Stevia rebaudiana Bertoni effect on the hemolytic potential of *Listeria monocytogenes*

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ABSTRACT

The effect of Stevia *Rebaudiana Bertoni* on the hemolytic potential of *Listeria monocytogenes* was studied by means the assessment of the **Listeriolysin O (LLO)** production. The three factors under study, stevia concentration in the range [0–2.5] (%) (w/v), incubation temperature [10 or 37] °C, and exposure time (0–65 h) significantly affected ($p \leq 0.05$) the hemolytic activity of *L. monocytogenes*. Results showed that at the lower incubation temperature the hemolytic potential of the bacterium was significantly reduced, from 100 % at 37 °C to 8 % at 10 °C (after 65 h of incubation) in unsupplemented substrate (0 % stevia). Irrespective of the temperature, [10 or 37] °C, supplementation of the medium with stevia at 2.5 % (w/v) reduced the bacterium's hemolytic activity by a maximum of 100 %. Furthermore, the time of exposure to 2.5 % (w/v) stevia concentration was also a significant factor reducing thehemolytic capability of *L. monocytogenes*. The possibility of reducing the pathogenic potential of *L. monocytogenes* (hemolysis) by cells exposure to stevia should be confirmed in real food matrices, opening a research niche with a valuable future impact on food safety.

Keywords: *Listeria monocytogenes*, virulence factors, Listeriolysin O, hemolysis, *Stevia rebaudiana* Bertoni, natural antimicrobials.

1. Introduction

Listeria monocytogenes is a Gram-positive, facultative, intracytosolic, foodborne pathogen in humans and animals, and the causative agent of listeriosis, a severe systemic illness with fatal consequences in elderly, pregnant, newborn, and immunocompromised populations (Mateus et al., 2013). *L. monocytogenes* is able to survive in a wide range of conditions being its presence and multiplication in fresh minimally processed vegetables, ready-to-eat products (RTE), and refrigerated or frozen foods of particular concern (Liu et al., 2016; Wagner et al., 2007). Among the most common food products implicated in listeriosis outbreaks, pork, fermented sausages, fresh products, soft cheeses, and ice cream have been the most important ones in recent years (CDC, 2015; FDA, 2015).

To understand the pathogenicity of this bacterium it is important to fully investigate the factors linked to *L. monocytogenes* adaptation to survive under stressful conditions (Gandhi and Chikindas, 2007), and the cellular mechanisms involved into injury repair after exposure to sublethal treatments (Wesche et al., 2009; Tamburro et al., 2015a),. Among the molecular mechanisms underlying *Listeria* virulence, hemolytic activity is classified as the most important virulence marker, being present only in the pathogenic species. The hemolytic capability of *L. monocytogenes* cells is controlled by **Listeriolysin O** (LLO, encoded by the hemolysin gene, *hly*). Listeriolysin O (LLO) is the pore-forming toxin that is responsible for lyses of primary vacuoles, subsequently producing the escape of *L. monocytogenes* into the cytosol of host cells, where it grows and multiplies (Ruan et al., 2016). According to the studies of Gaillard et al. (1986), in the absence of LLO activity the virulence of the bacterium is attenuated by over five logs in a murine model of listeriosis. The studies conducted by Birmingham et al. (2008) suggest the decisive role of LLO in promoting *L. monocytogenes* replication in spacious *Listeria*-containing phagosomes (SLAPs), leading to permanent host infection within host macrophages.

The production of listeriolysin O by pathogenic *L. monocytogenes* strains is one of the most interesting factors that is being studied with the application of new processing treatments

(Dutilly, 2011) or stressful environmental conditions because of the close relationship that has been shown to exist between this factor and the virulence exerted by the microorganism (Sampathkumar et al., 1999; Smith-Palmer et al., 2002; Tamburro et al., 2015b; Wesche et al., 2009).

Several studies have associated exposure of *L. monocytogenes* cells to stressful conditions with modification of expression of the hemolytic factor (LLO) (Arévalos-Sánchez et al., 2012; Buncic et al., 1996; Van Boeijen et al., 2010). However, up to date, scarce studies have been published regarding the influence that bacterial growth, processing factors, and storage conditions have on LLO production or inhibition.

In this context, it is clearly important to study the effect that novel technologies (e.g. High hydrostatic pressure (HHP)) (Bowman et al., 2008; Dutilly, 2011; Golberg, 2015; Scolari et al., 2015) and novel antimicrobial substances (e.g. phytochemicals from algae (Rafiquzzaman et al., 2015) and vegetable by-products (Sanz-Puig et al., 2015)) could have on the expression of virulence factors by this highly concerning bacteria. Alternative sources of natural antimicrobials can be found in vegetables, functional herbs, and superfruits (Gyawali and Ibrahim, 2014). Among them is *Stevia rebaudiana* Bertoni (stevia), a potent sweetener and acaloric plant with proven high antimicrobial capability against a wide range of foodborne pathogens (*Escherichia coli*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) (Belda-Galbis et al., 2014; Lemus-Mondaca et al., 2011), one of them being *L. monocytogenes* (Abou-Arab et al., 2010). Exposure of *L. monocytogenes* to sublethal concentrations of natural vegetable extracts and essential oils could lead to increases (Arevalos-Sánchez et al., 2012) and reductions (Smith-Palmer et al., 2002; Upadhyay et al., 2012) in listeriolysin O activity. Not only the production of LLO but also the analysis of transcription levels of virulence factors encoding genes are relevant aspects to study under the

exposure of the bacterium to sublethal doses of natural antimicrobials (Tamburro et al., 2015b).

To our knowledge, there are no previous studies focusing on the influence that *Stevia rebaudiana* Bertoni could have on production of listeriolysin O by exposed *L. monocytogenes* cells.

Consequently, the present study aims to evaluate the listeriolysin O activity of *L. monocytogenes* cells exposed to *Stevia rebaudiana* Bertoni in a reference medium, and the influence that time and temperature of exposure could have on the hemolytic capability of the bacterium.

2. Material and Methods

2.1 Microorganism

The present study was carried out with *Listeria monocytogenes* strain CECT 4032 provided by the Spanish Type Culture Collection. *L. monocytogenes* CECT 4032 is a food isolate (soft cheese, UK, 1986), causative agent of meningitis.

A culture was prepared as described by Saucedo-Reyes et al. (2009). Briefly, a fresh culture of *L. monocytogenes* (pre-incubated for 6 hours) was inoculated in Tryptone and Soy Broth (TSB) (Scharlau S.A., Barcelona, Spain), followed by incubation to stationary phase, then cooled in ice, washed twice with TSB, and dispensed in 2 mL sterile plastic cryogenic vials containing TSB supplemented with 20 % glycerol in a relation of 1:1. The final bacterial concentration of the vials was 5×10^9 CFU mL^{-1}. The prepared vials were stored at –80 °C.

2.2 *Stevia rebaudiana* Bertoni infusion process

106 *Stevia* infusion was obtained from dried leaves (Anagalide S.A., Spain). To prepare a stock
107 solution of 8.33 ± 0.01 % (w/v), 1000 mL of boiling mineral water was added to the dried
108 leaves (83.3 g), and the mixture was covered and allowed to infuse for 30 min. The infusion
109 was vacuum filtered using filter paper (Whatman® No. 1, Whatman International Ltd, UK) and
110 the filtrate was sterilized by filtration (0.20 μm; Minisart®, Sartorius, Spain). The sterile stock
111 solution was freezed at -20 °C up to its use.
112
113 **2.3. Listeriolysin O (LLO) test**

114 The listeriolysin O activity test was carried out in broth culture (TSB; Scharlab S.L., Spain),
115 following the modified Upadhyay et al. (2012) protocol. Fresh *L. monocytogenes* culture was
116 grown in TSB at 37 °C for 21 h. The culture was centrifuged (4000 g, 10 min), and washed three
117 times with TSB. The final concentration of the prepared culture was 1 x 10^9 CFU/mL.

118 Three independent TSB media were prepared, supplemented with stevia to achieve three final
119 concentration levels: 0 %, 1.25 %, and 2.5 % (w/v) (pH 7.44, 7.37, and 7.28, respectively). The
120 stevia concentration (2.5 % (w/v)) was selected on the basis of the sucrose concentration of
121 commercial fruit-based beverages and the sweetness equivalence of stevia and sucrose (Savita
122 et al., 2004).
123
124 Afterwards, *L. monocytogenes* was inoculated in the prepared matrices at a final concentration
125 of 1 x 10^4 CFU/mL. The inoculum concentration was selected according to the studies of Moors
126 et al. (1999) corresponding to 50% lethal dose for BALB/c mice. The study was carried out at
127 two temperatures, 10 °C (temperature of cold chain breakage) and 37 °C (optimum growth
128 temperature of the bacterium), for each of the matrices considered, and the study was
129 prolonged for a maximum time of 65 hours.

Bacterial counts were determined by means of serial dilution in each of the conditions studied (stevia concentration and incubation temperature), at 0, 17, 24, 48, and 65 h. Furthermore, five aliquots of each sample were taken at the intervals selected in the period studied (0, 17, 24, 48, and 65 h), and were centrifuged (12000 g, 15 min). The supernatant was separated and used for the listeriolysin O test.

Independently, sheep erythrocytes (Sheep Red Blood Cells (SRBC), Scharlau Chemie, Spain) were prepared according to the modified protocol of Upadhyay et al. (2012). The samples were centrifuged (600 g, 15 minutes) and washed three times with Phosphate Buffer Saline (PBS, Scharlab S.L., Spain). A dilution of 3 % (v/v) in PBS was prepared from the last pellet.

The listeriolysin O activity test was carried out by triplicate mixing 300 µL of each supernatant sample, 300 µL of PBS, and 300 µL of SRBC, and incubating for 30 min at 37 °C. A positive control equivalent to 100 % hemolysis was prepared by mixing 300 µL of SRBC, 300 µL of distilled water, and 300 µL of non-incubated medium. Similarly, a negative control (0 % hemolysis) was included in the present study by mixing 300 µL of SRBC, 300 µL of PBS, and 300 µL of the non-incubated medium. A spectrophotometric measurement (600 nm) was carried out after that time by means of a model PG 1800 spectrophotometer (Labolan S.L., Spain).

The percentage of hemolysis was calculated for each treated sample according to the following equation (Bhakdi et al., 1984):

$$\%Hemolysis = \left[\frac{(1 - OD_{sample})}{OD_t} x100 \right]$$

Where OD_{sample} is the difference between the optical density of the sample and the positive control; and OD_t is defined as the difference in optical density between the negative control

and the positive control. The spectrophotometric measurements were carried out by triplicate on three independent replicates for each studied condition.

2.5 Statistical analysis

The statistical analysis was performed with Centurion XV Statgraphics (STATGRAPHICS Centurion XV, version 15.1.03). The analysis included the average and standard deviation calculations for the three repetitions and a Variance Analysis (ANOVA) to detect significant differences.

3. Results and Discussion

Listeriolysin O production was evaluated under the conditions studied (temperature (°C), stevia concentration (w/v), exposure time (h)) by assessing the hemolytic capability of *L. monocytogenes* cells during the entire incubation period (65 h). The effect of stevia concentration on listeriolysin O delivery was evaluated at 10 and 37 °C. Figure 1 shows that both stevia concentration and growth temperature are significant factors affecting the hemolytic activity of *L. monocytogenes* (p-value ≤ 0.05).

It is well known that the growth kinetics of *L. monocytogenes* are affected at temperatures below the optimum (Kocaman & Sarimehmetoglu, 2016). According to the studies of Belda-Galbis et al. (2014) a significant elongation of the *Listeria innocua* lag time (λ), and a significant reduction of the maximum specific growth rate (μ_{max}) were observed when the incubation temperature was reduced from 37 to 10 °C. In this case, the exposure of *L. innocua* cells to Stevia *Rebaudiana Bertoni* acted as an additional hurdle factor decreasing the growth kinetics of the bacterium. Under sub-optimal temperatures it occurs the reduction in the metabolic activity, the protein and enzyme activity, the reduction in the membrane elasticity, the slow

down of transport systems, and also the delay of the gene expression process (Kocaman & Sarimehmetoglu, 2016).

In the present study, under low temperature conditions (10 °C) the hemolytic potential of the bacterium is significantly ($p \leq 0.05$) reduced. As can be seen graphically, after 65 h of incubation the 100 % hemolytic activity of *L. monocytogenes* cells observed at 37 °C falls below 10 % when incubated at 10 °C. These results are in agreement with those reported by Buncic et al. (1996), Datta et al. (1993), Doyle (2001), and Leimeister-Wächter et al. (1992), according to which listeriolysin O delivery to extracellular media was observed to decrease as the growth temperature fell below 37 °C, and was even more suppressed at a refrigeration temperature of 4 °C (Myers and Martin, 1994). Transcriptional regulation of *hly* is predominantly controlled by the factor A (*PrfA*) (de las Heras et al. 2011; Seveau, 2014). According to the studies of de las Heras et al. (2011) the *PrfA* expression or activity is mainly controlled by temperature. The translation of *PrfA* selectively occurs at 37 °C once the bacterium is within its animal host (Seveau, 2014).

Additional environmental stresses are also referred as responsable factors with important role controlling *PrfA* expression (being in this case stevia additionally affecting LLO production) (Seveau, 2014). The stevia concentration added to the medium also significantly affects the hemolytic potential of the bacterium (Figure 1). At 37 °C after 65 h of incubation, the *L. monocytogenes* hemolytic activity is reduced to 20 % under 1.25 % (w/v) stevia supplementation, and completely reduced (0 % hemolytic activity) in TSB substrate supplemented with 2.5 % (w/v) stevia. Meanwhile, at 10 °C, the hemolytic activity of *L. monocytogenes* is limited to [0–20] % in TSB supplemented with stevia in the range [0–1.25] % (w/v), and to 0 % in substrate supplemented with 2.5 % (w/v) stevia. In conclusion, and independently of the temperature effect, in the range 10–37 °C, the exposure of *L. monocytogenes* cells to substrate supplemented with 2.5 % (w/v) stevia completely reduced the hemolytic activity of the bacterium. Some authors have directly correlated the release of

202 listeriolysin O enzyme to the medium by *L. monocytogenes* cells with the concentration of *L.*
203 *monocytogenes* cells present in the substrate (bacterial load) (Buncic et al., 1996; Dewamitta
204 et al., 2010). In the present study, however, it was confirmed that the differences observed in
205 LLO production were not due to differences in bacterial concentration. After 65h of incubation,
206 maximum *L. monocytogenes* bacterial counts (10^9 CFU/mL) were achieved in [0–2.5] % stevia–
207 TSB media at 10 °C and 37 °C and exerted different hemolytic capability: the hemolytic activity
208 of 10^9 CFU/mL at 37 °C (in 0 % stevia–TSB medium) was 100 %, and the hemolytic activity of
209 the same bacterial load at 10 °C in unsupplemented substrate was 8 % (see Figure 1).
210 Although optimal conditions for listeriolysin O production (highly dependent on pH value) have
211 been defined as being in the range 5.5–6 (Bavdek et al., 2011), the results obtained in the
212 present study reveal that pH values close to 7 (7.44 (0 % stevia); 7.37 (1.25 % stevia); and 7.28
213 (2.5 % stevia)) do not condition listeriolysin O secretion under optimal temperature growth
214 conditions (37 °C). These results are in agreement with those obtained by Datta et al. (1993),
215 which indicate better listeriolysin O production with a pH value close to 7.

216 Both incubation temperature and also duration of exposure to stevia at different
217 concentrations are factors that influence the hemolytic capability of the bacterium. In this
218 study, the progression of hemolytic activity during the incubation period was evaluated at 10
219 and 37 °C in the stevia infusions studied. Complete (100 %) hemolytic activity was observed in
220 *L. monocytogenes* cells suspended in TSB–0 % stevia medium during the 65 h incubation period
221 at 37 °C. In stevia supplemented matrices, the higher the exposure time (above 17h) the lower
222 the LLO production. At 37°C, and after 17 h of incubation no significant differences in the
223 hemolytic activity of the bacterium were observed between 1.25 and 2.5 % stevia
224 supplemented substrates. Increasing cell exposure times to stevia (24, 48 and 65h), the higher
225 the stevia concentration, the lower the listeriolysin O production (see Figure 2). Also at 10 °C a
226 reduction in LLO production of *L. monocytogenes* cells was observed , depending on the
227 exposure time to stevia at different concentrations. The hemolytic capability of the bacterium

228 is reduced as much as the longer is the exposure time to stevia: from 15 % after 17 h of
229 exposure to 2 % after 65 h of exposure to a 2.5. % (w/v) stevia concentration (data not shown).
230 According to our results, the effect of stevia inhibiting LLO production is conditioned by
231 exposure time: the higher the exposure time, the higher the effectiveness of stevia in reducing
232 the hemolytic activity of *L. monocytogenes.*

233 The mechanisms by which exposure of *L. monocytogenes* cells to natural antimicrobial
234 substances drastically reduces listeriolysin O production still remain unknown. In spite of this,
235 and according to the studies of Smith-Palmer et al. (2002), under the exposure of *L.*
236 *monocytogenes* cells to essential oils some ribosomal and genetic modifications take place, by
237 means of modifications in the pathogenic potential of the bacterium. The results obtained by
238 Upadhyay et al. (2012) are in agreement with those obtained in the present study regarding
239 exposure to antimicrobial substances. According to Upadhyay et al. (2012), a significant
240 reduction in the hemolytic activity of *L. monocytogenes* was observed when this
241 microorganism was exposed to several natural antimicrobials, such as carvacrol, thymol, and
242 *trans*-cinnamaldehyde, after 12 h at 37 °C. In spite of the innovative results obtained up to
243 date in the reduction of the hemolytic potential of *L. monocytogenes*, additional studies are
244 required deepening in the expression of the listeriolysin (*hly*) in treated/not treated cells under
245 the expossure to natural antimicrobials, to relate the induced stressing conditions (e.g. low
246 temperature; increasing antimicrobial concentrations; food matrices) with the degree of
247 virulence factors expression and its upregulation. Future studies based on the reverse
248 transcription-quantitative polymerase chain reaction (RT-qPCR) technique could be used to
249 investigate the expression profile of the *hly* virulence gene and others (e.g. *plcA*, *iap*) (Rantsiou
250 et al., 2012) after the expossure of *L. monocytogenes* cells to stevia at different
251 concentrations, both in reference substrates and also adding this ingredient to novel real food
252 matrices.
253

254 The present study is providing additional and relevant information complementary to the
255 research articles published up to date regarding the capability of this ingredient to inactivate *L.*
256 *monocytogenes*, by itself or in combination with novel technologies (Rivas et al., 2016).
257 However, the observed reduction in the hemolytic activity of stevia-treated cells should be
258 verified in subsequent bacterial generations. According to the studies of Doyle (2001) the
259 listeriolysin O production capability could be recovered by bacterial populations grown in the
260 gut intestinal tract. So, the assessment of the possible reduction of the *hly* expression in the
261 subsequent generations of *L. monocytogenes* exposed cells to stevia is required. The molecular
262 mechanisms by which the stevia is reducing the hemolytic potential of the bacterium should
263 be investigated also *in vivo*, assessing the impact of regular stevia consumption on the
264 protection of the host against the *L. monocytogenes* cells production of the pore-forming toxin
265 (LLO).
266
267 **4.Conclusions**
268 The hemolytic capability of *L. monocytogenes* cells is affected by the addition of *Stevia*
269 *rebaudiana* Bertoni to the growth medium at different concentrations [1.25–2.5] % (w/v).
270 Under optimal growth conditions (37 °C, in reference medium), the addition of stevia at 2.5 %
271 (w/v) concentration nulled the hemolytic activity of the bacterium. Additionally, a significant
272 effect of incubation temperature on the hemolytic activity of *L. monocytogenes* was observed
273 being the lower the temperature (37 or 10 °C), the lower the production of LLO.

274 High applicability could be obtained from the present study as both storage at refrigeration
275 temperatures and addition of stevia to ready-to-serve or lightly pasteurized products are
276 possible future effective measures to reduce the pathogenicity of *L. monocytogenes* by
277 controlling the production of LLO. More studies should be carried out to consider the
278 effectiveness of *Stevia rebaudiana* Bertoni in real food matrices as an alternative way of
279 inhibiting production of LLO by the bacterium.

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464 **Figure 1**. Hemolysis percentages obtained after 65 h of *L. monocytogenes* cells exposure to
465 *Stevia Rebaudiana Bertoni* (0 %, 1.25 % and 2.5 % (w/v)) in TSB media at 10 °C and 37 °C
466 incubation temperatures.

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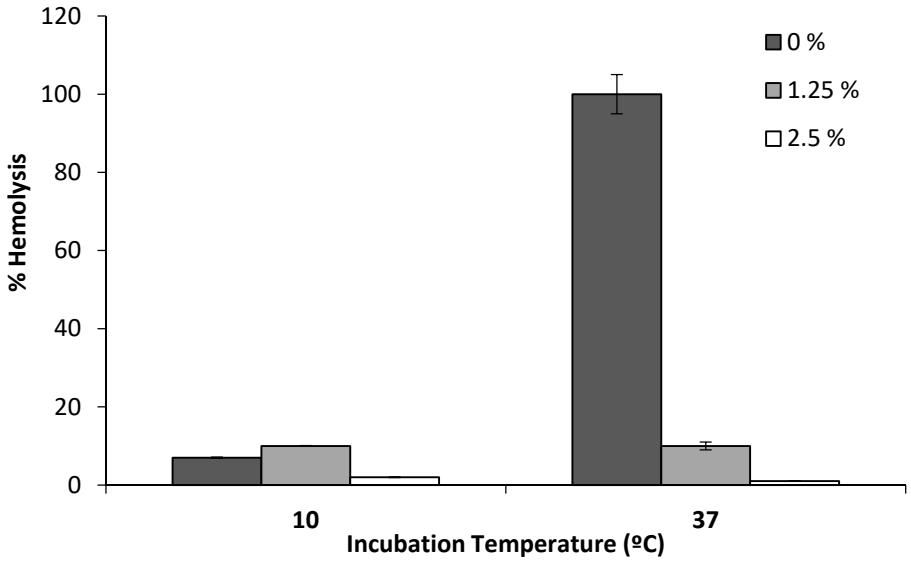

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469 **Figure 2.** Hemolysis percentages obtained after 17 and 65 h of *L. monocytogenes* cells
470 exposure to Stevia *Rebaudiana Bertoni* (0 %, 1.25 % and 2.5 % (w/v)) in TSB media at 37 °C.

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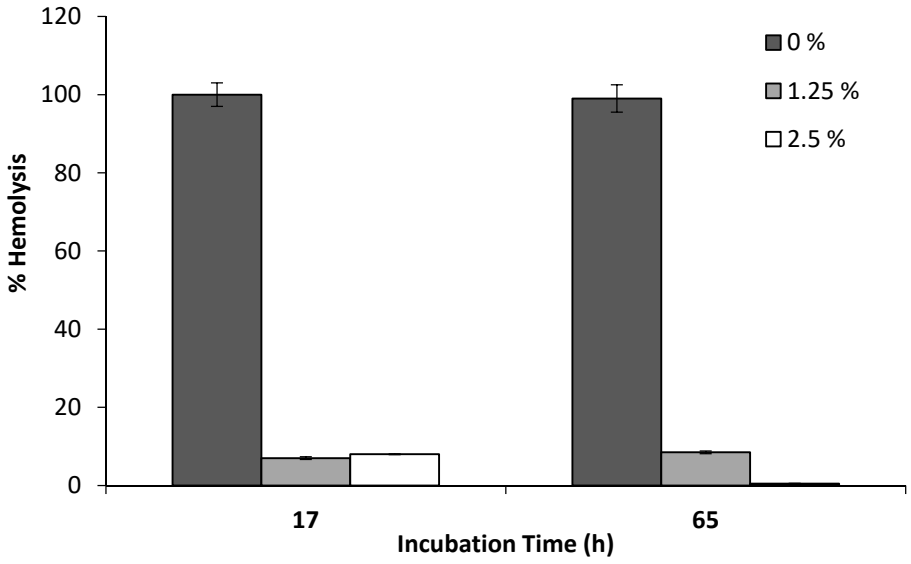

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