacConkey; MRS = de Man, Rogosa, and Sharpe; nd = not detected; NP = non pasteurized samples; P = pasteurized samples; PEMBA = Polymyxin-Pyruvate-Egg Yolk-Mannitol with Bromothymol Blue; SD = standard deviation of the mean; WCh, Wilkins Chalgren.

* Mean value of the samples where growth was detected.

TABLE 2. Toxin gene profiles and toxin production by the *B. cereus* strains isolated from pasteurized samples

		Toxin-encoding genes								Toxin production	
RAPD Pattern	Number of strains	*hblC*	*hblD*	*hblA*	*nheA*	*nheB*	*nheC*	*cytK1*	*cytK2*	Hbl	Nhe
I	11	-	-	-	+	+	+	-	-	-	+
II	6	-	-	-	+	+	+	-	+	-	+
III	13	-	+	-	+	+	+	-	-	-	+
IV	17	-	+	-	+	+	+	-	+	-	+
V	1	+	+	+	+	+	+	-	+	+	+
VI	1	-	-	-	+	+	-	-	-	-	+

RAPD = random amplification polymorphic DNA.

TABLE 3. Effect of Holder pasteurization on the concentration of carbohydrates in donor milk samples analyzed

					Range		
	n	Mean	SD	95% CI	Minimum	Maximum	*P*[*]
Lactose (g/L)							
NP	21	64.08	6.14	61.28 - 66.88	50.10	71.40	
P	21	65.50	7.20	62.22 - 68.78	51.14	75.96	
Change	21	1.42	2.89	0.10 - 2.74	-3.95	6.96	0.036
Glucose (mg/L)							
NP	21	206.45	78.79	170.59 - 242.32	25.50	327.90	
P	21	199.63	76.78	164.68 - 234.58	24.90	305.11	
Change	21	-6.82	14.75	-13.53 - 0.11	-34.44	19.90	0.047
Myo-inositol (mg/L)							
NP	21	196.45	104.40	148.93 - 243.97	58.30	459.00	
P	21	194.84	104.48	147.29 - 242.40	54.36	431.34	
Change	21	-1.61	19.95	-10.69 - 7.47	-30.53	53.33	0.716
Lactulose (mg/L)							
NP	21	nd[†]					
P	8	nd					
	13	18.96	6.14	15.25 - 22.67	9.98	33.98	

CI = confidence interval of the mean; NP = non pasteurized samples; P = pasteurized samples; SD = standard deviation of the mean.

[*] Paired Student's *t*-test.

† Below the detection limit of the method (10 mg/L).