Biocontrol of *Listeria monocytogenes* in a meat model using a combination of a bacteriocinogenic strain with curing additives

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ABSTRACT

The aim of this work was to evaluate the effect of meat curing agents on the bioprotective activity of the bacteriocinogenic strain, *Enterococcus* (*E.*) *mundtii* CRL35 against *Listeria* (*L.*) *monocytogenes* during meat fermentation. The ability of *E. mundtii* CRL35 to grow, acidify and produce bacteriocin *in situ* was assayed in a meat model system in the presence of curing additives (CA). *E. mundtii* CRL35 showed optimal growth and acidification rates in the presence of *CA*. More importantly, the highest bacteriocin titer was achieved in the presence of these food agents. In addition, the CA produced a statistical significant enhancement of the enterocin CRL35 activity. This positive effect was demonstrated *in vitro* in a meat based culture medium, by time-kill kinetics and finally by using a beaker sausage model with a challenge experiment with the pathogenic *L. monocytogenes* FBUNT strain. *E. mundtii* CRL35 was found to be a promising strain of use as a safety adjunct culture in meat industry and a novel functional supplement for sausage fermentation, ensuring hygiene and quality of the final product.

1. Introduction

Biopreservation has gained increased attention as means of naturally controlling shelf life and safety of food products. The usage of bacteriocinogenic cultures and/or their bacteriocins, *i.e.* antimicrobial peptides ribosomally synthesized that have antibacterial activity towards closely related strains (Pérez, Zendo, & Sonomoto, 2014), is a relevant bioprotective strategy to control spoilage and pathogenic microorganisms such as *L. monocytogenes*. During the fermentation and drying of cured fermented sausages, *L. monocytogenes* counts tend to decrease substantially. Nevertheless, despite the various hurdles in the dry sausage manufacturing process, this food borne pathogen is able to survive and it is frequently detected in the final product (De Cesare, Mioni, & Manfreda, 2007; Gandhi & Chikindas, 2007). Lactic Acid Bacteria (LAB) have a major potential for their use in biopreservation as they are safe for human consumption (GRAS status) and are the prevalent microbiota during storage in different fermented foods. Many studies have demonstrated bacteriocin antagonism against spoilage LAB, *Brochothrix thermosphacta* and *L. monocytogenes* in cooked, raw and fermented meat products (Vignolo, Castellano, & Fadda, 2015; Giello, La Storia, De Filippis, Ercolini, & Villani, 2018; Chikindas,

Weeks, Drider, Chistyakov, & Dicks, 2018; Oliveira, Ferreira, Magalhães, & Teixeira, 2018). Among LAB, enterococci are ubiquitous microorganisms, representing a large proportion of the native microbiota found in the gastrointestinal tract of humans and animals, but also in some fermented foods and related products including probiotics (Ananou et al., 2010; Fontana, Gazzola, Cocconcelli, & Vignolo, 2009; Todorov, Favaro, Gibbs, & Vaz-Velho, 2012). Most of these microorganisms are able to produce bacteriocins, which play an important role in food preservation. Therefore, they are interesting candidates for guaranteeing the safety of fermented food products (Gaaloul et al., 2015; Gómez-Sala et al., 2016). Nevertheless, the production of bacteriocin is not always accomplished in complex food matrixes such as cheese or fermented sausages. Many other authors have previously demonstrated the causes that may affect both bacteriocin production and activity in food matrices (Verluyten, Messens, & De Vuyst, 2004; Leroy and De Vuyst, 2005; Kouakou, Daouda, & Biego, 2016). In fact, absence of bacteriocin production was observed under production conditions of Greek Feta cheese (Aspri et al., 2017; Sarantinopoulos et al., 2002). Callewaert, Hugas, and De Vuyst (2000) reported that although different bacteriocin-producing *E. faecium* strains displayed strong anti-listeria activity, they were only partially competitive during

Spanish-style dry sausage fermentation. These examples demonstrate that bacteriocin production must be in tune with environmental conditions.

The aim of this work was to evaluate the effect of meat curing agents on the bioprotective activity of the bacteriocinogenic strain, *E. mundtii* CRL35 against *L. monocytogenes* during meat fermentation. *E. mundtii* CRL35 was isolated from an artisanal cheese of Northwestern Argentina (Tafí del Valle, Tucumán, Argentina) (Farias, Farias, Ruiz Holgado, & Sesma, 1996) and it has been the subject of deep biochemical, technological and molecular studies. In fact, its genome was recently sequenced and deeply analyzed (Bonacina, Saavedra, Suarez, & Sesma, 2014). This bacterium produces Enterocin CRL35, a pediocin-like bacteriocin with strong antilisterial and antiviral activity (Saavedra, Minahk, de Ruiz Holgado, & Sesma, 2004; Salvucci, Hebert, Sesma, & Saavedra, 2010; Salvucci, Saavedra, & Sesma, 2007; Wachsman et al., 2003). The technological features of *E. mundtii* CRL35 prove that this bacterium could be an efficient protective culture, able to enhance hygienic quality of fermented dairy products (Farias et al., 1996; Vera Pingitore, Todorov, Sesma, & Franco, 2012). More recently, a genomic overview of food-related and probiotic *Enterococcus* strains including *E. mundtii* CRL35 was reported (Bonacina et al., 2016). *In silico* analysis revealed that the strain does not contain any antibiotic resistance genes, including those related to vancomycin resistance, in agreement to a MIC value lower than 6 µg/mL. In addition, no coding DNA sequences related to known pathogenic markers of clinical relevant enterococcal species where detected in genomic islands (Bonacina et al., 2014; Bonacina et al., 2016). These results are consistent with other studies where virulence factors were mostly detected in *E. faecalis* and among clinical strains (Eaton & Gasson, 2001; Franz, Cho, Holzapfel, & Galvez, 2010). Whole genome-based and orthologous proteins-based phylogenetic analyses demonstrated no correlations between isolation source/probiotic properties and phylogenetic signal, neither at species or strain level; supporting the notion that the selection of enterococci to be used in food processing should be based on a careful determination of safety of each strain.

The present study contributes with solid research to postulate *E. mundtii* CRL35 as an effective bioprotective adjunct culture for cured fermented meat products. The ability of *E. mundtii* CRL35 to grow, acidify and produce bacteriocin *in situ* was assayed in a meat model system in the presence of curing additives (CA). Changes in bacteriocin activity by effect of CA were analyzed by means of time-kill kinetics of *Listeria* cells. In addition, challenge experiments using *E. mundtii* CRL35 and *L. monocytogenes* FBUNT in ground beef with and without curing additives were undertaken.

2. Materials and methods

2.1. Bacterial strain and culture conditions

E. mundtii CRL35, was isolated in 1996 from an artisanal cheese of Northwestern Argentina (Tafí del Valle, Tucumán, Argentina) by Farias et al. (1996) and belong to CERELA (Centro de Referencia para Lactobacilos-CONICET, Tucumán, Argentina) culture collection. Before use, an overnight culture (16 h) was grown in BHI (Brain Heart Infusion) broth at 30 °C, harvested by centrifugation (10,000 $\times g$, 20 min at 4 °C) (IEC Multi RF centrifuge, Thermo Electron Corporation, Altrincham, Cheshire, UK), washed with 20 mM phosphate buffer (pH 6.5) and resuspended in the same buffer, to achieve a bacterial concentration equivalent to an optical density (OD) of 10, measured in a spectrophotometer (Cecil 2021–2041 UV–Visible Spectrophotometer, Cambridge, UK) at 600 nm wavelength ($OD_{600nm} = 10$). This suspension was used to inoculate both the sarcoplasmic model and the beaker sausages.

Listeria innocua 7 and *L. monocytogenes* FBUNT were obtained respectively from the culture collection of INRA (Jouy-en-Josas, France) and the National University of Tucumán in Tucumán, Argentina. Both

strains are widely used as sensitive target organisms for enterocin CRL35 antimicrobial activity with similar enterocin CRL35 MICs values validating *L. innocua* 7 as surrogate microorganism for *L. monocytogenes* FBUNT (Saavedra et al., 2004; Salvucci et al., 2007, 2010; Salvucci, Saavedra, Hebert, Haro, & Sesma, 2012). Before routine use, they were activated in BHI (Merck, Darmstadt, Germany) by incubation at 30 °C for 16–18 h.

2.2. Meat model systems

2.2.1. Sarcoplasmic model (SM)

Ten grams of bovine *semimembranosus* muscle were homogenized with 100 mL of 20 mM phosphate buffer (pH 6.5) for 8 min in a Stomacher blender (Stomacher 400 London, UK). The homogenate was then centrifuged (14,000 x g, 20 min at 4 °C). To obtain the SM, the supernatant containing the sarcoplasmic proteins was filtered with filter-sterilized Whatman paper, through a 0.22 µm-pore-size filter (Steritop GP, Biopore, Buenos Aires, Argentina) and supplemented with 0.5% glucose and 0.01% Tween 80 according to Fadda, Vignolo, Ruiz Holgado, and Oliver (1998). When required, filter sterilized curing additives (3% NaCl, 0.02% $NaNO_2$ and 0.0075% ascorbic acid) were added alone or as a mixture. The sterility of the system was followed by plating on PCA (Plate Count Agar). This model was used as culture medium to evaluate growth kinetics, acidogenic ability and bacteriocin production of *E. mundtii* CRL35.

2.2.2. Beaker sausage (BS)

All operations were carried out in a laminar flow hood. Beef meat (*semimembranosus*) was obtained 24 h post-mortem from cooled carcasses, frozen and aseptically sampled by superficial burning, followed by removal of surface cuts using sterile knives and minced under aseptic conditions according to López, Sentandreu, Vignolo, and Fadda (2015). The sausage formulation included, when required, a solution of curing agents: 3% NaCl, 0.02% $NaNO_2$, 0.0075% ascorbic acid, 0.75% sucrose and 0.75% glucose, previously filtered-sterilized (0.22 µm) (Millipore, Billerica, USA). This meat model was used in the challenge assays with *L. monocytogenes* FBUNT to evaluate the effect of curing additives on the growth and antilisterial activity of *E. mundtii* CRL35.

2.3. Experiment 1: performance of E. mundtii CRL35 on the sarcoplasmic model with curing additives (CA)

The SM, with and without CA, was inoculated with approximately 6 log CFU/mL of *E. mundtii* CRL35 and incubated at 25 °C during 72 h under gentle stirring conditions to keep cells in suspension. A completely randomized 2 × 2 factorial design was applied with repeated measures at 0, 4, 8, 12, 24, 36, 48 and 72 h of incubation. The evaluated factors were: "*Curing Additives*" (absence, presence of food agents as a mixture, or separately added) and "*E. mundtii* CRL35" (presence or absence of this strain). Sterile sarcoplasmic model, with and without the curing additives, was used as control batches. All experiments were conducted in triplicate.

Viability of *E. mundtii* CRL35, pH and bacteriocin activity were evaluated in samples taken from 0 to 72 h of fermentation. For antimicrobial activity, the samples were centrifuged (12,000 × g, 10 min) and cell free supernatants (CFS) collected.

2.4. Experiment 2: time kill kinetics of Listeria cells to evaluate the effect of curing additives on enterocin CRL35 activity

For this trial a 2 × 2 factorial design with three independent replications was applied. In this case one evaluated factor was "*Curing Additives*" (presence or absence) and the other was "*Bacteriocin*" (none, synthetic or naturally produced in CFS). These assays were carried out as described by Salvucci et al. (2007). Briefly, *L. innocua* 7 cells in the exponential growth phase were collected and resuspended in 50 mM

HEPES plus 12.5 mM glucose, an energizing medium where cells are more sensitive (McAuliffe et al., 1998; Minahk, Farías, Sesma, & Morero, 2000). Then, 1×10^7 cells were taken and resuspended in 1 mL of the same buffer. The synthetic enterocin CRL35 (S-EntCRL35) (Saavedra et al., 2004), was synthesized by Genbiotech SRL (Buenos Aires Argentina). A total of 100 μL of S-EntCRL35 (46 pM) or an equivalent concentration of CFS with (+CA) or without (–CA) curing additives were added to the *Listeria* cell suspension (in a final volume of 1 mL). Samples were taken at appropriated times to determine *Listeria* cells viability. Three independent replicates of each time kill curve were performed.

2.5. Experiment 3: challenge experiment in the beaker sausage (BS) model

2.5.1. Experimental design

A completely randomized 2×2 factorial design with repeated measures at 0, 48 and 96 h, with two replications, was used. The "*Curing Additives*" constituted one factor that was evaluated as either presence or absence (BS + CA; BS – CA) and the other factor was "*Bacteria*" evaluated at three levels: presence of *L. monocytogenes* (Lm); presence of *E. mundtii* (Em); presence of *L. monocytogenes* + *E. mundtii* (Lm + Em). A linear mixed model was used for data analysis. *Curing Additives*, *Bacteria* and *Time* constituted the fixed factors and *replicates* were included as random effects.

One kg of Beaker Sausage (BS) was divided into two fractions: i. Fraction 1 without curing additives (BS–CA), ii. Fraction 2 thoroughly mixed with 3% NaCl, 0.02% $NaNO_2$, 0.0075% ascorbic acid, 0.75% sucrose and 0.75% glucose, previously filtered-sterilized (0.22 μm) (Millipore, Billerica, USA) (BS + CA). Both batches (with and without CA) were divided into three portions and inoculated as following: with *L. monocytogenes* alone (4 log CFU/g) (BS–CA + Lm; BS + CA + Lm); with *E. mundtii* CRL35 alone (8 log CFU/g) (BS–CA + Em; BS + CA + Em) or with *L. monocytogenes* (4 log CFU/g) combined with *E. mundtii* CRL35 (8 log CFU/g) (BS–CA + Lm + Em; BS + CA + Lm + Em) and mixed thoroughly. An un-inoculated BS batch, supplemented with antibiotics (20.000 UI/kg penicillin, 20 mg/kg streptomycin, 50 mg/kg amphotericin B and 0.01% Na azide) (Gibco, Grand Island, US) was used as a control according to López, Sentandreu, Vignolo, and Fadda (2015).

All batches were incubated at 25 °C and samples collected at 0, 48 and 96 h of fermentation.

2.6. Bacterial counts and pH measurements

In *Experiment 1*, for *E. mundtii* cell counts, serial ten-fold dilutions were plated on BHI agar (Merck, Darmstadt, Germany), and incubated for 48 h at 30 °C. Total aerobic mesophilic microorganisms were evaluated by plating on Plate Count Agar (PCA, Merck, Darmstadt, Germany). Viability was expressed as log of colony forming units per milliliter (log CFU/mL). The pH values of the samples were determined by using a Metrohn 692 pH/Ion Meter.

Viability of *L. innocua* 7, expressed as CFU/mL, was determined at the indicated times using BHI + 25 μg/mL nalidixic acid agar plates for the experiments of time–kill kinetics (*Experiment 2*).

The viability of inoculated microorganisms in beaker sausage experiments (*Experiment 3*) was measured using the following selective media: Oxford medium for *Listeria monocytogenes* FBUNT, Slanetz-Bartley agar for *E. mundtii* CRL35. Total aerobic mesophilic microorganisms were enumerated using PCA. Viability was expressed as log of colony forming units per gram (log CFU/g).

2.7. Detection of antimicrobial activity of E. mundtii CRL35

E. mundtii CRL35 bears only one bacteriocin gene cluster in its genome (Bonacina et al., 2014). The production and activity of this antilisterial peptide, was measured as described below and according to

the different experiments.

2.7.1. Titration of enterocin CRL35 by spot-on-lawn assay

The inhibitory activity of filtered sarcoplasmic model containing the enterocin CRL35 was determined by the well diffusion assay with some modifications (Salvucci et al., 2007). Briefly, 5 μL of serial two fold dilution of CFS at different sampled times (from 0 to 72 h) were spotted onto an indicator lawn of *L. innocua* 7 and incubated for 16–18 h at 30 °C. The indicator lawn was prepared by adding 10 μL of an overnight culture of *L. innocua* 7 to 10 mL of BHI agar (0.7%). The titer was defined as the reciprocal of the highest dilution giving a visible zone of inhibition of the indicator lawn and expressed in arbitrary units per milliliter (AU/mL).

Cell viability, expressed as CFU/mL, was determined at the indicated times for the experiments of time–kill kinetics with synthetic enterocin CRL 35 or CFS alone or in combination with curing additives against *L. innocua* 7 cells.

2.7.2. In situ antilisterial activity of E. mundtii CRL35

In the *experiment 3* using the beaker sausage model, the bioprotective activity of *E. mundtii* CRL35 was evaluated by measuring the viability of *L. monocytogenes* under the assayed conditions. In this experiment, a bacteriocin titration was not performed since a significant fraction of the peptide might be associated to the target cell or adsorbed to fat and meat particles with the consequent underestimation of its antimicrobial activity.

2.8. Statistical analysis

Data were statistically analyzed using the Infostat Statistical Software (Universidad Nacional de Córdoba, Argentina). Two-way analysis of variance (ANOVA) with the post-hoc LSD Fisher or Tukey test used to evaluate significant differences among samples. Data were analyzed using a mixed model. In this procedure, replicates were included as random effects and inoculation treatment (inoculated with strains or non-inoculated); curing additives addition (presence, absence or each additive separately added) and incubation time were included as fixed effects.

3. Results and discussion

3.1. Growth and acidogenic potential of E. mundtii CRL35 in cured meat environments

Results showed that *E. mundtii* CRL35 was able to optimally grow in the SM in the presence or absence of CA commonly used in sausage production (Fig. 1). Even thought, a slightly increased microbial growth rate was observed for the culture without curing additives, the maximal bacterial counts were attained at 24 h (8.8 log CFU/mL and 8.6 log CFU/mL) for both conditions (Fig. 1a). In agreement with the known tolerance of *Enterococcus* to stress conditions (Foulquié Moreno, Rea, Cogan, and De Vuyst, 2003; Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006), viability was not notably affected by the presence of the mixture of CA under the evaluated concentrations. This aspect was previously described for the bacteriocinogenic strain *E. faecalis* A-48-32, that proved to be well adapted to a sausage model (Ananou et al., 2005). However, other authors have reported negative effects of curing salts on bacteriocinogenic LAB growth (Leroy & De Vuyst, 1999; Verluyten et al., 2004). Nevertheless, the highest bacterial growth was observed between 24 and 36 h when the system was separately supplemented with each additive, mainly with NaCl (10.1 log CFU/mL) (Fig. 1b). On the other side, adequate acidification rates were achieved by *E. mundtii* CRL35 during growth in the SM with or without CA (Fig. 1a, b), in contrast to the known low acidifying potential reported for enterococci (Aymerich, Artigas, Garriga, Monfort, & Hugas, 2000; Sarantinopoulos, Kalantzopoulos, &
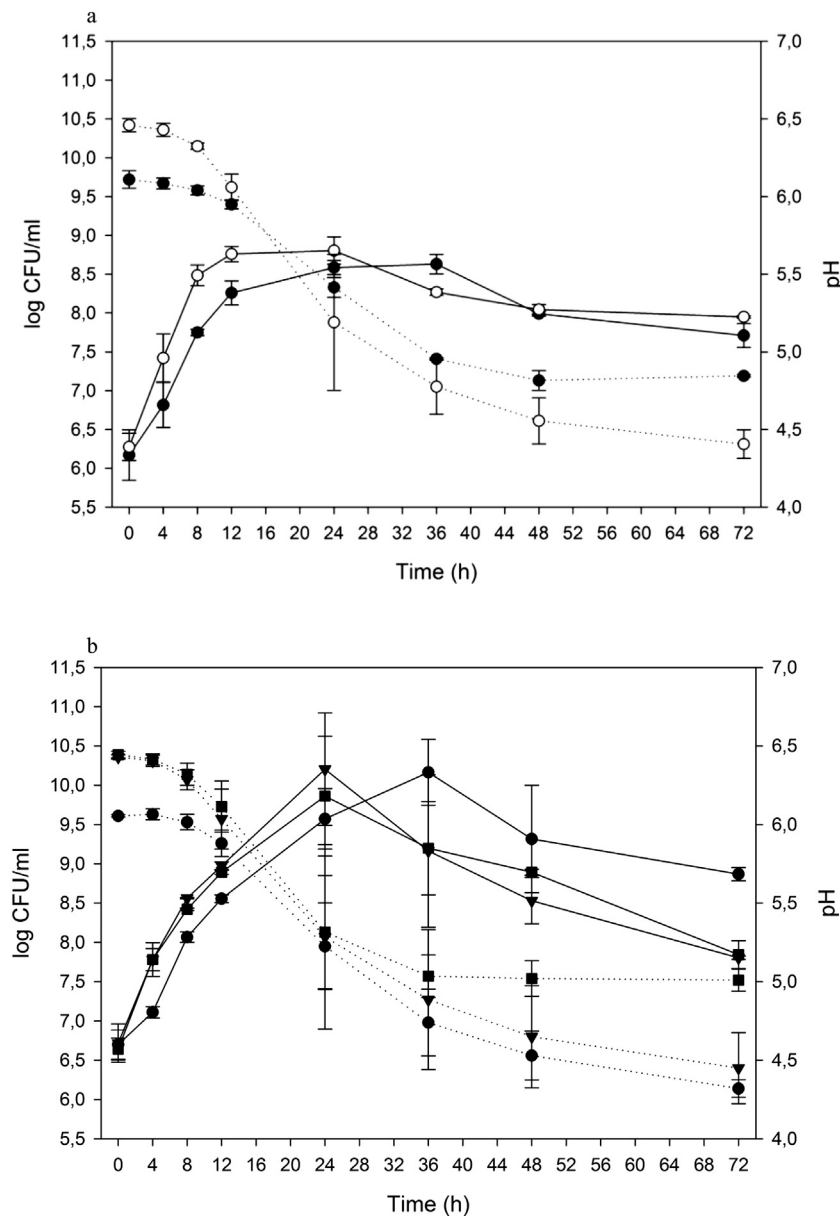
Fig. 1. Growth and pH evolution of *Enterococcus mundtii* CRL35 at 25 °C in Sarcoplasmic Model (SM) for 72 h. (a) SM without curing additives (CA) (o), SM with CA altogether (●), (b) SM with NaNO₂ (▲), NaCl (♦) and ascorbic acid (■). Growth (solid line) and pH (dashed line). Data concern three independent experiments.

Tsakalidou, 2002). In addition, no significant pH changes were observed with or without curing agents throughout the incubation period except at 72 h when a lower pH was detected in CA absence (Fig. 1a). Moreover, cell viability was not dramatically affected by the pH drop throughout the fermentation period. The maximal pH drop occurred in SM without CA and in SM supplemented with NaCl and ascorbic acid (Fig. 1a, b). These results are in accordance with the optimal growth of *E. mundtii* CRL35 under such conditions. The control batches (non-inoculated with and without CA) remained sterile over the incubation time (data not shown).

3.2. Inhibition of Listeria growth by bacteriocin production in SM supplemented with curing additives

A number of factors must be taken into consideration when choosing a strain for *in situ* or *ex situ* antimicrobial production. Particularly, presence and concentration of food additives that play an important role in bacteriocin effectiveness. In this study, high titers of enterocin CRL35 were produced throughout the time in SM without CA

(maximal titers around 4000 AU/mL) (Fig. 2) compared with other bacteriocinogenic LAB such as *Weissella paramesenteroides* (approximately 900 AU/mL) growing in a meat simulation model (Papagianni & Sergelidis, 2013). Noteworthy is the significant increase of bacteriocin activity observed in the presence of CA, especially, in the 24–36 h period. The highest activity (9.600 AU/mL) is in consistency with the maximal growth of *E. mundtii* CRL35 in SM (Fig. 1; Fig. 2). It should be mentioned that CA did not inhibit *Listeria* growth (one by one or as a mixture) (Fig. S1). A moderate decrease of enterocin CRL35 activity was noticed in the 48–72 h period, possibly due to the proteolytic degradation and/or to its adsorption to the producing cell (Kouakou et al., 2009; Leroy & De Vuyst, 2005). The herein presented findings contrast with the widespread assumption that adding salts to meat products might be one of the major causes of reduced efficacy of bacteriocin-producing starter cultures in the food environment. Indeed, many studies reported that curing salts negatively affect the bioprotective potential of LAB strains (Himelbloom, Nilsson, & Gram, 2001; Kouakou et al., 2009; Leroy & De Vuyst, 1999; Settanni & Corsetti, 2008; Todorov et al., 2012). Specifically, it has been shown that *E. faecium*
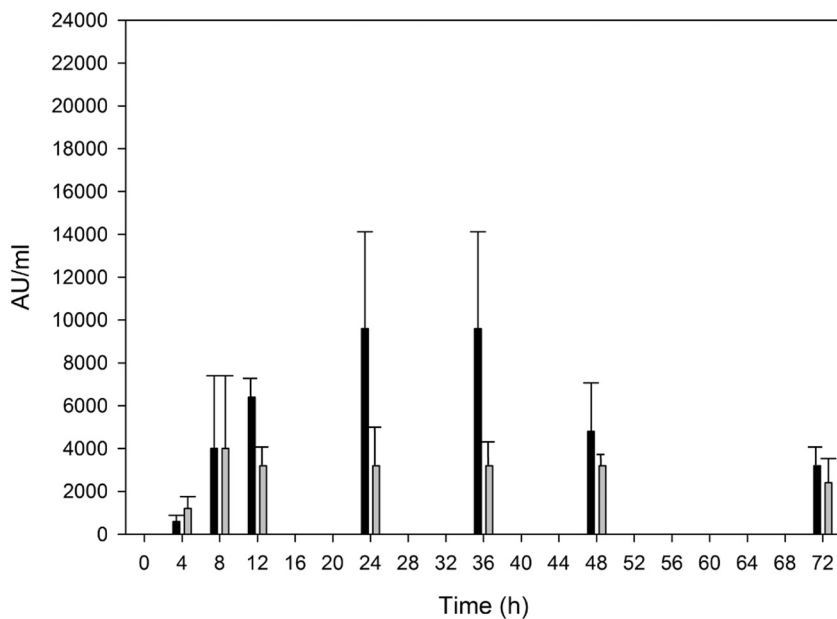
Fig. 2. Bacteriocin production of *E. mundtii* CRL 35 in SM with or without curing additives (CA) at 25 °C during 72 h. Black and grey bars correspond to bacteriocin titers produced in SM with or without CA mixture, respectively. Two- way analysis of variance (ANOVA) was carried out for bacteriocin titers (AU/mL) to evaluate the main effects. The interaction effects between *Treatment* (–CA; +CA) and *time* were also assessed. The *post hoc* Tukey test ($p < 0.05$) was applied to evaluate statistical significances between means.

CTC492 is able to grow and carry out dry sausage fermentation, albeit not being able to produce the A and B enterocins in the presence of salt, pepper and low pH (Aymerich et al., 2000). Moreover, Delgado, Brito, Fevereiro, Tenreiro, and Peres (2005) postulated that bacteriocin production, in the presence of NaCl, is closely related to the number of growing cells in the medium. Accordingly, specific sakacin K production was not affected by nitrite presence, but the bacteriocin titer indirectly decreased because of detrimental nitirite effects on cell growth (Hugas, Garriga, Pascual, Aymerich, & Monfort, 2002). The improved bioprotective potential of *E. mundtii* CRL35 in the presence of the curing mixture should not be associated to its growth as it was not significantly affected by CA. In consistency with such findings, Castro, Palavecino, Herman, Garro, and Campos (2011) found that environmental factors enhanced bacteriocin activity against target organisms. Specifically, ascorbic acid and EDTA were proposed as responsible for the enhanced bacteriocin activity of a *Lactobacillus* strain, isolated from a dry sausage, against *Staphylococcus aureus.* Although, the authors also established a negative effect of NaCl on bacteriocin production.

3.3. Effect of curing additives on the antilisterial activity of enterocin CRL35. Time-kill kinetics

Complementary experiments were aimed to evaluate enterocin CRL35 and curing additives interaction by means of time-kill kinetics.

Firstly, *Listeria* cells in buffer containing glucose were either incubated in the presence or absence CA mixture. Results showed no significant changes in *Listeria* counts in both conditions demonstrating that these compounds do not affect target cells by themselves (Fig. 3, control with and without CA). Subsequently, aliquots of *Listeria* cells were either incubated with synthetic enterocin CRL35 (S-Ent CRL35) or CFE (cell free meat extracts, containing enterocin CRL35), both supplemented or not with the CA mixture. Results showed a faster decrease in the number of *Listeria* cells when CA were added to the reaction mixture in both conditions (with S-Ent CRL35 and CFE) (see Fig. 3). Thereby, the killing efficiency of both S-Ent CRL35 and CFE-containing enterocin CRL35 was increased by the CA mixture. In fact, a rapid decrease in the number of viable cells (with a maximum of 54% cell death) was observed in the first minutes of incubation. CFS supplemented with the CA mixture showed similar *Listeria* death percentage (51%). On the other hand, in the absence of CA mixture, lower antilisterial activity of the S-EntCRL35 or CFE was observed, reaching only 26% of cell death (Fig. 3). These findings suggest an interaction
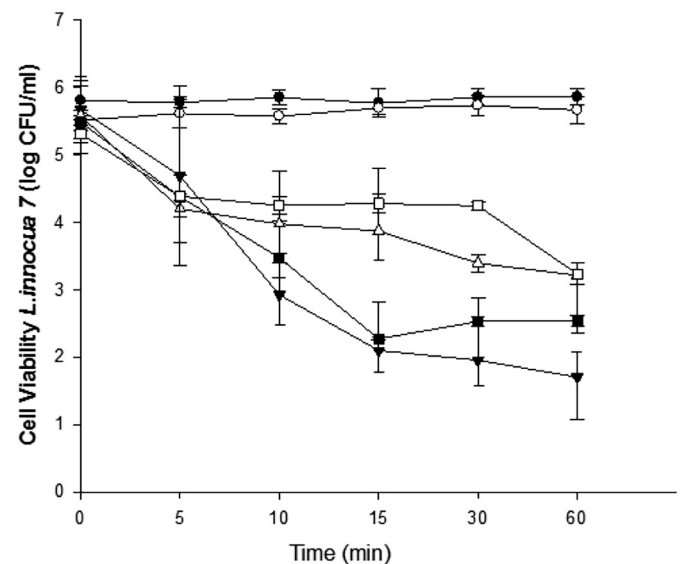

Fig. 3. Time-kill kinetics of S-Ent CRL35 (synthetic enterocin CRL35) and cell free meat supernatants (CFS) with and without CA mixture against *L. innocua* 7. *Listeria* cells (logCFU/mL) with (●) and without (○) CA mixture (Control); *Listeria* cells + S-Ent CRL35 with (▼) and without (△) CA mixture, *Listeria* cells + CFS with (■) and without (□) CA mixture. Error bars represent standard deviations. Data concern three independent experiments.

between the antimicrobial peptide and CA resulting in an increased antilisterial activity of the enterocin CRL35 (Fig. 3).

Several mechanisms have been proposed to explain the effects of CA on bacteriocin activity *i.e.* nitrite-triggered repression of bacteriocin expression (Benkerroum, Daoudi, & Kamal, 2003); nitrite binding to bacteriocin-targeting pole (Dykes & Hastings, 1998; Ennahar, Sashihara, Sonomoto, & Ishizaki, 2000); sodium chloride inhibition of the bacteriocin induction factor binding to its receptor (Nilsen, Nes, & Holo, 1998) or the enhancement of *Listeria* resistance through nitrite induced stress (Gravesen et al., 2002). The results herein presented show that curing agents, at the concentrations and conditions assayed, improve enterocin CRL35 antimicrobial activity. Although, further studies must be done to elucidate molecular mechanisms involved in this phenomenon; a possible speculation would be the stabilization of the peptide by the effect of salts or ascorbic acid.

a

E. mundtii CRL35

b

L. monocytogenes FBUNT
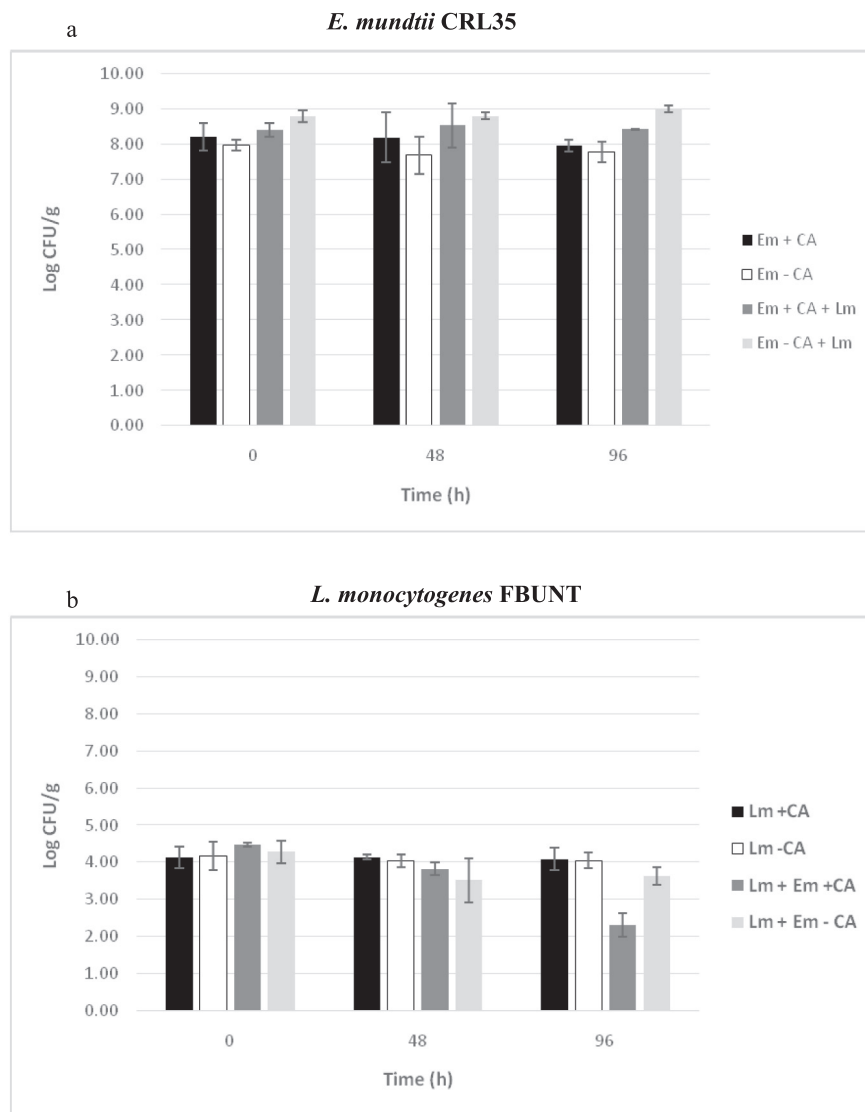
Fig. 4. Viability of *L. monocytogenes* FBUNT and *E. mundtii* CRL35 grown alone or in co-culture in the beaker sausage model, with or without curing additives (CA) during 96 h at 25 °C. (a) **E. mundtii CRL35 (Em) counts**: Em + CA (black bars), Em–CA (white bars), Em + *L. monocytogenes* FBUNT (Lm) + CA (dark grey bar), Em + Lm–CA (light grey bar). (b) **L. monocytogenes FBUNT (Lm) counts**: Lm + CA (black bars), Lm–CA (white bars), Lm + Em + CA (dark grey bars), Lm + Em–CA (light grey bars). Data concern three independent experiments.

3.4. *E. mundtii CRL35 and the enterocin CRL35 effectiveness in a solid meat matrix. Challenge experiments in beaker sausage model*

Challenge experiments in a beaker sausage model aimed to conduct the research in conditions that are similar to those of the meat processing industry. BS represents a useful model to evaluate the performance of *E. mundtii* CRL35 and enterocin CRL35 effectiveness in a solid meat matrix when a pathogenic strain such as *L. monocytogenes* FBUNT is co-inoculated. The beaker was inoculated with 8 log CFU/g of *E. mundtii* CRL35, and as shown in Fig. 4a it is able to maintain its viability throughout the incubation time (96 h; 25 °C). This applies to both conditions, when it was inoculated alone or co-inoculated with *L. monocytogenes* FBUNT. In addition, the presence of CA does not affect *E. mundtii* CRL35 viability (Fig. 4a). On the other hand, *Listeria* cells did not show significant differences growing alone in the meat matrix supplemented or not with CA in agreement with the *in vitro* assay where *Listeria* viability was not affect in presence of CA (Fig. 4b; Table 1; Fig. S1). When the pathogen was in competition with *E. mundtii* CRL35 in the meat matrix without CA (BS–CA), a slight *L. monocytogenes* inhibition was observed at 96 h (0.66 log CFU/g decrease) (Fig. 4b). However,

Table 1

Viability of *L. monocytogenes* FBUNT (Lm) grown individually or co-inoculated with *E. mundtii* CRL35 (Em) in the beaker sausage model, with (+) or without (−) curing additives (CA) during 96 h at 25 °C. LSD Fisher test (alpha = 0.05).

Batch	CA	Time	Mean[a]	SE
Lm + Em	+	0	4.47[*] A	0.21
Lm ± Em	−	0	4.28 A B	0.21
Lm	−	0	4.18 A B	0.21
Lm	+	48	4.14 A B	0.21
Lm	+	0	4.12 A B	0.21
Lm	+	96	4.09 A B	0.21
Lm	−	96	4.05 A B	0.21
Lm	−	48	4.04 A B	0.21
Lm + Em	+	48	3.82 A B	0.21
Lm + Em	−	96	3.62 A B	0.21
Lm + Em	−	48	3.51 B	0.21
Lm + Em	+	96	2.30 C	0.21

[a] Mean values are the result of three determinations ($n = 3$).
[*] Results are expressed as log CFU/g (mean), SE (standard error). Means with common letters are not significantly different ($p > 0.05$).
LSD Fisher (Alfa = 0.05). Procedure for the correction of p-values: Benjamini & Hochberg.

the antilisterial activity of *E. mundtii* CRL35 was enhanced when BS model was supplemented with the curing mixture (BS + CA), reaching a significant decrease of *L. monocytogenes* viable cells at 96 h (> 2 log CFU/g of BS) (Fig. 4b; Table 1). These results demonstrate that the presence of both, bacteriocinogenic *E. mundtii* CRL 35 and CA mixture, are necessary to achieve the highest inhibitory activity over *L. monocytogenes* in ground meat. It can be postulated that enterocin activity plays a major role in the antagonistic action towards *Listeria* cells, although other inhibitory compounds and/or interaction mechanisms cannot be discarded (Giello et al., 2018). In consistency with the Sarcoplasmic Model and time kill kinetics assays, challenge experiments in the Beaker Sausage model demonstrate the enhancing effects of CA on the bioprotective action of *E. mundtii* CRL35 against the pathogenic *L. monocytogenes* FBUNT strain. These results are promising because they refer to a LAB strain capable of exerting a higher bioprotective effect under the curing conditions used in fermented sausage production.

4. Conclusion

Taken together, these results indicate a high performance of *E. mundtii* CRL35 in a cured meat environment, involving effective bioprotective activity, adequate growth and technological potential evidenced by the optimal pH decrease. The curing additives are proposed as improving factors for bacteriocin activity not related to the growth of the producing strain. In fact the evaluated curing agents (NaCl, NaNO$_2$ and ascorbic acid) produced a statistical significant enhancement of the antilisterial activity of enterocin CRL35. This positive effect was demonstrated *in vitro* in a meat based culture medium, by time-kill kinetics and finally by using a beaker sausage model with a challenge experiment with the pathogenic *L. monocytogenes* FBUNT strain. It should be taken into account that other soluble inhibitory products from *E. mundtii* CRL35 metabolism and mechanisms of cell-cell interaction could also contribute to the whole antagonistic activity produced towards *Listeria* cells. *E. mundtii* CRL35 is therefore a promising strain that can be used as an adjunct culture to play a significant role as a novel functional supplement in sausage fermentation, ensuring hygienic quality of the final product.

Acknowledgements

Competing interests

Authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.02.043.

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