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Anti-*Listeria monocytogenes* effect of bacteriocin-incorporated agar edible coatings applied on cheese

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ABSTRACT

Bacteriocins synthesised by lactic acid bacteria are natural alternatives to control *Listeria monocytogenes*, a foodborne pathogen. Additionally, antimicrobial biopolymer coatings have significant benefits in food packaging and preservation. The effect of anti-*L. monocytogenes* enterocins synthesised by *Enterococcus avium* DSMZ17511 was evaluated when supported on agar edible films and applied as antimicrobial coatings on different cheese matrices artificially contaminated with *L. monocytogenes* 01/155. A decrease of at least 1 log unit in viability of the pathogen was detected. The wetter matrix of soft cheese facilitated rapid diffusion of the antimicrobials, while the drier matrix of semi hard cheese produced a gradual release with prolonged inhibition of the pathogen. Also, DSMZ17511 antimicrobial peptides, only at very high concentrations, exhibited mild cytotoxicity against L929 and Caco-2 cell lines. Therefore, application of these enterocin agar coatings could be an effective, low cost, natural and safe alternative to control *L. monocytogenes* in cheeses.

1. Introduction

Listeria monocytogenes is a pathogen widely distributed in nature and considered a serious food safety problem. This pathogen can cause listeriosis; a disease with a low incidence compared with other foodborne illnesses, but with a high mortality rate (Melo, Andrew, & Faleiro, 2015). This ubiquitous pathogen is capable of overcoming most common microbiological barriers applied during food processing (acidity, refrigeration, desiccation, etc.; Luber et al., 2011). As a result, listeriosis is mainly acquired by the ingestion of contaminated ready to eat (RTE) food ($<10^3$ cfu mL^{-1}), especially when kept under refrigeration for long periods (FAO/WHO, 2004). Despite control actions implemented to prevent this food-borne disease, a steady increase of listeriosis reports is still being detected in European Countries (ECDC, 2018; Goulet, Hedberg, Le Monnier, & de Valk, 2008).

Among the natural strategies proposed for control of this foodborne pathogen are bacteriocins; extracellular cationic peptides excreted by producer cells that inhibit or stop the growth of bacteria from closely related species, commonly by adsorption to specific receptors on the outer surface of the sensitive bacterium, followed by metabolic, biological and morphological changes (Jack, Tagg, & Ray, 1995). Bacteriocins synthesised by lactic acid bacteria (LAB) have gained interest due to their potential as natural and safe food biopreservatives (Davidson, Cekmer, Monu, & Techathuvanan, 2015). In particular, bacteriocins synthesised by the genus *Enterococcus*, called enterocins, are distinguished for their strong listericidal effect (Eijsink, Skeie, Middelhoven, Bente Brurberg & Nes, 1998). Despite the GRAS (Generally Recognised As Safe) status of LAB, their metabolites do not receive this status directly, so it is necessary to ensure their toxicological safety (Khan, Flint, & Yu, 2010). In fact, nisin is the only bacteriocin that has achieved this approval, in more than 50 countries (Delves-Broughton, 2005).

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Other bacteriocins, like pediocin PA-1/AcH (ALTA™2341, MicroGARD™) and lacticin 3147, are commercialised as raw concentrates obtained by cultivation of the producer strain in a food-grade substrate (such as milk or whey). These bacteriocin preparations still need to be considered food additives from the legal view-point (Balciunas et al., 2013).

Although direct introduction constitutes the standard method for application of antimicrobials in food products, this technique could cause undesirable interactions with food components and an inefficient distribution in the food matrix, reducing antimicrobial effectiveness. Inclusion of bacteriocins in edible functionalised coatings is an interesting option since only the minimum amount of antimicrobial is used and, it is not incorporated as a direct food additive (Yildirim, 2011). The films are preferably formed from natural polymers (polysaccharides, proteins, polyesters, lipids) acting as a continuous matrix as well as a support for substances such as antimicrobials, antioxidants, dyes, flavourings, etc., becoming "active or functionalised films" (Cagri, Ustunol, & Ryser, 2004). In addition to the biodegradability of its components, active coatings distinguish for the surface retention of the active agents, controlled diffusion, retention of a high concentration of the additive where it is necessary and, the possibility of acting together with other conservation methods (refrigeration, controlled atmosphere, etc.).

Cheese is a RTE product, usually not exposed to heat treatment before consumption, and kept under refrigeration temperatures, conditions that allow the survival and growth of psycrotrophic bacteria, such as *L. monocytogenes* (Melo et al., 2015). This microorganism is able to survive during the elaboration, fermentation, ripening and storage of various dairy RTE products (Bhatti, Veeramachaneni, & Shelef, 2004). Indeed, contaminated soft-textured cheeses have been implicated in several listeriosis reported outbreaks (Koch et al., 2010; Silk et al., 2013).

Previous work showed the antimicrobial activity of *Enterococcus avium* DSMZ17511 (ex PA1) cell free supernatants (CFS) against different *L. monocytogenes* strains (Audisio, Terzolo, & Apella, 2005; Ibarguren, 2010). In the present study, the antimicrobial compounds present in *E. avium* DSMZ17511 CFS were supported on food grade agar films applied as coatings on cheeses and evaluated for the control of *L. monocytogenes*, a serious problem associated with these RTE products.

2. Materials and methods

2.1. Strains and culture conditions

E. avium DSMZ17511 (ex PA1), previously isolated and characterised (Audisio et al., 2005), was used as the bacteriocin producer strain. *L. monocytogenes* 01/155 (GenBank Access N°JN086994) was utilised as the antimicrobial activity indicator strain. The cultures were activated by successive transfers in brain-heart infusion broth (BHI; Britania, Buenos Aires, Argentina) and incubated at 37 °C for 16–20 h. When agar media was required, 1.5% (w/v) agar (Britania) was added to the culture media. All strains were maintained at −20 °C in BHI broth with the addition of 10% (v/v) glycerol.

2.2. Enterocin solution

E. avium DSMZ17511 was cultured in BHI broth (37 °C, 16 h) and the cells were removed from the fermentation broth by centrifugation (10,000× *g*, 15 min, 10 °C), neutralised to pH 6.0 with 0.1 N NaOH and filter-sterilised using cellulose acetate membrane filters (0.22 μm, Sartorius AG, Göttingen, Germany). The resulting cell free supernatant (CFS) was considered the enterocin solution (ES). A fraction of this ES was concentrated by precipitation with 75% $(NH_4)_2SO_4$. The resultant precipitate was re-suspended in sterile distilled water (approximately 10% of starting ES volume) and labelled ESp. A fraction of this ESp suspension was desalted by 3 cycles of centrifugation (6000× *g*, 30 min, 25 °C) using Millipore Amicon® Ultra-15 centrifugal filter tubes (3 kDa cut off; Merk Milllipore Ltd., Cork, Ireland), to achieve a retained volume equivalent to 10% of the starting ESp volume. This concentrated fraction was considered the enterocin concentrated solution (ESc). Also, a fraction of non-cultured media (BHI) was treated the same as the ES, and the obtained concentred BHI (BHIc), was used as a control in some experiments. ES, ESc, BHI and BHIc were kept at 4 °C until used.

The antimicrobial titres of the ES and ESc were determined by the serial dilution method and expressed in arbitrary units (AU) mL^{-1} (Daba et al., 1991; Ibarguren, Raya, Apella, & Audisio, 2010a), using *L. monocytogenes* 01/155 as the indicator strain. The titre determined for ES and ESc were 3200 AU mL^{-1} and 409,600 AU mL^{-1}, respectively.

2.3. Characterisation of antimicrobial compounds present in the ES

2.3.1. SDS-PAGE analyses

To determine the antimicrobial compounds molecular size, the peptides present in the CFS of *E. avium* DSMZ175111 were separated by Tricine-SDS-PAGE, as described by Schägger (2006), modified by Ibarguren et al. (2010a). A Low Range Rainbow™ Molecular Weight Marker was used, with masses ranging from 3.5 to 38 kDa (GE Healthcare, Buckinghamshire, UK). After the run, the gel was fixed with an isopropanol (20%, v/v) - acetic acid (10%, v/v) solution, exhaustively washed with distilled water and overlaid on BHI agar inoculated with *L. monocytogenes* 01/155 (approximately 1×10^7 cfu mL^{-1}) for detection of antimicrobial activity. After incubation (24 h, 37 °C) the presence of inhibition halos was examined.

2.3.2. PCR amplification of enterocin genes

The presence of enterocin-encoding genes was studied in *E. avium* DSMZ17511 by PCR amplification with primers for the following well-known enterococcal bacteriocins: enterocin A; enterocin B; enterocin P; enterocin 31 and enterocin L50 A/B (du Toit, Franz, Dicks, & Holzapfel, 2000). Chromosomal DNA isolation and PCR reactions were performed as described by Ibarguren et al. (2010a). *Enterococcus faecium* CRL1385 was used as positive control for enterocin A, enterocin B and enterocin P (GenBank Access N° GQ369791, GQ369790, FJ57726, respectively).

2.3.3. In vitro cytotoxicity

The 3-(4,5-dimetildiazol-2-yl)-2,5 diphenyltetrazolium (MTT) test was used to assess ES and ESc in vitro antimicrobial compounds cytotoxicity against L929 cells (murine fibroblast CCL.2 ATCC) and Caco-2 cells (epithelial cells derived from human colon adenocarcinoma HTB-3 ATCC). Adherent cells were cultured in 96-well microplates, incubated for 16–20 h (37 °C, 5%, v/v, CO_2) until monolayers were formed. Cells were treated with 5 μL of either ES (3200 AU mL^{-1}) or ESc (409,600 AU mL^{-1}) and maintained in incubation conditions for 24 h. After this period, the medium was discarded and replaced with 100 μL of medium supplemented with MTT solution (final concentration: 0.5 mg mL^{-1}) and the plates were incubated in darkness (37 °C, 4 h). The resulting dark blue formazan precipitate was dissolved in 100 μL dimethylsulfoxide (DMSO) and absorbance was measured spectrophotometrically (A_{570nm}). Cells incubated with culture medium were used as a control of cell viability (100%). Also, the culture medium (BHI) used to obtain the ES/ESc was included as a control treatment.

The percentage of viable cells after treatment with the peptides was calculated according to Paiva et al. (2012) as $A_t/A_c \times 100$, where A_t and A_c are A_{570nm} of treated and control cells (100% survival), respectively.

A_{570nm} measures of three independent assays were expressed as mean ± standard deviation and subjected to analysis of variance (ANOVA) and Tukey's test. The differences were considered significant for $P < 0.05$.

2.4. Evaluation of the anti-L. monocytogenes effect of agar-ES coatings applied on different cheese matrices

2.4.1. Film preparation

Prior to cheese trials, the amount of agar to be used for the coatings was defined. A commercial food-grade agar obtained from a local pastry supplier (BM, Salta, Argentina) was used as the edible film matrix. Films were prepared by dissolving different amounts of agar (5%, 2.5%, 1.25% and 0.8%, w/v) in boiling water with the addition of glycerol as a plasticiser in a 20% (w/w) ratio relative to the polymer. To obtain active films, 5 mL of *E. avium* DSMZ17511 ES (3200 AU mL^{-1}) was added to each film-forming solution and poured into plastic Petri dishes (Ø = 9 cm). The final titre of enterocins was approximately 250 AU cm^{-2} of dry film, calculated according to Ibarguren, Vivas, Bertuzzi, Apella, and Audisio (2010b). In addition, films formulated with agar of each concentration (5%, 2.5%, 1.25% and 0.8%, w/v), pure solvent (distilled water), and glycerol were prepared as controls. Once cast, the films were oven-dried with air circulation at 35 °C and stored at 25 °C in closed containers.

The inhibitory activity of the antimicrobial compounds supported on the different films was evaluated against *L. monocytogenes* 01/155 using an adaptation of the agar diffusion technique (Ibarguren et al., 2010b). Briefly, strips of 1 cm^2 of active films with ES incorporated or controls, previously sterilised under UV light, were placed on a *L. monocytogenes* 01/155 lawn (10^7–10^8 cfu mL^{-1}) and the presence of inhibition halos was determined after incubation for 24 h at 37 °C.

2.4.2. In situ evaluation of the antimicrobial effect of bioactive agar coatings applied on cheeses artificially contaminated with *L. monocytogenes*

Antimicrobial tests were carried out on two type of cheese matrices: commercial Tybo cheese (semi-hard and semi-fat cheese) and handcraft goat cheese (soft to semi-hard and semi-fat to fat cheese), acquired in the local municipal market. In both cases, cheeses were aseptically fractionated with a punch into pieces, 2 cm diameter and approximately 5 g each.

According to results in section 2.4.1, food grade agar solution (0.8%, w/v) added with glycerol 20% (w/w, relative to the polymer) as plasticiser, was used as the coating. For active coatings, ES was added to the agar solution to obtain a final concentration of approximately 250 AU cm^{-2} of dry film. Both type of cheese (Tybo and Goat Cheese) were arranged into different groups: (A) uncoated control, (B) agar coated and (C) active agar coated (ES added). For coating, each fractionated cheese was immersed for 10 s in the corresponding warm agar suspension (with or without SE addition), and placed, by groups, on plastic grids to facilitate drainage of excess agar suspension and drying of the film (Fig. 1A,B). The coated cheeses were dried 2 h at 30 °C and then, 24 h at 25 °C, and immediately used for the following experiment.

Once dried, groups of 20 cheeses from the three systems (A, B and C) were contaminated by immersion for 10 s in a suspension of *L. monocytogenes* 01/155 in peptone water (approximately 10^5 cfu mL^{-1}). In addition, groups of 20 cheeses of each system, not contaminated with the pathogen, were included as controls (labelled A_c, B_c and C_c) (Fig. 1C). Also, a fraction of the suspension of *L. monocytogenes* 01/155 was set as the microorganism growth control. Samples of cheeses of each system were individually placed in wells of sterile microplates (BD Falcon™, 12 wells; BD Biosciences, Bedford, USA). Five microplates (one for each survival sampling point) were prepared (each with three uncontaminated systems and three other systems contaminated with the pathogen, each system in duplicate) (Fig. 1C), as well as two tubes with the control suspension of *L. monocytogenes* 01/155. All microplates and both tubes, were placed in hermetic containers and stored under refrigeration (4–8 °C) and, the survival of the pathogen was followed on each cheese system over 2 weeks (sampling at 0, 2, 6, 8, and 14 days). Soaked cotton was placed in the containers to maintain the cheese moisture in the refrigerator during the test.

For *L. monocytogenes* counts, a homogenate of each cheese sample (approximately 5 g) was prepared in 45 mL of sterile peptone water using sterile bags. Decimal dilutions were prepared from this homogenate and plate counts of the pathogen were done using *Listeria* Oxford modified agar (Biokar, Paris, France).

The analysis of the survival of *L. monocytogenes* in contaminated cheeses consisted of two replicates with the experiment performed in duplicate (n = 4). The results were expressed as mean ± standard deviation.

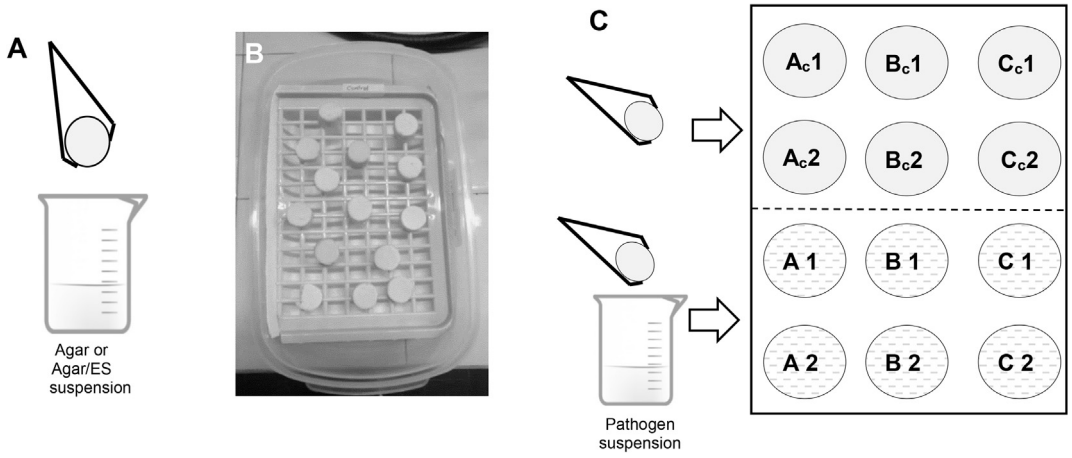

Fig. 1. Cheese coating by immersion in warm agar suspensions (A), coated cheeses arranged in individual containers over drainage racks (B) and diagram of the microplate used for the *L. monocytogenes* 01/155 survival test cheeses (C). In panel C, codes are: A_c, cheeses without treatment; B_c, cheeses coated with 0.8% (w/v) agar film; C_c, cheeses coated with 0.8% (w/v) agar/ES functionalised film; A, B and C (1 and 2 indicate replicas) correspond to the same systems contaminated by immersion in a suspension of *L. monocytogenes* 01/155.

The general microbiological status of the samples was also controlled during the experiment.

3. Results

3.1. Characterisation of antimicrobial compounds present in the ES

The analysis of *E. avium* DSMZ17511 ES proteins by Tricine-SDS-PAGE, and further antimicrobial assays against *L. monocytogenes* 01/155, showed a broad band below 8.5 kDa (Fig. 2A). This band could not be resolved even by lowering the sample loading volume; suggesting that this strain synthesised one or more antimicrobial compounds with a molecular mass <8.5 kDa. Additionally, the study of different structural enterocin genes in *E. avium* DSMZ17511 revealed the presence of both *entA* and *entB* genes. Specific PCR fragments of 126- and 162-bp, corresponding respectively to enterocins A and B, were amplified (Fig. 2B). *E. faecium* CRL1385 was used as a positive control for *entA* and *entB* (data not shown). No specific PCR fragments were observed for bacteriocins P, L50 A/B, or 31.

3.2. In vitro cytotoxicity evaluation of the antimicrobial peptides present in the ES

Analysis performed on Caco-2 cells did not show significant differences between the values for cell viability (A_{570nm}) of ES treatments compared with the negative control (Fig. 3A). Although no marked cytotoxicity was observed, there was a decrease in the percentage of viable cells treated with concentrated culture media (BHIc) and concentrated antimicrobial solution (ESc), with values of 94% and 82%, respectively. In the case of the L929 cell line, no cytotoxicity was observed for the amounts of ES tested (Fig. 3B). In contrast, all the treatments (ES, ESc, BHI and BHIc) produced an increase in A_{570nm}, with percentages of viable cells of 263%, 248%, 209% and 167%, respectively, suggesting an induction in cell proliferation by some component of the BHI culture medium.

3.3. Evaluation of the anti-*L. monocytogenes* effect of active coatings applied on different cheese matrices

3.3.1. Film preparation

The antimicrobial activity of the films made with different agar concentrations was analysed by the agar diffusion technique. No significant differences were observed in the size of the inhibition halos, with values equal to 23, 22.5 and 22 mm, for 2.5% (w/v), 1.25% (w/v) and 0.8% (w/v) agar films, respectively (Fig. 4). This result indicates that these agar concentrations do not affect the diffusion of the bacteriocins supported on this food grade matrix. Also, in all cases, inhibition halos were observed around as well as below each agar film.

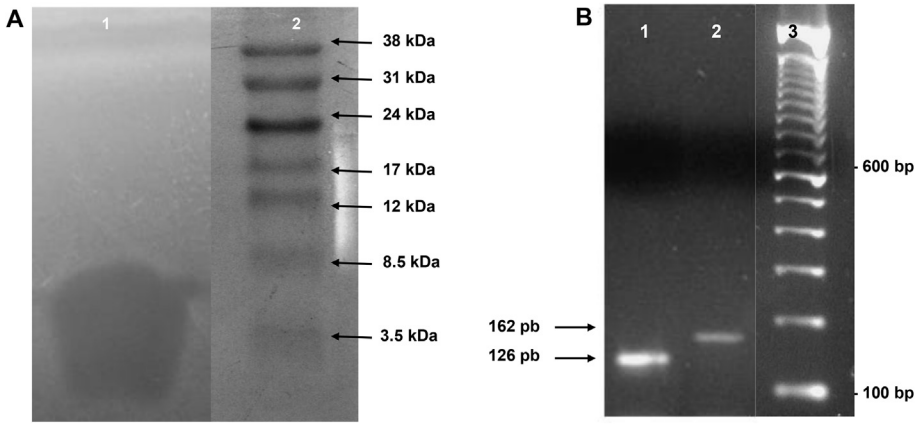

Fig. 2. Tricine-SDS-PAGE gel (A) showing the zone of growth inhibition of *L. monocytogenes* 01/155 by the band of the ES from *E. avium* DSMZ17511 (lane 1; lane 2 is the molecular mass marker) and amplification of enterocin A and B structural genes (B) from DNA of *E. avium* DSMZ17511: lanes 1, DSMZ16511 (*ent A*); lane 2, DSMZ16511 (*ent B*); lane 3, 100 bp ladder.

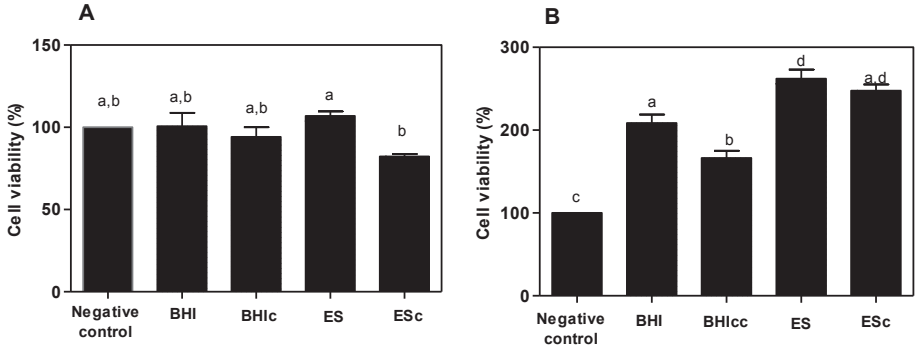

Fig. 3. Cellular viability (A_{570nm} measurements according to the MTT technique): (A) Caco-2 cells and (B) L929 cells, after incubation with the different treatments; different letters over the bars indicate significant differences among the treatments. Abbreviations are: BHI, culture medium used to obtain ES; BHIc, concentrated culture medium by precipitation with ammonium sulphate (75%) and filtration-centrifugation; ES, *E. avium* DSMZ17511 CFS without treatment; ESc, *E. avium* DSMZ17511 CFS concentrated by precipitation with ammonium sulphate (75%) and filtration-centrifugation; Negative control, cells incubated with MEM culture medium (cell control).

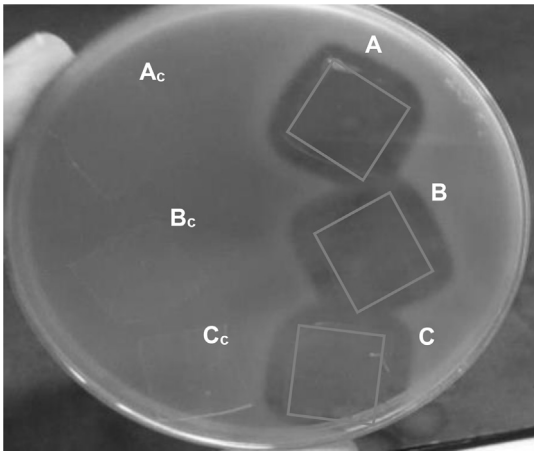

Fig. 4. Effect on *L. monocytogenes* 01/155 of *E. avium* DSMZ17511 ES supported on films of A) 2.5% (w/v) agar, B) 1.25% (w/v) agar, C) agar 0.8% (w/v) agar, according to agar diffusion technique; A_c, B_c, and C_c are control films prepared without ES addition.

3.3.2. Evaluation of the anti-*L. monocytogenes* effect of the active agar coatings applied on artificially contaminated cheeses

As no significant differences were detected among the agar films, the matrix with the lowest proportion of polymer (0.8%, w/v) was selected, since the same effect could be achieved at a lower cost. On this basis, different Tybo and handcraft goat cheese systems were prepared to examine the effect of bioactive 0.8% (w/v) agar coatings applied on cheeses contaminated with *L. monocytogenes*.

For the tests with Tybo commercial cheese (Fig. 5A), the pathogen remained viable in the peptone water suspension used as a growth control, during the 2 weeks of experiment. In the first 2 days, viability slightly increased and subsequently remained stable approximately 6 log cfu mL^{-1} until the end of the test (Fig. 5A, solid lines). In cheeses without agar coating and contaminated with *L. monocytogenes* 01/155 (system A), growth of the pathogen remained practically constant (approximately 4.2 log cfu mL^{-1}) throughout the trial (Fig. 5A, dashes). In the case of cheeses coated with 0.8% (w/v) agar film without addition of ES (system B), growth of the pathogen increased during the first 2 days and the viability remained constant in approximately 4.5 log cfu mL^{-1} until the end of the experiment (Fig. 5A, dotted line). For cheeses coated with active film (system C) viability of *L. monocytogenes* 01/155 decreased from the beginning of the experiment. During the first 8 days, a reduction of approximately 1–1.5 logarithmic orders was observed compared with the controls. From day 10 onwards, the difference in the viability of the pathogen compared with the controls increased, and the growth of bacteria decreased to values below 2 log cfu mL^{-1} (Fig. 5A, line of dashes and double points).

In the trial with artisanal goat cheese, viability of the control pathogen suspension maintained approximately 7 log cfu mL^{-1} throughout the experiment (Fig. 5B, solid lines). For cheeses without agar coating and contaminated with *L. monocytogenes* 01/155 (system A), as well as for cheeses coated with the 0.8% (w/v) film agar without addition of SE (system B), the behaviour of the pathogen was similar (Fig. 5A, dashes and dot line, respectively). Viability remained approximately 5 log cfu mL^{-1} during the first week and subsequently increased until reaching a growth similar to the peptone water control; even though viability of *L. monocytogenes* in coated cheeses (system B) always remained slightly below the values recorded for uncoated cheeses (system A). For cheeses coated with the agar film with ES incorporated (system C), a difference of approximately 1–1.5 logarithmic orders in viability of *L. monocytogenes* 01/155 was observed throughout the experiment, in relation to the control cheeses (Fig. 5A, line of dashes and double points). Initial viability of 5 log cfu mL^{-1}, decreased to approximately 4.4 log cfu mL^{-1} between days 2–8, and returned to approximately 5 log cfu mL^{-1} after 2 weeks of assay.

In addition, both for commercial Tybo cheese and artisanal goat cheese, the possible growth of *L. monocytogenes* 01/155 was controlled in cheeses not contaminated with this pathogen (A_c, B_c and C_c systems), without viable cells of the pathogen being observed during the trial.

4. Discussion

E. avium DSMZ17511 antimicrobial substances were previously characterised as thermoresistant compounds of proteinaceous nature with marked anti-*Listeria* inhibitory effects (Audisio et al., 2005; Ibarguren, 2010). The approximate molecular mass of these antimicrobial metabolites was determined by Tricine-SDS-PAGE electrophoresis. The broad band observed between 3 and 8 kDa in the lawn of *L. monocytogenes* 01/155, suggests the presence of more than one compound with antimicrobial activity in the ES of *E. avium* DSMZ17511. Since the heat-resistant anti-*Listeria* peptides under study showed common characteristics with class II

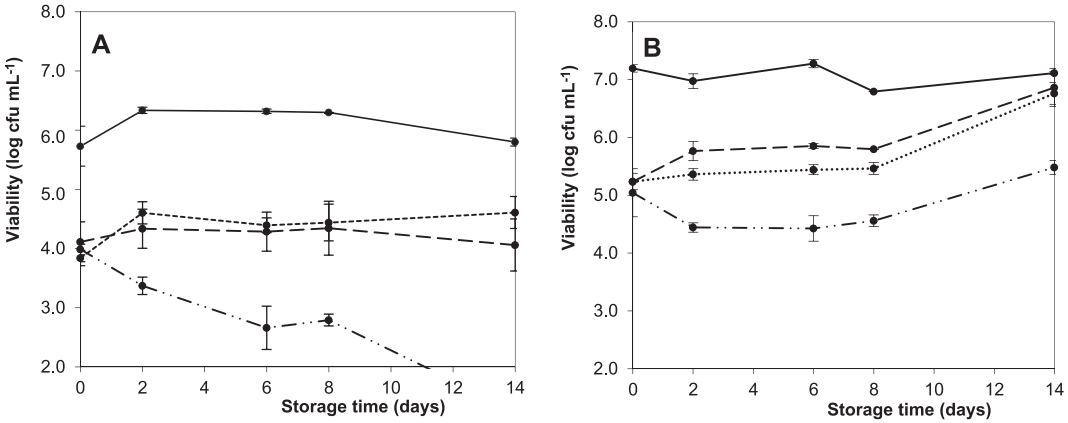

Fig. 5. Viability of *L. monocytogenes* 01/155 in (A) Tybo commercial cheese, (B) Goat artisanal cheese: contaminated with the pathogen (dashed line), coated with agar films (0.8%, w/v) previous contamination (dotted line) and coated with agar films (0.8%, w/v) with added *E. avium* DSMZ17511 ES (approximately 63 AU cm^{-2}) previous contamination (dashed and dotted line). Control of pathogen growth in peptone water (solid line).

enterocins (Drider, Fimland, Hechard, McMullen, & Prevost, 2006; Franz, van Belkum, Holzapfel, Abriouel, & Galvez, 2007), the presence of different structural enterocin genes was also analysed. The amplification of specific PCR fragments of 126- and 162-bp (Fig. 2B), indicated that *E. avium* DSMZ17511 contained both *entA* and *entB* enterocin genes.

Toxicological safety of DSMZ17511 antimicrobial compounds was also analysed. In vitro cytotoxicity assays are useful to initiate preliminary research about basal toxicity, indicating the ability of a compound to cause cell death or loss of viability because of damage to several cellular functions (Fotakis & Timbrell, 2006). The compounds present in *E. avium* DSMZ17511 ES (3200 AU mL^{-1}) and ESc (409,600 AU mL^{-1}) exhibited mild cytotoxicity against Caco-2 cells, but only at high concentration (409,600 AU mL^{-1}); while L929 cells growth was stimulated rather than inhibited in contact with both, ES and ESc. Likewise, a concentration dependant cytotoxicity was observed for bacteriocin bovicin HC5 (Paiva et al., 2012), bacteriocin GM3 (Devi Avaiyarasi, David Ravindran, Venkatesh, & Arul, 2016) and enterocin DD14 (Caly et al., 2017) when tested against different mammalian cells. Even nisin, a well-known commercial bacteriocin, has been reported to be mild toxic to Caco-2 human cells (Maher & McClean, 2006). These cytotoxicities have been associated with a high level of hydrophobicity in the antimicrobial peptides, which can promote interaction with the mammalian cell membranes (Fitzgerald, Omary, & Triadafilopoulos, 1997). However, the results obtained for *E. avium* DSMZ17511 strain are positive since mild cytotoxicity was detected in concentrations considerably higher than those suitable for food biopreservation.

To examine the antimicrobial effect of *E. avium* DSMZ17511 metabolites, bioactive agar coatings with addition of ES were applied to cheeses artificially contaminated with *L. monocytogenes*. The integrity and biological activity of these coatings had been evaluated satisfactorily prior to their application to cheeses (Ibarguren et al., 2014). The aim of this experiment was to observe the behaviour of the active film against *L. monocytogenes* in a RTE food susceptible to contamination with this pathogen and, in turn, to evaluate the effect of different cheese matrices on the antimicrobial activity of the *E. avium* DSMZ17511 ES.

Different matrices have been evaluated as structural components of active coatings designed for the control of *L. monocytogenes* in cheeses, such as corn protein (Ünalan, Arcan, Korel, & Yemenicioğlu, 2013), sodium caseinate (Cao-Hoang, Chaine, Gregoire, & Wache, 2010), serum (Ramos et al., 2012), starch (Marques et al., 2017), with the addition of commercial antimicrobials such as nisin, natamycin, among others. The immobilisation of the antimicrobials synthesised by *E. avium* DSMZ 17511 was previously successfully tested in other polymer matrices (gelatine, gluten and brea gum) (Ibarguren et al., 2010b). In this case, agar was selected as the matrix for antimicrobial coatings, since films obtained with this biopolymer are clear, transparent, strong and flexible (Phan The, Debeaufort, Voilley, & Luu, 2009a). In addition, we observed that agar films had good stability and consistency in contact with an aqueous medium (Ibarguren et al., 2014), even using low proportions of the polymer (0.8%, w/v), which means a lower cost of the product. In addition to being harmless to humans, agar does not constitute a substrate easily metabolised by microorganisms that could be, for example, present on the surface of the cheese, either as saprophytic or spoilage microbiota. Although this matrix has been previously evaluated as material for formation of films and edible coatings (Phan The, Debeaufort, Voilley, & Luu, 2009b), and as a support for silver nanoparticles applied as food biopreservatives against *L. monocytogenes* and *Enterococcus coli* (Rhim, Wang, & Hong, 2013); to our knowledge, the direct application of agar films functionalised with antimicrobials in cheeses has not been reported previously.

Coatings were applied by direct immersion of each fractionated cheese in the polymer matrix; thus a uniform coating was obtained using a simple, fast and low cost technique (Andrade, Skurtys, & Osorio, 2012). Dipping-coatings also avoid exposures to excessive heat (Cho, Lee, & Han, 2009) and can be applied at any stage of the food supply chain (Rodrigues, Han, & Holley, 2002).

A non-purified fraction of the CFS (ES) of *E. avium* DSMZ17511 was used for the elaboration of agar active coatings. Application of purified CFS entails additional costs and possible reduction of the bacteriocins antimicrobial activity (Marques et al., 2017; O'Bryan et al., 2018). Therefore, commercial use of purified bacteriocins is rare (Deegan, Cotter, Hill, & Ross, 2006). Also, CFS can include a variety of antimicrobial substances, thus various biologically active substances are incorporated and could exert possible synergistic effects on the final product (Hartmann, Wilke, & Erdmann, 2011).

The application of agar edible coatings on cheeses showed that the immobilised bacteriocin maintained its anti-*L. monocytogenes* activity, with a decrease of at least 1 log cycle in the viability of the pathogen, compared with growth in the contaminated control cheese, during the first week of trial. During the second week, the inhibition varied according to the cheese matrix. The viability of the pathogen decreased below 2 log cfu mL^{-1} for semi-hard semi-fat cheese (Tybo cheese), while for soft fat cheese (handcraft goat cheese) the counts of *L. monocytogenes* 01/155 were higher, although a difference of approximately 1 log cycle compared with the controls remained during the entire trial. In both cases, release of the antimicrobial occurred in the first three hours of contact, allowing quick action to delay bacterial cell growth during storage (Marques et al., 2017). The wetter matrix of the artisanal cheese apparently facilitates more rapid diffusion of the antimicrobials, favouring the development of the pathogen. In turn, the drier matrix of Tybo cheese allows a gradual release of the antimicrobial compounds, producing a more effective and prolonged inhibition of the pathogen.

In addition, *L. monocytogenes* counts were also controlled on the groups of non-contaminated cheeses. No viable culturable pathogen cells were detected; also, visible microbial contamination was not observed during the refrigeration storage period. The non-proliferation of contamination or deterioration microbiota also endorses the protective action of the agar coatings.

5. Conclusion

The application of agar coatings (0.8%, w/v) functionalised with enterocins synthesised by *E. avium* DSMZ17511 constitutes a promising alternative for control of *L. monocytogenes* in cheeses. Although further studies to determine toxicological and sensory properties of coated cheeses are needed, these active agar coatings represent a simple, effective and low cost option, for potential application as a biopreservation tool in cheeses or other RTE food. Also, this is a natural, biodegradable and a safe alternative that meets food consumers low processed expectations.

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