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Title: 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.

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

3

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25

26 **ABSTRACT**

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

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

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

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

48

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

51 **INTRODUCTION**

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

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

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

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

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

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

99

## 100 MATERIAL AND METHODS

101

### 102 Breast milk samples

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

118

### 119 Bacterial cultures and identification of isolates

120 Proper peptone water dilutions of 21 pairs of milk samples (21 before and 21  
121 after pasteurization) were plated onto Brain Heart Infusion (BHI, Oxoid, Basingstoke,  
122 UK; a general-purpose medium suitable for the cultivation of non-fastidious bacteria,  
123 yeasts and moulds), Columbia Nalidixic Acid Agar (CNA, BioMerieux; a highly  
124 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 37°C 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<sub>2</sub>, L<sub>1</sub> 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:isoamyl 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  
181 110°C for 24 h in a Pyrex screw-cap vial with PTFE-faced septa. The hydrolysate was  
182 filtered through Whatman N° 40 filter paper, and, 0.5 mL of filtrate were applied to a  
183 previously activated Sep-Pak C<sub>18</sub> 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<sub>8</sub> 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- $\beta$ -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 38–40°C in a rotary evaporator. The dried  
206 mixtures were treated with 100  $\mu$ L N,N-dimethylformamide and held at 70°C for 1 h to  
207 obtain a constant anomeric composition. Then, 100  $\mu$ L of N-trimethylsilylimidazole  
208 were added to silylate the carbohydrates and the reaction was completed in 30 min at  
209 70°C. 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  $\mu$ l 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  $\times$  0.32 mm  
214 inside diameter, 0.5  $\mu$ m 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 235°C for 9 min, followed by  
217 an increase up to 270°C at rate of 15°C/min and keeping this temperature for 15 min.  
218 Temperatures of injector and flame ionisation detector were 300°C 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

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

226

## 227 **Statistical Analysis**

228 Microbiological data, recorded as colony forming units (CFU) per mL of milk,  
229 were transformed to logarithmic values before statistical analysis. Quantitative  
230 biochemical data were expressed as mean  $\pm$  standard deviation (SD) and 95%  
231 confidence interval (CI) of the mean. Values were tested for normality of distribution.  
232 Correlations between lactose, glucose, and *myo*-inositol concentration and lactation time  
233 were determined by the Spearman method. The effect of Holder pasteurization on sugar  
234 concentrations was evaluated with paired Student's *t*-tests. The influence of the lactation  
235 period (transition milk, mature milk, and late lactation milk) in lactose, glucose, and  
236 *myo*-inositol concentration was analyzed by one-way ANOVA followed by  
237 Bonferroni's multiple comparison tests. Statistical tests were considered significant at *P*  
238  $< 0.05$ . The SAS system (Statistical Analysis Systems Institute Inc., Cary, NC, USA)  
239 was used to perform these analyses.

240

241

## 242 **RESULTS**

243

### 244 **Bacterial counts in the milk samples and identification of the isolates**

245 To evaluate the effect of Holder pasteurization on the viability of the milk  
246 bacteria, 21 samples of donor milk were cultured before and after heating at 62.5°C for  
247 30 minutes. In all cases, inoculation of non-pasteurized milk samples in BHI, CNA, BP,

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

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

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

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

274

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

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

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

287

## 288 **Effect of pasteurization and lactation period on the concentrations of furosine and** 289 **carbohydrates**

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

293 Lactose, glucose, *myo*-inositol, and lactulose concentrations in donor milk  
294 samples are presented in Table 3. Mean (SD; 95% CI) concentration of lactose in non-  
295 pasteurized milk samples was 64.08 (6.14; 61.28 – 66.88) g/L. Glucose and *myo*-  
296 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  $\pm$  SD) increased by  $1.42 \pm 2.89$  g/L (paired Student's *t*-test,  $P =$   
307  $0.036$ ) and mean glucose and *myo*-inositol concentrations decreased by  $6.82 \pm 14.75$   
308 mg/L and  $1.61 \pm 19.95$  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  $18.96 \pm 6.14$  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

322 concentrations were slightly higher in mature milk (15-180 days) samples than in  
323 transition milk (< 15 days) and in late lactation milk (180-250 days of lactation), but  
324 these differences were not statistically significant. In contrast, the lactation period had a  
325 significant effect on *myo*-inositol concentration (one-way ANOVA, *F*-value = 11.65, *P*-  
326 value = 0.0006). The concentration of *myo*-inositol in transition milk ( $341.35 \pm 95.22$   
327 mg/L, n = 4) was significantly higher than in mature ( $198.04 \pm 80.14$  mg/L, n = 8, *P* <  
328 0.05) and late lactation ( $130.64 \pm 53.44$  mg/L, n = 9, *P* < 0.001) milk samples (Figure  
329 1).

330

## 331 **DISCUSSION**

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

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

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

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

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



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

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

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

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

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

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

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

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

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572 **Legend to figures**

573

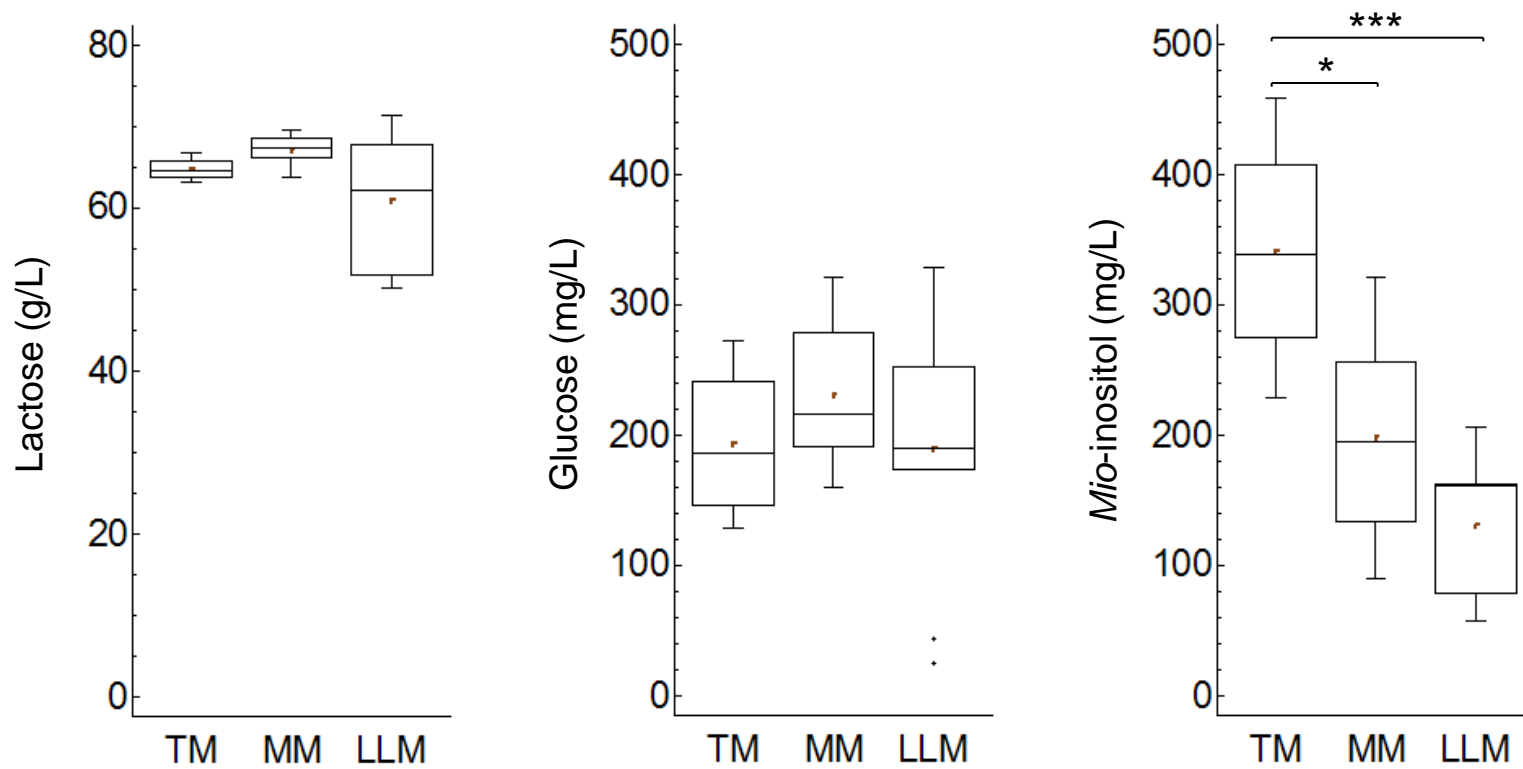
574 **FIGURE 1.** Lactose, glucose, and *myo*-inositol concentration in transition (TM, <15  
575 days, n = 4), mature (MM, 15-180 days, n = 8) and late lactation (LLM, >180 days, n =  
576 9) raw donor milk. The results of a one-way ANOVA and Bonferroni's post hoc tests  
577 are shown by asterisks.

578 \* =  $P < 0.05$ ; \*\*\* =  $P < 0.001$ .

579

580

FIGURE 1



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 <sup>†</sup>			2.00	3.44

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

7 \* Mean value of the samples where growth was detected.  
8  
9

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

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

11 RAPD = random amplification polymorphic DNA.

12

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

		Range						
		n	Mean	SD	95% CI	Minimum	Maximum	<i>P</i> <sup>*</sup>
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 <sup>†</sup>					
	P	8	nd					
		13	18.96	6.14	15.25 - 22.67	9.98	33.98	

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

17 <sup>\*</sup> Paired Student's *t*-test.

18 <sup>†</sup> Below the detection limit of the method (10 mg/L).