Antibacterial activity of bovine milk lactoferrin on the emerging foodborne pathogen *Cronobacter sakazakii*: effect of media and heat treatment

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ABSTRACT

Cronobacter sakazakii is a pathogen transmitted by food, with high osmotic resistance and tolerance to desiccation, which affects mainly to newborns, infants and immunocompromised adults. *C. sakazakii* infection in infants has been associated with consumption of powdered milk. The purpose of this study was to evaluate the antibacterial activity of native and iron-saturated bovine lactoferrin (bLF) (from 0.5 to 5 mg/ml) on non-desiccated and desiccated *C. sakazakii* (10^4 CFU/ml) in different media (phosphate buffer, bovine skim milk and whey). In general, native bLF was the only effective form that inhibited growth of *C. sakazakii* in all media, its activity increasing with concentration and time of incubation. These results suggest that the antibacterial effect of bLF on *C. sakazakii* is mainly due to iron sequestration. However, iron-saturated bLF showed some effect by reducing the viability of *C. sakazakii* in whey. There has not been observed an increased sensitivity of desiccated bacteria to native bLF in phosphate buffer. However, although the antibacterial activity of native bLF against non-desiccated *C. sakazakii* was drastically reduced in milk or whey compared to phosphate buffer, there was a certain activity when it was assayed against desiccated cells in those media. The effect of some heat treatments on the antibacterial activity of native bLF was evaluated and only those of 72℃ for 15 s, 85℃ for 15 s, and 63℃ for 30 min maintained its whole activity.

Keywords: bovine milk lactoferrin, *Cronobacter sakazakii,* antibacterial activity, heat treatment, UHT milk, whey

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1. Introduction

   Cronobacter sakazakii is an emerging pathogen transmitted by food that has been associated with meningitis (Burdette & Santos, 2000), sepsis (Simmons, Gelfand, Haas, Metts, & Feruson, 1989), bacteremia (Noriega, Kotloft, Martin, & Schwalb, 1990) and necrotizing enterocolitis (Van Acker et al., 2001), mainly affecting to newborns, infants, and immunocompromised adults (Lai, 2001). Recently, Iversen et al. (2008) reclassified *Enterobacter sakazakii* as a new genus, *Cronobacter*, in which fives species were included: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii* and *C. dublinensis*. Although the outbreaks caused by this pathogen are scarce, infections with *C. sakazakii* are often accompanied by a high rate of mortality that can reach 80% (Lehner & Stephan, 2004; Kim & Beuchat, 2005). *C. sakazakii* is a Gram-negative, motile, non-spore forming, ubiquitous, facultative anaerobic bacteria, belonging to the family *Enterobacteriaceae*. It can grow over a wide temperature range (6-47ºC) and is inactivated at 70℃. The consumption of cont aminated powdered infant formula (PIF) has been mainly associated with the majority of outbreaks caused by *C. sakazakii* (Burdette & Santos, 2000; Lai, 2001; Van Acker et al., 2001). The Codex Alimentarius Commission (CAC) of the United Nations provides regulations relevant to PIF, such as that *Cronobacter* spp. should be absent in 30 samples of 10 g in finished PIF products (CAC, 2008). The European Union officially introduced similar microbiological standards (European Commission, 2007).

   C. sakazakii is usually inactivated during pasteurization of PIF (Nazarowec-White & Farber, 1997a). Therefore, the presence of this bacterium in milk can be caused by post-processing environmental contamination, addition of contaminated ingredients (Nazarowec-White & Farber, 1997b) or colonization by *C. sakazakii* of utensils used in milk preparation. It has been shown that *C. sakazakii* have a remarkable resistance in dry media for periods at least of two years (Caubilla-Barron & Forsythe, 2007). This feature represents a competitive advantage, facilitating their prevalence in products with low water content (Edelson-Mammel, Porteus, & Buchanam, 2005). *Cronobacter* spp. may accumulate solutes such as trehalose which protects the microorganism against osmotic stress by stabilizing its membrane (Breeuwer, Lardeau, Peterz, &

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67 Joosten, 2003). Heat treatment of water at ≥70°C for reconstitution of PIF has

68 been recommended by FAO and WHO (2004). However, this treatment may

69 adversely affect the sensory quality and nutritional value of this essential food

70 for baby development. The heat treatment necessary to reduce the number of

71 *Cronobacter* cells in milk and to avoid its proliferation could be decreased by

72 combining it with antimicrobial compounds. According to WHO

73 recommendations, great interest has recently grown in using natural

74 antimicrobials, such as lactoferrin (LF), to avoid proliferation of *C. sakazakii* in

75 infant formula. From a regulatory point of view, bovine lactoferrin (bLF) is

76 considered safe under the proposed uses and levels in a variety of foods for

77 nutritional applications (EFSA, 2012a,b), i.e., infant and follow-on formulas,

78 dietary food, dairy products, yoghurts, and chewing gums (European

79 Commission, 2012a,b).

80   Lactoferrin is a glycoprotein of the transferrin family present in the majority of

81 external secretions and mucosal surfaces, milk being its main source.

82 Lactoferrin binds two atoms of iron and due to this capacity several functions

83 have been attributed to it, such as antibacterial, antioxidant, antitumoral and

84 immunomodulatory (Sánchez, Calvo, & Brock, 1992a).

85   Almost all bacteria require iron for their growth; therefore LF devoid of iron is

86 capable of preventing its utilization by some bacteria (Orsi, 2004). A large

87 number of studies have demonstrated the bacteriostatic and bactericidal effect

88 of LF, against a wide range of Gram-positive and Gram-negative bacteria

89 (Farnaud & Evans, 2003). However, other mechanisms besides iron holding

90 can be involved in the antibacterial activity of LF, such as blocking microbial

91 metabolism of carbohydrates or destabilizing the bacterial cell wall (Sánchez,

92 Calvo, & Brock, 1992a).

93   The aim of this study was to evaluate the antibacterial activity of bLF on the

94 emerging foodborne pathogen *C. sakazakii* and the influence of different

95 factors. Thus, iron saturation and concentration of bLF, desiccation of bacterial

96 cells, media, incubation time and heat treatment have been evaluated. The

97 effect of heat treatment is especially important since denaturation of bioactive

98 whey proteins may result in loss of their biological functions.

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2. Materials and methods

2.1. Culture of C. sakazakii and preparation of desiccated cells

A freeze-dried culture of *C. sakazakii* CECT 858, equivalent to strain ATCC 29544, was supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain). After reviving freeze-dried culture, it was stored at -70℃ in sterile cryopreservation vials. Working cultures were obtained by transferring a porous bead of stock culture into 10 ml of Trypticase soy broth (TSB), incubating at 37℃ for 24 h and transferring a loop to Trypticase soy agar (TSA). After 24 h at 37℃, an isolated colony was transferred to 10 ml o f TSB and was incubated at 37℃ for 24 h.

Desiccated cells of *C. sakazakii* were prepared as described by Al-Nabulsi et al. (2009), dispensing a volume of 1 ml of freshly prepared suspension from a single colony of *C. sakazakii* in 50 μl portions on a sterile Petri dish. The plate was placed at 40℃ in an incubator to be air-dried. After 2 h, the plate was placed in a desiccator at room temperature for 4 d, and afterwards, 2 ml of 0.2% (w/v) peptone water were added to the plate to collect desiccated cells, mixed with 8 ml of 0.2% peptone water and serial decimal dilutions in 1% peptone water were used to yield a suspension of 10^4 CFU/ml for antibacterial activity assays.

2.2. Preparation of native bLF solutions

Native bLF was kindly provided by Tatua Nutritionals Company (Morrinsville, New Zealand) and had an iron-saturation below 10%. The purity of bLF was checked by SDS-PAGE, which showed a single band corresponding to a protein of about 80 KDa and purity higher than 90%. The stock solution of bLF was prepared from the native protein in ultrapure water at 20 mg/ml and sterilized through a low-binding protein 0.22 μm filter. After filtration, the absorbance was measured at 280 nm and the concentration of bLF determined by considering a molar extinction coefficient ($E^{1\%}$) of 1.27 ml/cm/g. The final concentration of bLF solutions was adjusted to 1, 2, 5 and 10 mg/ml.

2.3. Preparation of iron-saturated bLF solutions

130 Native bLF was saturated with iron by adding ferrinitrilotriacetate (FeNTA)

131 solution as described previously (Ismail & Brock, 1993). Afterwards, bLF was

132 subjected to Sephadex G-25 chromatography to remove unbound iron. The

133 iron-saturated bLF solution was filtered through 0.22 µm and the concentration

134 was determined, considering a $E^{1\%}$ of 1.51 ml/cm/g. The concentration of

135 solutions was adjusted to 1, 2, 5 and 10 mg/ml.

2.4. Effect of heat treatment on bLF

137 To study the effect of different heat treatments on the antibacterial activity of

138 bLF against non-desiccated *C. sakazakii*, native or iron-saturated bLF were

139 dissolved at a concentration of 5 mg/ml in phosphate buffer solution, composed

140 by 15 mM monopotassium phosphate, 8 mM dibasic sodium phosphate, 14 mM

141 NaCl and 2 mM KCl, pH 7.4 (PBS). A volume of 650 µl of bLF solutions was

142 subjected to different heat treatments in sterile glass vials of 12.0 mm outer

143 diameter and 11.6 mm inner diameter. Treatments were performed in a water

144 bath with agitation and temperature controlled with an accuracy of ± 0.1℃. The

145 vials were removed at several times and immediately immersed into an ice-

146 water bath. Heat treatments performed were: 63℃ fo r 30 min, low-temperature

147 long-time pasteurization (LTLT); 72℃ for 15 s, hig h-temperature short-time

148 pasteurization (HTST); 72℃ for 15 min, that was ch osen as an intermediate

149 treatment between HTST and high pasteurization; 85°C for 15 s and 85℃ for

150 10 min, treatments used normally in the manufacture of some dairy products.

151 Samples of heat treated bLF were subjected to electrophoresis and radial

152 immunodiffusion to evaluate the degree of protein denaturation.

2.5. SDS-PAGE electrophoresis and radial immunodiffusion

154 Heat treated bLF was analyzed by SDS-PAGE. A volume of 50 µl of the

155 treated samples was added to 40 µl of 10 mM Tris 1 mM EDTA, pH 8, and 10 µl

156 of 25% SDS and treated at 100℃ for 5 min. Afterwar ds, 1 µl of each sample

157 was applied to a 7.5 % polyacrylamide gel and electrophoresis developed in a

158 Phast System equipment (Pharmacia Biotech, Uppsala Sweden). After the

159 electrophoresis, the gels were stained with Coomassie blue type R.

160 The immunochemical reactivity of heat-treated bLF was determined by radial

161 immunodifusion, as described previously (Sánchez et al. 1992b), by using 0.4,

162 0.2, 0.1 and 0.05 mg/ml bLF standards. After diffusion for 72 h, the gel was

163 washed with saline buffer, stained with Coomassie blue type G, destained and

164 diameters of radial precipitate measured.

165 *2.6. Antibacterial activity assay*

166 The media used to evaluate the antibacterial activity of native and iron-

167 saturated bLF were: commercial ultra-high temperature (UHT) bovine skim milk,

168 bovine whey obtained from UHT milk by ultrafiltration with 100,000 MWCO

169 hollow fiber, and PBS. Whey and PBS were filtered through 0.22 µm.

170 Non-desiccated or desiccated *C. sakazakii* were diluted in 1% peptone water

171 to achieve 10^4 CFU/ml. A volume of 100 µl of those suspensions was added to

172 each well of a microtiter plate with 100 µl of UHT skim milk, whey or PBS

173 containing native or iron-saturated bLF at a final concentration of 0.5, 1, 2.5 or 5

174 mg/ml. The final concentration of bacteria in the well was approximately 10^4

175 CFU/ml. Control samples consisted of media without bLF.

176 Heat treated bLF was assayed at 2.5 mg/ml in PBS against non-desiccated

177 bacteria (10^4 CFU/ml). In this assay, non-treated native bLF at 2.5 mg/ml was

178 included as reference.

179 The plates were incubated at 37℃ for 4 and 8 h. Th e number of viable cells

180 was determined by serially diluting the content of each well in 1% peptone water

181 and plating on TSA plates which were incubated at 37℃ for 24 h. Each well

182 was seeded by duplicate.

183 *2.7. Statistical analysis*

184 Experiments were performed three times using freshly prepared samples.

185 Mean and standard deviations were calculated from all the data obtained in the

186 experiments performed. Data were statistically evaluated by t test and ANOVA

187 according to Duncan test using the SPSS 19.0 package for Windows.

188 **3. Results**

189 *3.1. Activity of native and iron-saturated bLF against non-desiccated and*

190 *desiccated C. sakazakii in PBS*

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191   The results showed that native bLF in PBS exerted antibacterial activity
192   against non-desiccated *C. sakazakii*, from 0.5 to 5 mg/ml, being significantly
193   different from the control at concentrations above 1 mg/ml at 4 and 8 h (Fig.
194   1A). This antibacterial activity increased with concentration and time of
195   incubation, and after 8 h, the 5 mg/ml bLF solution reduced the bacterial counts
196   7.98 log cycles, respect to the control. Iron-saturated bLF did not reduce
197   significantly the growth of non-desiccated *C. sakazakii* at any concentration and
198   incubation period (data not shown).

199   The results obtained against desiccated *C. sakazakii* (Fig. 1B) showed a
200   significant inhibitory effect of native bLF, except for the concentration of 0.5
201   mg/ml, at 4 and 8 h. In general, the inhibitory effect of native bLF on desiccated
202   cells appeared later respect to non-desiccated bacteria. The antibacterial effect
203   of native bLF at 4 h was lower on desiccated bacteria than on non-desiccated
204   bacteria for the same concentrations of protein. However, native bLF at 5 mg/ml
205   produced practically the same reduction on the growth of desiccated *C.*
206   *sakazakii* at 8 h, than on non-desiccated cells. The iron-saturated bLF did not
207   show any antibacterial activity on desiccated bacteria (data not shown).

208   *3.2. Activity of native and iron-saturated bLF against non-desiccated and*
209   *desiccated C. sakazakii in bovine skim milk and whey*

210   The antibacterial activity of native bLF against *C. sakazakii* was drastically
211   reduced when the protein was assayed in bovine milk or whey, compared with
212   PBS (Table 1). However, certain activity was observed for some concentrations
213   of native bLF, being significant at 1, 2.5 and 5 mg/ml on desiccated *C. sakazakii*
214   cells in whey after 4 h and at 5 mg/ml after 8 h, being the highest reduction of
215   2.96 cycles. In milk the only significant activity was found for native bLF at 5
216   mg/ml at 4 and 8 h on desiccated cells. The inhibitory activity of native bLF on
217   non-desiccated cells was lower than one logarithmic cycle and only observed
218   for 5 mg/ml.

219   Furthermore, significant activity was found for iron-saturated bLF at 5 mg/ml
220   on desiccated cells in whey at 4 and 8 h of incubation, with reductions of 1.49
221   and 1.86 cycles, respectively (data not shown).

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222 *3.3. Effect of heat-treatment on the activity of native and iron-saturated LF*
223 *against non-desiccated C. sakazakii in PBS*

224   Treatments of 72℃ for 15 s, 85℃ for 15 s and 63℃ for 30 min did not affect
225 the antimicrobial activity of native bLF (Table 2). However, native bLF treated at
226 72℃ for 15 min and at 85℃ for 10 min, showed lowe r antibacterial activity than
227 that observed for bLF without treatment. Iron-saturated bLF did not show any
228 antibacterial activity.

229   Samples of heat treated bLF in PBS were subjected to SDS-PAGE to
230 evaluate their aggregation (Fig. 2). Some LF aggregates were observed in the
231 stacking gel, especially for samples treated at 72°C for 15 min, 85℃ for 10 min
232 and 85℃ for 15 s. This can be due to the establish ment of interactions between
233 bLF molecules under heating, consequently losing its antibacterial activity.

234   The estimated concentrations of heat treated bLF determined by its
235 immunoreactivity respect to the non-treated bLF are shown in Table 3. The
236 treatments that affected bLF immunoreactivity in higher extent were 85℃ for 10
237 min and 72℃ for 15 min, decreasing it to 4 and 10% , respectively. After
238 treatment of bLF at 63℃ for 30 min its immunoreact ivity decreased to 38%,
239 although its antibacterial activity was still high as shown in Table 2. After
240 treatment at 85℃ for 15 s the immunoreactivity was of 66% and at 72℃ for 15
241 s of 100% with respect to non-treated bLF.

242 **4. Discussion**

243   The antibacterial activity of LF has been largely demonstrated against a wide
244 range of Gram-positive and Gram-negative bacteria (Jenssen & Hancock,
245 2009). During the last years some bacteria have appeared as emerging
246 pathogens, as is the case of *C. sakazakii* (Iversen et al., 2008), and therefore,
247 there is little knowledge of LF activity against them. Natural antimicrobials have
248 been proposed as food preservatives in the last years, by combining them with
249 non-thermal treatments or lower treatments, in order to maintain the nutritional
250 and organoleptic characteristics of food (Masschalck, Van Houdt, & Michiels,
251 2001; Del Olmo, Calzada, & Nuñez, 2012).

252   Although the most recognized mechanism for LF antibacterial activity is iron
253 sequestration, it has been shown that iron-saturated LF is also active on some

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254 bacteria. This activity is related to its capacity to interact with the bacterial
255 membrane, subsequently producing its destabilization, altering the bacterial
256 metabolic processes and finally causing their death (Sánchez, Calvo & Brock,
257 1992a). In the present work only native bLF has been proved as inhibitory for *C.*
258 *sakazakii*, therefore being iron sequestration the most probable mechanism of
259 that activity. However, a certain inhibitory effect of iron-saturated bLF was
260 observed on desiccated *C. sakazakii* cells in bovine whey. This could be due to
261 changes in the bacterial membrane produced by desiccation and/or to the
262 contribution of some whey component to the inhibitory mechanism.

263   *C. sakazakii* has been reported to be highly persistent in formulas with low
264 water activity for long periods (Gurtler & Beuchat, 2007) and others indicated
265 that capsulated strains of *C. sakazakii* were still recoverable from dry infant
266 formula after two and a half years (Caubilla-Barron & Forsythe, 2007). Those
267 studies carried out on desiccated bacteria intended to reproduce the conditions
268 that might occur in PIF contaminated with *C. sakazakii* prior to transformation
269 into powder. In an earlier study, Al-Nabulsi et al. (2009) reported that
270 desiccation enhanced the sensitivity of *Cronobacter* spp. to the LF inhibitory
271 activity, though they also found that nisin was less active on those desiccated
272 cells. In contrast, the results obtained in this study did not show higher
273 sensitivity of desiccated *C. sakazakii* to the effect of native bLF compared with
274 non-desiccated cells. On the contrary, the desiccated cells reacted later to the
275 activity of bLF and were inhibited at similar levels to the non-desiccated cells
276 only at the highest concentration of bLF and after 8 h. This could be explained
277 by different mechanisms reported for *C. sakazakii* to tolerate desiccation, such
278 as synthesis of high levels of trehalose or glycine betaine (Breeuwer, Lardeau,
279 Peter & Joosten, 2003), or production of exopolysaccharides (Jung, Choi, &
280 Lee, 2013).

281   The composition of media limits the activity of antimicrobials, specially when
282 those are complex food matrices. This has been shown in studies carried out in
283 meat (Venkitanarayanan, Zhao & Doyle, 1999; Del Olmo, Morales, & Nuñez,
284 2009) or carrot juice (Chantaysakorn & Richter, 2000) in which the reduction of
285 antibacterial activity of LF or its hydrolysates was attributed to the presence of
286 high levels of divalent cations (Branen & Davidson, 2000; Al-Nabulsi et al.,

287 2009; Del Olmo, Morales & Nuñez, 2009). In previous studies we evaluated the

288 activity of native bLF (Conesa et al, 2010), human recombinant lactoferrin from

289 *Aspergillus awamori* (Conesa et al., 2008) and from rice (Conesa et al., 2009)

290 on *Listeria monocytogenes* and *Escherichia coli* 0157: H7 in skim milk and

291 whey. We found that skim milk and whey acted by protecting the bacteria

292 reducing LF antibacterial activity. In the present work, we have also observed

293 that bLF antibacterial activity against *C. sakazakii* is low when it was evaluated

294 in bovine skimmed milk or whey. However, there was some activity of native

295 and even iron-saturated bLF when were assayed on desiccated cells in milk or

296 whey. Therefore, these results show that it is worthwhile investigating the

297 procedures to improve LF antibacterial activity in milk and whey by modifying or

298 eliminating some interfering components.

299   Considering that bLF is being used as a bioactive ingredient in some

300 processed foods, it is essential to study the effect of heat treatment on its

301 antibacterial activity. In the present study we found that heat treatments of 72℃

302 for 15 s, 85℃ for 15 s and 63℃ for 30 min maintained the whole antibacterial

303 activity of native bLF against *C. sakazakii.* Lactoferrin subjected to the highest

304 treatments*,* 72℃ for 15 min and 85℃ for 10 min, diminished in a great extent

305 its activity, though still having some effect. The results we previously obtained of

306 the activity of heat treated bLF and human recombinant LF from rice and

307 fungus, on three different bacteria: *Escherichia coli* O157:H7, *Salmonella*

308 Enteritidis and *Listeria monocytogenes* were quite coincident with those

309 obtained in the present work, as the only heat treatment that counteracted

310 almost completely the antibacterial activity of LF was 85℃ for 10 min (Conesa

311 et al., 2008; Conesa et al., 2009; Conesa et al., 2010). Therefore, we can

312 confirm that native bLF is very resistant to the most common pasteurization

313 treatments, maintaining its antibacterial activity against *C. sakazakii.*

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399   *turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis*
400   sp. nov., *Cronobacter* genomospecies 1, and of three subspecies,
401   *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter*
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1 **FIGURE AND TABLE CAPTIONS**

2 **Figure 1.** Effect of the concentration of native bLF on the growth of non-
3 desiccated (A) and desiccated (B) *C. sakazakii* in PBS after 4 and 8 h of
4 incubation at 37℃. Each value represents the mean ± standard deviation of
5 nine replicates from three independent experiments. *Significant differences for
6 *p*<0.05 with respect to the control.

7 **Figure 2.** SDS-PAGE of heat-treated native bLF. Lanes contain the following
8 samples:1 and 7, non-treated bLF (5 mg/ml); 2, bLF treated at 63℃ for 30 min;
9 3, bLF treated at 72℃ for 15 s; 4, bLF treated at 72℃ for 15 min; 5, bLF treated
10 at 85℃ for 10 min; 6, bLF treated at 85℃ for 15 s . Molecular weight markers:
11 β-galactosidase (116 kDa), transferrin (76 kDa), glutamate deshidrogenase (53
12 kDa).

13

14 **Table 1.** Effect of the concentration of native bLF on the growth of non-
15 desiccated and desiccated *C. sakazakii* in bovine whey and skimmed milk after
16 4 and 8 h of incubation at 37℃. Each value represe nts the mean ± standard
17 deviation of nine replicates from three independent experiments. *Significant
18 differences for *p*<0.05 with respect to the control.

19 **Table 2.** Activity of heat-treated native and iron-saturated bLF (final
20 concentration of 2.5 mg/ml) on the growth of non-dessicated *C. sakazakii* in
21 PBS at 4 and 8 h of incubation at 37℃. Values repr esent the mean ± standard
22 deviation of data from three independent experiments and three replicates at
23 each experiment. Significant differences for *p*<0.01 and **p*<0.001 with respect
24 to the control. [a]n.d: not detected.

25 **Table 3.** Effect of heat treatment on the concentration of native bLF determined
26 by radial immunodifusion using polyclonal specific antibodies. Immunoreactivity
27 is expressed as the relative concentration respect to the non-treated bLF.

28

29

Table 1.

Bovine whey	Non-desiccated *C. sakazakii* (log CFU/ml)		Desiccated *C. sakazakii* (log CFU/ml)	
	4 hours	**8 hours**	**4 hours**	**8 hours**
Control	5.91 ± 0.26	8.30 ± 0.06	6.05 ± 0.07	8.17 ± 0.31
LF 0.5 mg/ml	5.87 ± 0.25	8.14 ± 0.09	6.01 ± 0.40	7.99 ± 0.40
LF 1 mg/ml	5.75 ± 0.32	8.03 ± 0.07	6.01 ± 0.07	8.03 ± 0.32
LF 2.5 mg/ml	5.71 ± 0.22	7.97 ± 0.09	5.91 ± 0.12	7.90 ± 0.37
LF 5 mg/ml	5.55 ± 0.34*	7.70 ± 0.15	5.95 ± 0.10	7.91 ± 0.37
Bovine milk	Non-desiccated *C. sakazakii* (log CFU/ml)		Desiccated *C. sakazakii* (log CFU/ml)	
	4 hours	**8 hours**	**4 hours**	**8 hours**
Control	6.00 ± 0.09	8.20 ± 0.09	6.19 ± 0.05	8.32 ± 0.14
LF 0.5 mg/ml	5.92 ± 0.09*	8.10 ± 0.06	5.32 ± 0.09	8.08 ± 0.07
LF 1 mg/ml	5.78 ± 0.13*	8.00 ± 0.37	5.88 ± 0.11	7.81 ± 0.21
LF 2.5 mg/ml	5.80 ± 0.09*	7.90 ± 0.32	5.74 ± 0.21	7.57 ± 0.43
LF 5 mg/ml	5.82 ± 0.06*	7.82 ± 0.09*	4.62 ± 1.37*	6.20 ± 2.67*

Table 2.

	Native lactoferrin (log CFU/ml)		Iron-saturated lactoferrin (log CFU/ml)	
	4 hours	**8 hours**	**4 hours**	**8 hours**
Control	5.61 ± 0.31	7.75 ± 0.14	6.10 ± 0.11	8.61 ± 0.13
Non-treated LF	nd[a]	nd	6.04 ± 0.13	8.47 ± 0.14
LF 63℃ 30 min	nd	nd	5.94 ± 0.18	8.46 ± 0.09
LF 72℃ 15 s	nd	nd	5.98 ± 0.19	8.44 ± 0.05
LF 85℃ 1 5 s	nd	nd	5.98 ± 0.17	8.44 ± 0.04
LF 72℃ 15 min	4.83 ± 1.49*	4.12 ± 3.48**	5.99 ± 0.14	8.12 ± 0.23
LF 85℃ 10 min	4.32 ± 1.93*	3.56 ± 3.57**	5.99 ± 0.17	8.45 ± 0.05

Table 3.

Heat treatment	% Immunoreactivity
Non-treated LF	100
LF 63℃ 30 min	38
LF 72℃ 15 s	100
LF 85℃ 15 s	66
LF 72℃ 15 min	10
LF 85℃ 10 min	4

Figure 1.
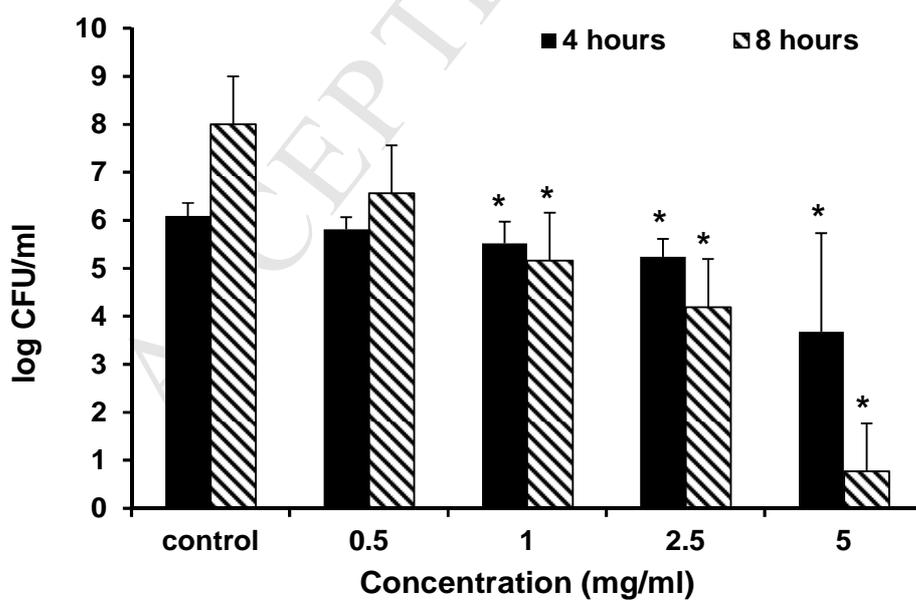
A

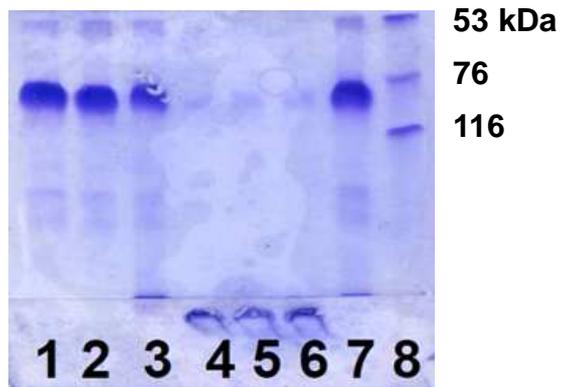**B**

Figure 2

53 kDa
76
116

1 2 3 4 5 6 7 8
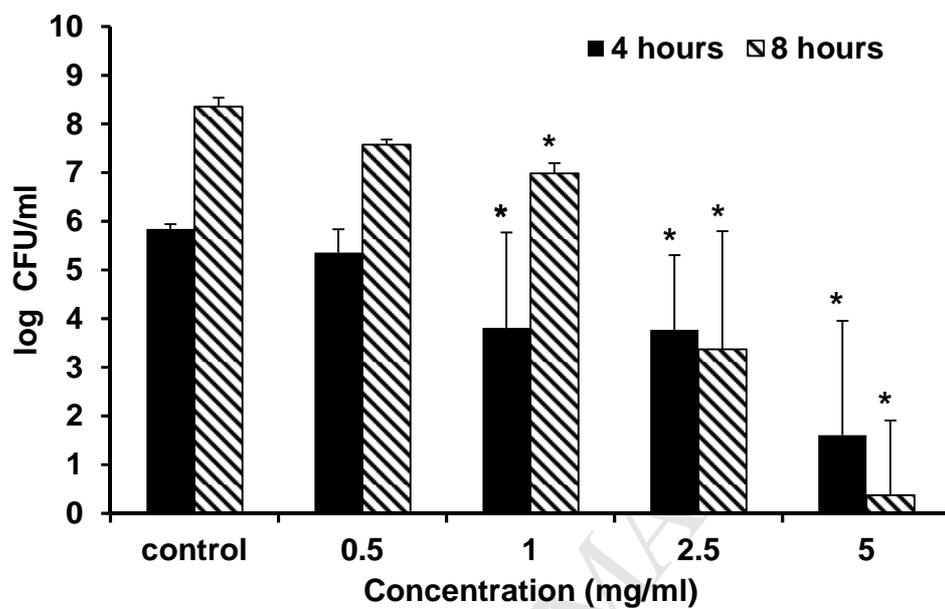
1   **Highlights**

2

3   • Native bovine lactoferrin at 5 mg/ml reduces 8 log cycles *C. sakazakii* growth.

4   • Milk and whey diminish lactoferrin antibacterial activity against *C. sakazakii.*

5   • Pasteurization maintains lactoferrin antibacterial activity against *C. sakazakii.*

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