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PEPTIDES PRODUCED BY NATIVE *Lactococcus lactis* Ella8 IMPAIRS MEMBRANE INTEGRITY OF FOOD PATHOGENS

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Abstract

Previously, we have isolated a Lactococcus lactis UTNElla8, a native strain originated from wild-type fruits of humid mesothermal region of Esmeraldas province, Ecuador and demonstrated its antimicrobial activity. Nonetheless, its mode of action remain unknown. This study was aimed to elucidate the mode of action of peptides of Ella8 against foodborne pathogens. The addition of partial purified Ella8 peptides (PPElla8) at the final concentration 1 MIC to the E. coli suspension cells at exponential (OD_{605} = 0.7) growth phase resulted in a decrease with 1.2 (log) order of magnitude compared to the control in the absence of the peptide. PPElla8 has marginal activity (>1 log difference) against Salmonella cell culture (0.79 log difference). No effect on cell viability was observed when the peptides were combined with EDTA suggesting that the presence of a chelating agent does not interfered with the antimicrobial activity. The membrane of E. coli was disrupted by the PPElla8 causing the release of β-galactosidase by the target cell. Moreover, we showed that the integrity of the target cell membrane was affected by PPElla8 leading to the leakage of DNA/RNA molecules and cellular death. The positive effect of PPElla8 towards the delay on rottenness and molds forming on tomato fruits stored at the room temperature demonstrated its bioprotective potential.

Key words*: antimicrobial peptides, Lactococcus, preservation, food spoilage, bacteriolytic, tomatoes*.

INTRODUCTION

The food industry is experiencing increased pressure from consumer demands to develop minimally processed food products with a long shelf life without the use of chemical preservatives.

Nowadays, at global level, many families eat outside their home, and, in this context, Ecuador is not an exception, many artisanal ready-to eat foods are sold on the street by ambulatory vendors. Thus, most foods are prone to contamination due to inadequate handling and storage, poor cooking, contaminated equipment or improper personal hygiene, therefore, the risk of sickness is higher (Cortese et al., 2016; Rios et al., 2016; Castellano et al., 2017; Perez-Parra et al., 2017; Tenea et al., 2018). For example, according with the epidemiological studies, reported by the Ministry of Health for 2015, 39% of reported cases of hepatitis A correspond to people aged between 5 and 9 years and 54% correspond to male people (Pérez Parra et al., 2017). On the other hand, the current legislation linked with food safety and quality standards clearly indicates that the processed products must be free of contaminants when used for human consumption (NTE INEN 1529-8, 2015-xx, Ecuador).

For more than a decade the lactic acid bacteria have been used by humans as health benefits as probiotics (Baradaran et al., 2012) including their capacity to combat the spoilage growth, due to the production of small peptides (bacteriocins o bacteriocin-like molecules) and metabolites (hydrogen peroxide) that actuate as potential natural preservative (Yang et al., 2014; Angmo et al., 2016; Rai et al., 2016; Özoguland Hamed, 2018). But, the effectiveness of individual bacteriocinsis direct related on the producer LAB strain. Among them, *Lactococcus* was identified as producer of nis in, the only bacteriocin recognized by FDA to be used as food additive (Cotter et al., 2013; Punyaunppa-path et al., 2015). Recently, we reported the antimicrobial potential of some lactic acid bacteria from wild-type fruits of different subtropical niches of Ecuador (Tenea et al., 2018). Several *Lactococcus lactis* strains

producing antimicrobial substances with wide range capacity to inhibit foodborne pathogens were identified; their activity was not linked with the presence of nis in encoding genes. One strain, *L. lactis* subsp. *lactis* Ella8, harbored the lacticin 3147, a two peptide lantibiotic bacteriocin, showing a wide spectrum activity against both Gram-positive and Gram-negative targets (Tenea et al., 2018). In order to understand its mode of action, in this research the effect of peptides produced by Ella8 against some common ubiquitous microorganisms founded in local food was evaluated along with its protective effect in fresh tomatoes fruits for further use as self-life extender.

MATERIALS AND METHODS

Bacterial strains

Lactococcus lactis subsp. *lactis* Ella8 (registered at Gen Bank with annotation No. MG675578.1) was isolated from tropical wild-type fruits of *Morus nigra* of Esmeraldas Province, a forest of semiarid climate in a costal Northeast of Ecuador (Tenea et al., 2018). As standard, *Lactobacillus fermentum* CNCM1-2998 (Lac) recovered from an available commercial probiotic, Lacteol Fort (Lactobacillus LB, AxcanPharma, France) was used as reference (Coconnier et al., 2000). The indicator strains were: *E. coli* ATCC 25922, *Salmonella enterica* ATCC 51741 and *Shigella sonnei* ATCC 25931.

Isolation of peptides

The peptides (PP) were obtained as previously described (Garzón et al., 2017). Briefly, the LAB strains grown in MRS broth at 37°C for 24 h were used to extract cell free supernatant (CFS) by centrifugation at 13,000 x *g* for 30 min (4°C) followed by filtration using 0.22 µm porosity syringe filter (# STF020025H, ChemLab Group, USA). To obtain PP, the 60% ammonium sulfate was added to CFS, incubated overnight with refrigeration without stirring and centrifuged at 8,000 x *g* for 30 min at 4°C. The PP were recovered in 25 mM ammonium acetate (pH 6.5), desalted by using a midi dialysis kit (cat # PURD10005-1KT, Sigma-Aldrich, USA) pre-equilibrated with phosphate buffer (pH 7.0) and stored at - 20°C before use.

Effect of PPElla8 on indicator cells viability

The PP (1MIC determined as previously reported by Garzon et al., 2017) was added to the indicator strain culture (100 ml) at exponential growth phase (OD_{605} = 0.7) followed by incubation at 37°C for 6 hours and the plate-agar method was used to determine the viable cell counts at certain incubation time intervals (Tenea et al., 2018). The effect of EDTA (20 mM) on bacteriocin activity was tested as previously described by Chopra et al. (2015). Log reduction was calculated as the difference between log10 (CFU) of the untreated cells (no bacteriocin, no EDTA) and the treated cells (bacteriocin added). Log reduction of < 1 was considered insignificant. The experiments were repeated three times and untreated indicator strain culture has been used as control.

Cell membrane integrity

If the bacterial membrane is compromised, the release molecules can be monitored by measuring the absorbance at 260 nm. Briefly, the *E. coli* bacterial suspension was grown overnight in LB medium, harvested by centrifugation and washed twice with 1X PBS (phosphate buffered saline, pH 7.5). The cells were treated independently with Ella8 and Lac peptides at the final concentration 1 MIC following by incubation at 30°C. One flask was maintained as control (untreated with peptides). Cell culture were centrifuged after 1, 2, 4 and 24 h of incubation, the supernatants filtered, and optical density was measured using the spectrophotometer (Nova60, Millipore, Merck).

Cytoplasmic membrane permeabilization

Inner membrane permeabilization of *E. coli* was investigated by using ONPG (o-nitro-phenyl-L-D-galactoside, # N1127, Sigma, USA) as substrate as previously described (Patra et al., 2015). Briefly, bacteria grown to logarithmic phase in LB medium containing 2% lactose were collected and washed twice with 10 mM sodium phosphate buffer (pH 7.5) and 100 mM NaCl. PP of Ella8 and Lac were added to bacterial suspension at optical density 0.6, incubated 5 min at 30°C, then ONPG at final concentration of 30 mM was added to each cell suspension. The hydrolysis of ONPG to O-nitrophenol (ONP) over time (120 min) was monitored at 420 nm (Patra et al., 2015).

Ella8 peptide mass determination

The dialyzed Ella8 peptide weight was estimated relative to molecular marker (Takara Clearly protein ladder, # 3454A, Takara, Bio Company). Tricine-SDS-PAGE method using pre-casted acrylamide gels (12%) and Thermo Fisher OWL (10 x 10) vertical electrophoresis system were used. The gel was stained with Takara CBB Safe Stain (cat # T9320A, Takara, Bio Company) for 4 h then distained with a solution of 30% methanol (v/v) and glacial acetic acid, 10% (v/v) until the bands become clear.

Effect of Ella8 peptides on tomatoes fruit during storage

The tomato fruits chosen at the stage four according with the color chart of tomato ripening (USDA standards) were purchased by a local vender, washed with 5% bleach solution for 5 min, then twice with distillate water and left to dry under laminar flow cabinet before treatment. Each tomato was immersed for 5 min in peptide solution of Ella8 and Lac (final concentration 1 MIC), manually rotating each fruit for 5 min to assure complete cover age and contact of surface with the wash solution, and then left to dry overnight under the safety cabinet.

The fruits were packed in trays covered with plastic alimentary film. For each treatment two tomatoes were used. Tomatoes without any treatment were used as control. The treated and no treated samples were stored at room temperature (18-24°C) and refrigeration (5°C). After regular interval of storage, the appearance of visible signs of rottenness or molds were monitored during storage. The experiment was repeated three times at one month interval with tomatoes purchased from same distributer.

RESULTS AND DISCUSSIONS

The addition of PPElla8 and PPLac to growth phase of *E. coli* suspension cells resulted in a decrease with 1.2 (log) order of magnitude compared to the control in the absence of the peptide (Figure 1). A significative decrease of 1.07 log towards *Shigella* while minor effect (0.79 log difference) was detected against *Salmonella* (data not shown).

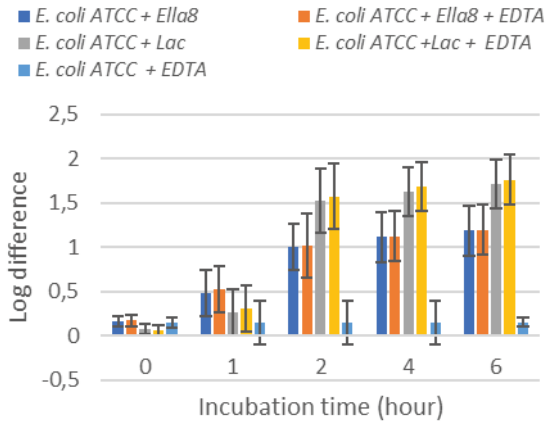

Figure 1. Effect of peptides on *E. coli* cell density. Bars represent log reduction calculated as difference between cells with and without bacteriocin; Log reductions <1 were considered insignificant

Similarly, the viability of *E. coli* and *Salmonella* decreased with 1.71 and respectively 1.27 log at 6 hours when PPLac was added to the suspension cells. No effect was registered against *Shigella* indicated that the inhibitory effectiveness depend on the peptide potential. Previously, we showed that the PPGt28 secreted by *Lactococcus lactis* Gt28 strain totally inhibit the *E. coli* and *Salmonella* cells at the exponential growth and the addition of EDTA enhanced the inhibitory effect (Tenea et al., 2018). In the current study, the peptides of Ella8 strain had the capacity to diminish the cell population of *E. coli* at exponential stage of growth without combining with an extra destabilizing membrane factor.

Cell membrane integrity of indicator strains

The release of cellular components with strong UV absorption at 260 nm indicates that occur when cells are exposed to drugs (e.g. antibiotics, antimicrobial peptides) applied in different doses and exposure time indicating the membrane damage (Patra et al., 2015). When *E. coli* suspension cells were treated with PPElla8 and PPLac, DNA/RNA molecules were detected in agarose gel electrophoresis at different incubation time (Figure 2), indicating that the damage of the target cell membrane occurred.

Early study indicated that some bacteriocins are able to interact with the bacterial membrane forming ion channels, leading to the increase of cytoplasmic membrane permeability and hence to bacterial death (Miao et al., 2016).

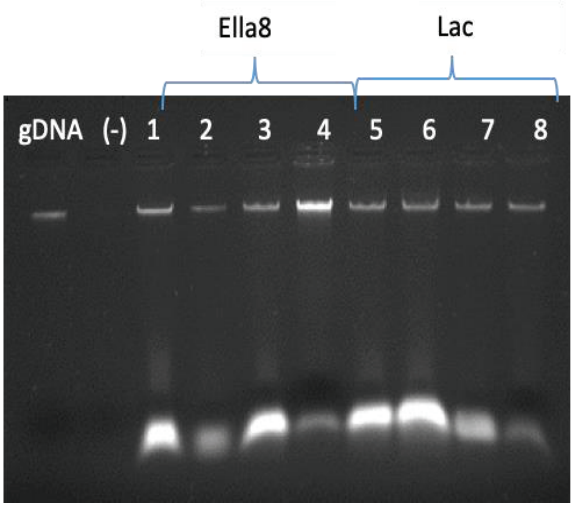

Figure 2. Detection of DNA/RNA molecules released when *E. coli* was treated with peptides. Legend: gDNA: positive control of *E. coli* gDNA; (-) negative control (no DNA/RNA molecules); Ella8, Lac: treatment with peptides at 1, 2, 4 and 24 h of incubation

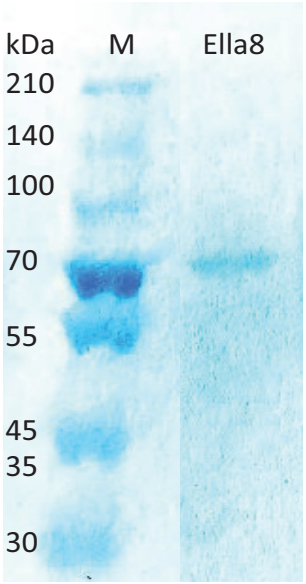

Figure 3. SDS-PAGE peptides release by *Lactococcus lactis* Ella8 M: molecular marker (Clearly protein ladder, Takara Bio USA)

However, our results indicated that the bacteriocin Ella8 damaged the cellular membrane of target Gram-negative bacteria leading to leakage of cytoplasmic molecules and then cellular death. Previously we showed that the bacteriocin produced by Ella8 is similar to lacticin 3147, a two peptide bacteriocin, however, the mechanism of action requires the interaction of Ltnα and Ltnβ, for optimal bactericid al activity in contrast with single peptide nisin. When using Tricine-SDS-PAGE analysis a larger peptide product of 70 kDa was identified (Figure 3). According with previous research, lacticin 3147 two peptide were estimated to 33 kDa and 28 kDa (Ryan et al., 1999). In our study, the protein was larger. Up to now no such larger peptides has been identified in *L. lactis* strains. It might be linked with posttranscriptional modification that can slow the migration in SDS gel or was not fully denatured being more stable at high temperature, therefore we shall further investigate its amino-acid composition.

Inner membrane permeabilization of *E. coli*

The ability of Ella8 to permeate the membrane of *E. coli* was evaluated as a function of cytoplasmic β-galactosidase release, with bacteria grown in lactose containing medium. In general cytoplasmic β-galactosidase cannot pass through the integral cell membrane of bacteria, however, when compromised the endoenzyme β-galactosidase, could permeate the cytoplasmic membrane.

When cells were treated with Ella8 it was a lag time of 30 min followed by a progressive release of β-galactosidase up to 120 min to reach unvarying condition (Figure 4). A progressive increase was observed when PPLac was used, noβ-galactosidase activity was detected in culture medium of bacterial cells that does not contained peptides. Alike, the peptide F1 produced by *Lactobacillus paracasei* increased the cell membrane permeability of *S. aureus* (Miao et al., 2016).

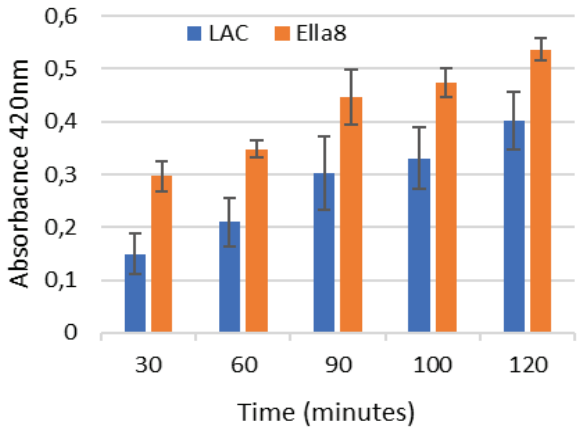

Figure 4. Time dependence of permeation of *E .coli* cells treated with peptides; Abbreviation: Ella8: peptides produced *L. lactis* Ella8; Lac: peptides produced by *L. fermentum*

Our results indicated that peptide Ella8 increased the cell membrane permeability of *E. coli* which led to the leakage of cytoplasmic content to cell culture medium. This result was

in concordance with the study of Ibrahim et al. (2000) implying that ovotransferr in peptide (OTAP-92) induced the cytoplasmic permeability of *E. coli* cells.

Ella8 peptides protect tomato fruits and delay its deterioration

In general vegetables without any preventive treatment are easily perish. The storage with refrigeration, freezing and canning techniques have been developed for the preservation and prolongation of the shelf life of vegetables. The common tomatoes fruits are daily consumed in Ecuador, but, due to the template climate and inappropriate storage conditions they are susceptible to contamination by various microorganisms, therefore, in this research the protective effect of bacteriocins produced by Ella8 and Lac strain was evaluated during storage.

The fruits were coated independently with peptides and no treated samples (control) were packed and stored at room temperature or refrigeration and evaluated for the delay in rottenness, wrinkles and visible molds forming. However, the first visible sign of the rottenness was observed in the control tomatoes after 7 days of storage at the room temperature, while in the treated peptides no signs of deterioration was observed (Table 1).

Table 1. Effect of peptides on tomatoes fruits after refrigeration

Treatment	1	9	17	21	28
PP Ella8	Ideal	Normal	Normal	Normal	Normal
PP LAC	Ideal	Normal	Wrinkles	Wrinkles	Wrinkles
Control	Ideal	Rootness	Rootness, Wrinkles	Rootness, Wringkes, Rootness	Total contami-nation Molds

The samples stored at the refrigeration were preserved up to 20 days, but the fruits treated with peptides became softer and showed superficial skin damage, leading to a dry, dull appearance and the formation of wrinkles. Figure 5 showed the visible damage of tomato skin in control sample no peptide treated in comparison with the tomato Ella8 treated.

Early research on tomato covered with a food packaging biofilm containing the bacteriocin sonorensin indicated the protective effect up to day 7 with refrigeration (Chopra et al., 2015).

Figure 5. Appearance of tomato at 7 days after storage with refrigeration. Control: untreated; Ella8: tomato treated with PPElla8

The edible coatings and biopolymers films forming a barrier between the food and the environment, improve the safety, quality, and functionality of food products without changing organoleptic and nutritional properties (Valdés et al., 2017). In other study, application of pentocin MQ1 to bananas fruits indicated the improvement of microbiological quality as well as the shelf-life of fruit in both room temperature and refrigeration (Wayah and Philipe, 2018). In the current study the tomato fruits were dipped in the peptide-containing solution, dried for 24 hours to assure that the covered the fruit, thus we suggested that the delay on the forming of spoilage was related with the presence of active component that might penetrate the fruit membrane. Covering or coating the fruit with active component might be a promising approach for active protection but at this point we don't know exactly the concentration of active substance that was adsorbed by the fruit. A direct interaction of active component with the surface of the food matrix might be a suitable alternative of biofilm as the bacteriocin can diffuse better to the surface of food and protected for further contamination.

CONCLUSIONS

Altogether, our results indicated that the peptides produced by *L. lactis* Ella8 displayed a bacteriolytic mode of action. The membrane of *E. coli* was disrupted by the PPElla8 causing there lease of β-galactosidase by the target cell. Moreover, we showed that the integrity of the target cell membrane was affected by PPElla8 leading to the leakage of DNA/RNA molecules and cellular death. The positive effect of PPElla8 towards the delay on rottenness and

molds forming on tomato fruits stored at the room temperature demonstrated its bioprotective potential.

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