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El potencial antimicrobiano de bacteriocinas producidas por cepa *Lactobacillus plantarum* GT4aislada de frutas nativas

Antimicrobial potential of bacteriocin produced by *Lactobacillus plantarum* GT4 isolated from native fruits

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Resumen

Las bacterias ácido lácticas son conocidas por su potencial uso en la conservación de alimentos. En este estudio se utilizaron frutos silvestres de la selva tropical amazónica ecuatoriana para aislar e identificar nuevas especies de bacterias ácido lácticas bacteriocinógenicas. Entre ellas, la cepa Gt4 de *Lactobacillus plantarum* (GenBank No. KY041689) que mostró una capacidad altamente inhibidora; esta se estudió adicionalmente. En el ensayo de difusión en agar, el extracto crudo (CE) y bacteriocina precipitada (PP) de Gt4 mostraron una actividad antimicrobiana elevada hacía varias bacterias patógenas. Su principio activo fue de naturaleza proteínica, ya que no se registró actividad al tratar con enzimas proteolíticas y también mostró estabilidad a temperaturas elevadas. La adsorción de la bacteriocina a las células patogénicas se incrementó cuando se trató con EDTA, NaCl, Tween 20, a pH ácido y temperatura de 37°C y 45°C. Se registró una disminución en la adsorción cuando las celulas se trataron con SDS. En base a Tricine SDS-PAGE, el peso molecular estimado fue de 10 kDa y mostró el modo de acción bactericida.

Palabras clave: bacterias ácido lácticas, péptidos antimicrobianos, patógenos transmitidos por alimentos, bacteriocina

Abstract

Lactic acid bacteria are known for their potential use in food preservation. Wild-type fruits of Ecuadorian Amazon tropical rainforest were used to isolate and identify new bacteriocinogenic lactic acid species. Among several isolates, *Lactobacillus plantarum* strain Gt4 (GenBank No. KY041689) showing highly inhibitory capacity was further characterized. Using agar-well diffusion assay, the crude-extract (CE) and precipitated bacteriocin (PP) derived from Gt4 strain displayed elevated antimicrobial activity towards several pathogenic bacteria. Its active principle was proteinaceous since the bacteriocin was inhibited by proteolytic enzymes and showed grater thermostability. Adsorption of the bacteriocin to pathogenic cells was increased when treated with EDTA, NaCl, Tween 20, at acidic pH and temperature of 37°C and 45°C. A decrease in adsorption was registered when cells were treated with SDS. Based on Tricine SDS-PAGE the estimated molecular weight was 10 kDa showing bactericidal mode of action.

Keywords: lactic acid bacteria, antimicrobial peptides, foodborne pathogens, bacteriocin

Introduction

Natural food preservation might be a satisfactory approach for the control of spoilage bacteria growth in raw material. Among many bacteria that produced bacteriocins or peptides that confer antimicrobial capacity, lactic acid bacteria are of interest due to their GRAS (Generally Regarded as Safe) status and efficiency on conferring the food safety and security without altering the product quality (Corsetti et al., 2004; Reis et al., 2012). Several lactobacilli species produce active peptides known as bacteriocin-like proteins of low molecular weight that target the pathogenic cells by binding to their surface receptors (Atrih et al., 2001; Deraz et al., 2007; Zambou et al., 2013). Among them, *Lactobacillus plantarum* produce active peptides having broad range inhibitory capacity (Yang et al., 2012). However, the antimicrobial activity being specie related, the current investigations are focusing on identification of novel peptides producing strains (Deegan et al., 2006; Zendo, 2013; da Silva et al., 2016).

In Ecuador, the diseases associated with the presence of pathogens in food, as consequences of defective storage conditions or poor manufacturing practices was early reported (Gaona, 2013). Therefore, new methods or strategies to reduce the contamination by pathogenic microorganisms are compulsory. With the aiming of selecting new lactic acid bacteria producer of antimicrobial peptides, for further exploited as natural ingredients in food preservation, we performed a large scale screening of bacteriocinogenic lactic acid bacteria of native microbiota of Ecuador (Benavidez et al., 2006; Tenea and Yépez, 2016). Among them, *Lactobacillus plantarum* assigned Gt4 showing highly inhibitory activity against two food pathogens was further characterized. In this study, the antimicrobial spectrum, the resistance to enzymes, and high heat stability, the molecular weight of the released peptides along with its possible mode of action were evaluated.

Materials and methods

Bacterial strain

Mature fruits of *Chrysophyllium oliviforme* (tropical plant) collected from Sucumbíos Provence (Amazon region of Ecuador) were used to selected and purify individual colonies. Out of thirty purified colonies Gt4 having elevated antimicrobial potential was selected for further analysis. The strain was identified as *Lactobacillus plantarum* based on API50CHL strips (Biomerieux, Marcy l'Etoile France, cat # 50300) and 16S rRNA gene sequencing conducted using manufacturer guidelines (Macrogen Inc., Korea, custom-service). The Gt4 was registered at GenBank under the accession number KY041689 (Garzón et al., 2017).

Inhibitory spectrum

Antimicrobial activity was performed using the agar-well diffusion method (Garzón et al., 2017). The in-

dicator strains: *E. coli* ATCC 25922, *E. coli* ATCC 10536, *Shigella sonnei* ATCC 25931, Salmonella enterica subsp. enterica ATCC 51741, *Salmonella enterica* subsp. *enterica* serovar Abaetetuba ATCC 35640, *Streptococcus thermophilus* ATCC 19258, *E. coli* UTN Ec1 (isolated from local fresh cheese), *Salmonella* UTN Sm2 (isolated from cooked chicken), *Enterobacter aerogenes* UTN Eag1, *Shigella* sp. UTN Shg1 (laboratory collection) were used. Briefly, the Gt4 strain was grown for 24 hours in MRS broth at 30°C and the crude extract (CE) collected by centrifugation at 13,000 x g for 20 minutes at 4°C, was filtered using 0.22µm porosity syringe filter. The indicator strain (100µl) grown in broth medium (7 log CFU/ml) were mixed with 3.5 ml of soft MRS agar (0.75%), overlaid on the nutrient agar plates and incubated at 37°C for 2-4 hours. The CE (100µl) was transferred onto the wells (6 mm) on overlaid agar, incubated at 37°C and subsequently examined for inhibition zone at different intervals of time (24-48 hours). The experiments were run in triplicate the mean value of the inhibition zone was estimated.

Effect of temperature and enzymes on peptides activity

Crude extract was precipitated with 40% and 60% ammonium sulfate, incubated overnight at 4°C, centrifuged at 8000 x g for 30 minutes, recovered in ammonium acetate 25mM, filtered and stored at (-) 80°C before use. The precipitated bacteriocin was treated with proteinase K (30 U/mg) and lysozyme (40 U/mg) (Sigma-Aldrich Corporation, USA) at the final volume of 1 mg/ml, incubated for 2 hours at 37°C and 5 min at 100°C to inactive the enzyme and the residual activity was measured. In other batch aliquots of bacteriocin were incubated for 10 minutes at 60, 80, 90, 100 and 121°C before performing the disk diffusion bioassay. As control, the precipitated bacteriocin and crude extract without any treatments has been used. Residual activity was determined towards *E. coli* ATCC 25922 strain.

Effect of bacteriocin Gt4 on target bacterial growth

The treatments were performed as following: Set A: crude-extract of Gt4 at the final concentration of 3200 AU/ml; Set B: same concentrations of precipitated peptides; Set C: precipitated peptides combined with 20mM EDTA were added independently to 3 hours old culture (OD_{605} = 0.2) of indicator strain *E. coli* ATCC 25922 (Deraz et al., 2007). Incubation was performed at 37°C for 7 hours and optical density (OD_{605}) was measured every hour using spectrophotometer (Nova60, Millipore, Merck) followed by plate-agar method to determine the viable cell counts. As control, untreated indicator strain culture have been used.

Adsorption of bacteriocin Gt4 to the target indicator cells

Adsorption of bacteriocin to indicator cells was performed following a method described by Yildirim et al. (2002). Briefly, the target cells, *E. coli* ATCC 25922 and *Salmonella enterica* ATCC 51741 were grown overnight in LB medium, then centrifuge at 8000 x g, 20 min at 4°C and the cells were suspended in 5mM of phosphate buffer (pH 6.5) to an OD 600=1.0. Each suspension was mixed with the bacteriocin at equal volume and incubated at 37°C for 1 h. After removal of the cells at 8000 x g, 20 min, the activity of unbound cells was determined. The percentage of adsorption was determined as following: =100 x [1 - (AU/ml in cell-free supernatant - AU/ml in control A)]/ (AU/ml in control B), of which Control A= consisted of 0.1 ml of dH_2O instead of bacteriocin, and control B = had 0.1 ml of dH_2O instead of cell suspension. The effect of pH, temperature and chemicals on adsorption were evaluated. The experiments were run in triplicates.

Molecular weight determination

Precipitated peptides were analyzed using Tricine-SDS-PAGE method with pre-casted acrylamide gels (4-20%) and Thermo Fisher OWL (10x10) vertical electrophoresis system. The gel was stained with Takara CBB Safe Stain (based on Coomassie brilliant blue G-250, cat # T9320A, Takara, Bio Company) for 4 hours, distained with a solution of 30% methanol (v/v) and glacial acetic acid, 10% (v/v) until the bands become clear. The molecular weight was estimated relative to broad range protein molecular weight marker (cat # V849A, Promega Corporation, US).

Results

Antimicrobial spectrum

The results indicated that the bacteriocin producing Gt4 strain displayed broad spectrum of inhibitory activity (Table 1). As observed the activity was grater when crude extract rather than precipitate peptides counterpart was used, suggesting that the inhibitory activity in vitro depends at least in part, by the presence of organic acids.

Table 1. Inhibitory spectrum of bacteriocin-produced by Gt4 strain.

Indicator bacteria	Inhibition zone (mm)	
	CE	PP
Salmonella enterica subsp. *enterica* (Kauffmann and Edwards) Le Minor and Popoff ATCC 51741	20.66 ± 0.94[a]	13.33 ± 0.47[a]
E. coli ATCC 25922	18.00 ± 0.00[b]	13.33 ± 0.47[a]
Salmonella UTN Sm2 (laboratory collection)	16.66 ± 0.94[c]	12.66 ± 0.94[ab]
Shigella sonnei ATCC 25931	16.33 ± 0.47[c]	12.66 ± 0.94[ab]
Enterobacter aerogenes UTN Eag1(laboratory collection)	15.33 ± 0.47[cd]	12.66 ± 0.94[ab]
Shigella sp. UTNShg1 (laboratory collection)	14.33 ± 0.47[d]	12.00 ± 0.00[b]
Salmonella enterica subsp. enterica serovar Abaetetuba ATCC 35640	14.00 ± 0.00[d]	11.66 ± 0.94[c]
E. coli UTN Ec1(laboratory collection)	14.00 ± 0.00[d]	12.33 ± 0.33[ab]
Streptococcus thermophilus ATCC 19258	13.00 ± 0.00[e]	12.66 ± 0.94[ab]
E. coli ATCC 10536	11.66 ± 0.47[f]	11.33 ± 0.47[c]

* Data are means ± standard error. Mean in the same column that are followed by different small letters are significant different ($p < 0.05$); CE: crude-extract, PP-precipitated peptides.

Sensibility of precipitated bacteriocin to enzymes and heat

The results indicated that the active compounds are proteinaceous as no activity was registered after the treatment with proteinase K (Table 2). A slightly increase in inhibitory activity was observed after treatment with lysozyme suggesting the non-lipid and no carbohydrate moiety content of released peptides.

Table 2. Characterization of bacteriocin-producing Gt4

Treatment	Zone of inhibition (mm)
	E. coli ATCC25922
Enzymes (1 mg/ml)	
PP + Proteinase K	-
PP + Lysozyme	13.33 ± 0.47
PP	12.41 ± 0.47
CE	16.50 ± 0.41
Heat (10 min)	
60°C	12.95±0.40
80°C	12.94±0.47
90°C	12.84±0.27
100°C	12.00±0.00
121°C	12.00±0.00

Moreover, the bacteriocin activity was maintained stable after exposure for 10 min to high heat (Table 1). The activity was not lost after autoclaving 121°C demonstrating the valuable characteristic if applicable further in food processing.

Effect of Gt4 on *E. coli* growth

When crude-extract corresponding to 3200 AU/ml was added to target *E. coli* a 2 log CFU/ml reduction of viable cells was recorded at 7 hour of incubation, while combining crude-extract with EDTA the same level of inhibition was recorded but minimizing the incubation time (5 hours). Figure 1 illustrates the difference between the viable cells with and without bacteriocin added in different forms (CE and PP with / without EDTA). Same level of reduction was registered when PP was added to the *E. coli* cells, while combining with EDTA the reduction increased (2.87 log CFU/ml) at 7 hours of incubation. EDTA only slightly reduced the viable cell counts.

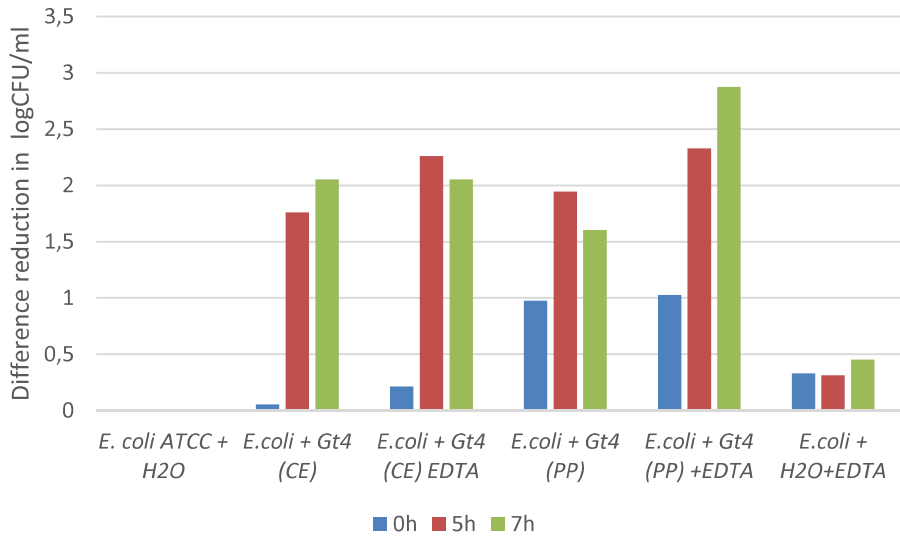

Figure 1. The effect of bacteriocin Gt4 on *E. coli* growth. Bars represent the log CFU/ml difference reduction between treatment and control (without bacteriocin); CE: crude-extract. PP-precipitated peptides

Adsorption of bacteriocin to the target cells

The bacteriocin Gt4 was adsorbed 84% to the cells of *E. coli* and 73% to *Salmonella* (Table 3). The optimum adsorption was recorded at pH 2 towards *E. coli* and pH4 towards *Salmonella*. Temperature has also a positive impact on adsorption with the maximum registered at 37°C and 45°C towards E.coli and respectively, *Salmonella*. Nonetheless, the adsorption was strongly influenced by Triton X100 (11% towards *Salmonella*) and SDS (55% towards *E. coli*).

Treatment	Adsorption (%)	
	E. coli ATCC 25922	*Salmonella enterica* ATCC 51741
pH		
2	87	77
4	80	80
6	84	73
Heat (°C)		
4	69	65
15	80	94
30	94	92
37	100	95
45	100	100
Chemicals (1%)		
NaCl	92	88
Triton X-100	50	11
EDTA	100	95
SDS	55	66
Tween 20	90	81

Molecular weight estimation

The molecular weight of precipitated bacteriocin was determined using tricine SDS-PAGE electrophoresis. The results indicated that the Gt4 has 10 kDa as only one band was detected in the polyacrylamide gel (Figure 2).

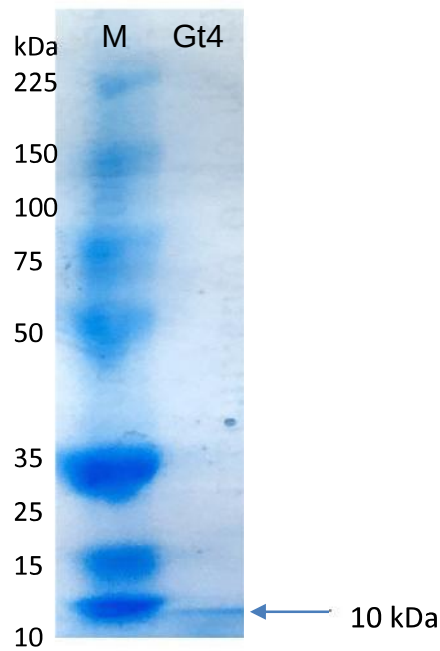

Figure 2. SDS-PAGE showing the 10 kDa fragment corresponding to bacteriocin Gt4. M-Molecular marker (Promega)

Discussion

The bacterial community of tropical plants, a biological reservoir of natural resources is poorly investigated. It is considered that microorganisms from this region might provide a newly source of functional compounds to be explored (Tenea and Yépez, 2016). The Ministry of Public Health, Ecuador reported considerable human illness related to food contaminants such as salmonellosis y shigellosis. Most artisanal minimally processed foods, typical dishes appears to pose significant number of pathogens, therefore the risk of developing diseases is elevated. Previously, a full scale identification of lactic acid bacteria isolated from tropical fruits were performed for further exploring their benefits in food industry as natural preservatives (Benavidez et al., 2016; Garzón et al., 2017). Antimicrobial activity against pathogenic microorganisms is one of the important properties of a probiotic lactic acid bacteria contributing to their colonization and competitive edge over other bacteria found in the same niches (Todorov et al., 2013). *Lactobacillus plantarum* is known as one of the most versatile LAB specie with a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria (Hernandez et al., 2005; Georgieva et al., 2009; Ali and Musleh, 2015; Arena et al., 2016). In this study, the bacteriocin producing Gt4 strain showed wider inhibitory activity against all pathogenic bacteria tested. Besides, the organic acids in crude-extract, locally produced in the bacterial growth, might establish the appropriate micro environmental condition to activate the antagonistic mechanism of produced peptides against harmful microorganisms founded in the same ecological niche. Additionally, the presence of organic acids might be beneficial if use CE in food due to their enzymatic resistance and higher solubility compared with the bacteriocin-like peptides. Similar with other investigation (Banerjee et al., 2013) the maximum production of Gt4 was detected at the end of logarithmic phase of growth (data not shown). The initial pH of bacterial culture was 6.0 declined at 3.8 to 4.0 at 24 hours of incubation which correlates with the optimum production of bacteriocin registered at the stationary phase. Sensitivity to proteolytic enzymes and resistance to heat are considered significant properties of bacteriocins making them suitable for human consumption (Yang et al., 2012; da Silva et al., 2014). In other study it has been shown that the exposure to higher temperatures decreases the inhibitory activity (Deraz et al., 2007). When the incubation time increased up to 75 minutes the effectiveness in the inhibitory activity was maintained stable demonstrating the valuable characteristic if applicable further in food processing (data not shown). The results indicated that overall activity was influenced by both active peptides and organic acids, suggesting the effect of possible synergistic interactions between all components presented in the bacterial crude extract. The results indicated that Gt4 act in a bactericidal manner against *E. coli* as a significant reduction cells viability overtime was registered. The inhibition increase when bacteriocin Gt4 was combined with EDTA suggesting that EDTA enhanced the killing effect of Gt4. Early study revealed that EDTA enhanced the activity of bacteriocins toward at least one Gram-negative strain in a concentration-dependent manner (Martin-Visscher et al., 2011). Based on SDS-PAGE results the Gt4 bacteriocin was small size (<10 kDa), heat stable, activated by chemicals, characteristics similar to bacteriocin belonging to Class II, but further analysis is required to confirm this statement. Adsorption of Gt4 to target cells was strongly influenced by heat and chemicals. Previous study showed that the adsorption of bacteriocin AMA-K produced by *L. plantarum* AMA-K to *Listeria innocua* LMG13568 was influenced by temperature (Todorov, 2008). Similarly, buchnericin-LB produced by *Lactobacillus buchneri* was adsorbed to Gram-positive but not Gram-negative bacteria and the adsorption was pH dependent. Also, the treatment of cells with some anions, detergents or organic solvents did not affect the binding of bacteriocin to the target cells (Yildirin et al., 2002). However, in this study the adsorption was positively influenced by some detergents and anionic salt. Alike temperature and acidity strongly influenced the adsorption. The differences in adsorption might be related with the target indicator bacteria. Thus, is important to evaluate the optimum adsorption parameters to further identify its mode of action against the target pathogenic cells.

Conclusions

This study demonstrated the valuable potential of bacteriocin produced by *L. plantarum* Gt4 strain to display a broad inhibition range effective and stable at higher temperature; regarded safe as been degraded by proteolytic enzymes, and reveal bactericidal mode of action against indicator bacteria. We shall further investigate the effectiveness of bacteriocin Gt4 on different food matrices.

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