Microbiol. Biotechnol. Lett. (2019), 47(2), 183–194
http://dx.doi.org/10.4014/mbl.1808.08014
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Potential Control of Foodborne Pathogenic Bacteria by *Pediococcus pentosaceus* and *Lactobacillus graminis* Isolated from Fresh Vegetables

C.J. González-Pérez[1], I. Vargas-Arispuro[2], E. Aispuro-Hernández[1], C.L. Aguilar-Gil[1,5], Y.E. Aguirre-Guzmán[2], A. Castillo[3], A. Hernández-Mendoza[4], J.F. Ayala-Zavala[1], and M.A. Martínez-Téllez[1]*

[1]*Coordination of Food Technology of Vegetal Origin, Research Center for Food and Development (CIAD) Ave. Gustavo E. Astiazaran #46, 83304. Hermosillo, Sonora, Mexico*
[2]*Coordination of Food Sciences, CIAD,* [3]*Animal Science Department, Texas A&M University, USA*
[4]*Coordination of Food Technology of Animal Origin, CIAD,* [5]*Center of Innovation and Agroalimentary Development of Michoacan, Morelia, Michoacan, Mexico*

Received: August 21, 2018 / Revised: October 27, 2018 / Accepted: November 5, 2018

The consumption of fresh vegetables has been related to recurrent outbreaks of foodborne diseases (FBD) worldwide. Therefore, the development of effective alternative technologies is necessary to improve the safety of these products. This study aimed to isolate and identify epiphytic lactic acid bacteria (LAB) from fresh fruits and leafy vegetables and characterize their antagonistic capacity due to their ability to produce bacteriocins or antibacterial compounds. For this, 92 LAB isolates from fruits and leafy vegetables were screened for antagonistic activity. Two strains with the highest and broadest antagonistic activities were selected for further characterization; one from cantaloupe melon (strain CM175) and one from cilantro leaves (strain C15). The cell-free supernatants (CFS) of CM175 and C15 were found to exhibit antagonistic activity against FBD-causing pathogens. The CM175 and C15 strains were identified as *Pediococcus pentosaceus* and *Lactobacillus graminis*, respectively. Notably, the *P. pentosaceus* CM175 CFS stopped the growth of *Salmonella* Typhimurium, *Salmonella* Saintpaul, *Staphylococcus aureus*, and *Listeria monocytogenes*, and delayed *Escherichia coli* O157:H7 growth. Moreover, *L. graminis* C15 CFS delayed the growth of all indicator pathogens, but did not completely stop it. Organic acids and bacteriocin-like molecules were determined to be possibly exerting the observed antagonistic activity of the identified LAB strains. Thus, application of the antagonistic compounds produced by *Pediococcus pentosaceus* and *Lactobacillus graminis* could be a novel and ecological strategy in developing antimicrobial biopreservatives for the food industry and mitigating FBD by reducing the biological contamination in fruit and vegetable orchards, mainly via their potential in controlling both gram-negative and gram-positive pathogenic bacteria.

Keywords: **Foodborne diseases, lactic acid bacteria, bacteriocin, antimicrobial activity**

Introduction

Foodborne diseases (FBD) have been a common health and economic problem. Worldwide, an estimated 420,000 deaths are associated with these illnesses each year [1]. In the United States of America (USA) it is estimated that one person in six has experienced this problem [2]. Although the consumption of fruits and vegetables brings great nutritional benefits and are an important element in the diet of people, it may also represent a potential threat of health hazards mainly when risks of contamination are not understood and no efforts are

***Corresponding author**
Tel: +52-662-289-2400
E-mail: norawa@ciad.mx

done to reduce the sources and level of contaminants [3]. The incidence of outbreaks related to contaminated horticultural products is a recurrent issue often linked to the consumption of both leafy and fruit vegetables such as lettuce, cilantro, cantaloupe melon and cucumber [4]. More than 15 outbreaks due to consumption of fruits and vegetables contaminated with pathogenic bacteria have been documented in the USA from 2010 to 2018, with more than 2500 cases of infections and more than 734 hospitalizations, including 50 deaths [5]. In the year 2018 a multistate outbreak of *Escherichia coli* O157:H7 infections linked to consumption of romaine lettuce resulted in 210 infected people, 96 hospitalizations and five deaths [6]. The most recent outbreak related to the consumption of cantaloupe contaminated with *Salmonella* Typhimurium and *S.* Newport was documented in 2012, which reached 261 cases with 94 hospitalizations and 3 deaths [7]. In addition to *Salmonella* spp. and *E. coli* O157:H7, there are several pathogenic bacteria causing similar or even greater economic and health problems.

Among the pathogenic agents most frequently associated with FBD are both gram-positive and -negative bacteria, viruses and parasites [8]. *Salmonella* spp., *E. coli* O157:H7, *Listeria monocytogenes*, *Campylobacter* spp. and *Staphylococcus aureus* have been linked with vegetables- and fruits-related foodborne illnesses [4]. These bacteria could cause serious health problems like bleeding diarrhea, vomiting, abdominal pain, dizziness, and even death; they can also develop antibiotic resistance and enhance the problem [9]. Due to the high incidence, persistency and severity of infections, the development of novel and effective alternatives is required to reduce the FBD cases and, consequently, decrease the mortality associated with this type of illnesses.

It has been reported that some members of the lactic acid bacteria (LAB) group exert antagonistic activity against pathogenic bacteria by secreting different metabolites such as hydrogen peroxide, organic acids and bacteriocins. Similar antagonistic characteristics are shared by different bacteria of this group, however very specific and particular antagonistic spectra may be found among members of the same genus and even among the same species, which highlights the importance of characterizing the antagonistic particularities of related bacteria [10, 11]. Therefore, the screening of LAB characteristic antagonistic metabolites and their capacities could positively impact the field of food safety, by leading to the discovery of compounds that can potentially be used for combating growth of either specific bacterial targets or for a broader spectrum of pathogenic bacteria in fresh products [12]. Particularly, the antagonism related to the production of bacteriocins has received a singular attention due to two main reasons: first, its mode of action resembles that of antibiotics, causing the formation of pores in the membrane of sensitive bacteria and interrupting gene expression [13] and second, bacteriocins share relevant features that allow their use in the food industry; they are resistant to surfactants, susceptible to proteases, active in a wide pH range and they are often thermostable [14].

The production of bacteriocins varies according to the bacterial strain. Most of the known bacteriocins are effective against related bacteria, e.g. nisin produced by *Lactococcus lactis* subspecies *lactis*; effective only against gram-positive bacteria [15, 16]. Some LAB strains are able to produce more than one bacteriocin [17] and interestingly, different strains of the same genus and species have been reported to exert distinct antagonistic spectra due to the production of bacteriocins with an unusual broader activity against several pathogens [18–21]. Therefore, research focused on the screening of bacteriocin-producing strains from underexploited sources, such as fresh fruits and vegetables and the characterization of their corresponding bacteriocin-mediated antagonistic activities should be encouraged. We hypothesize that epiphytic LAB from vegetables have antagonistic activity against FBD-causing bacteria, exerted by the production and secretion of antibacterial metabolites. The objectives of this work were to isolate and identify epiphytic LAB from fresh leaf and fruit vegetables, and to characterize their antagonistic capacities against FBD-causing bacteria due to the production of bacteriocins or other extracellular compounds.

Materials and Methods

Vegetable material

Epiphytic bacteria from fresh vegetables were isolated from 25 mallets of cilantro (as a leaf vegetable model) and 25 cantaloupe melons (as a fruit model) collected

http://dx.doi.org/10.4014/mbl.1808.08014

from San Miguel de Horcasitas, Sonora, Mexico (29°27'46.32"N–110°44'9.35"W) and from the Guaymas Valley, Sonora, Mexico (28°14'44.27"N–110°39'2.8"W), respectively. Sampling, handling, and transport of samples were carried out using sterile conditions and equipment during the process. The samples were transported to the laboratory in cold (2–4℃) and dark conditions for their analyses in less than 24 h.

Isolation of lactic acid bacteria from cantaloupe melon fruits and cilantro leaves

From each melon, a 30 cm^2 sample of the epicarp was taken, and from each mallet of cilantro were taken 25 g. Samples were placed in 99 ml of 0.1% peptone water for their homogenization in a stomacher (400 Circulator, Seward Stomacher®, England) at 260 rpm for 1 min, and serial dilutions were made up to the 1×10^{-4}. A Petri dish containing De Man, Rogosa and Sharpe (MRS) agar (DIFCO, USA) was inoculated with each dilution and was incubated at 37℃ for 48 h. Afterwards, the colonies grown on the plates were inoculated in MRS broth and incubated at 37℃ for 24 h and an aliquot of each culture was preserved at -80℃, in the presence of 20% glycerol, according to Shan *et al*. [22] with modifications.

Pathogenic bacterial strains and culture conditions

Salmonella Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 6538 were provided by the Laboratory of Emerging Technologies of CIAD; *Salmonella* Saintpaul 476 and *Escherichia coli* O157:H7 K3999 were provided from collections of the FDA/CFSAN. All strains were grown in Tryptic Soy Broth (TSB) (DIFCO) at 37℃.

Antagonistic activity

The antimicrobial activity was measured through a slightly modified well diffusion agar test described in Hernandez *et al*. [23], and microplate turbidimetric growth inhibition assay described in de Lima Marques *et al*. [24]. Pathogenic bacteria were used as indicator.

To prepare cell-free supernatant (CFS), lactic acid bacteria were grown in 30 ml of MRS broth at 37℃ for 24 h, then the supernatant was recovered by centrifugation (10,000 ×*g*, 10 min, 4℃), sterilized by microfiltration (Durapore®, 0.22 μm size; Millipore Co., USA) and lyophilized (Labconco Freezone 4.5, USA). Lyophilized samples were reactivated in 20 mM sodium phosphate buffer pH 6.5 ± 0.2, with the tenth part of the initial volume.

For well diffusion agar test, indicator bacteria solutions were used to inoculate brain heart infusion (BHI) (DIFCO, USA) soft agar (1%) medium in a final concentration of 1×10^5 CFU/ml, 18 ml were placed in a Petri dish (90 mm diameter) and refrigerated at 4℃ for 30 min and wells of 5 mm diameter were made with sterile Pasteur pipettes. In each well, 50 μl of CFS of LAB was added and incubated at 37℃ for 24 h. The antimicrobial activity was determined by measuring the diameter (mm) of the clear zone around the well, using a Vernier. LABs showing an inhibition zone against at least four of the pathogenic bacteria tested were used for further growth inhibition assay in microplate with culture media.

A 96-well microplate was utilized for microplate turbidimetric growth inhibition assay. Five microliters of indicator bacteria in solution at a concentration of 1×10^6 CFU/ml was utilized to inoculate BHI broth with 0, 5, 10 or 15 μl of CFS for a final volume of 300 μl in each well and then, the microplate was placed in a FLUOstar Omega microplate reader (BMG LabTech, Germany) with incubation at 37℃, which reads Optic Density (OD) at 600 nm every 30 min with a 10 s shaking before each reading. To verify sterility three wells were filled with BHI broth without indicator bacteria, and other three wells with BHI broth and CFS (data not shown). OD values were plotted versus time (h) using the SigmaPlot 12.0 software (Systat software, USA).

Kinetic parameters of growth inhibition

Growth rate values (expressed as h^{-1}), maximum culture density (OD max), doubling time (min) and the duration of the growth lag phase (min) were calculated with the GrowthRates 3.0 software using the optical densities recorded during the growth curves of indicator bacteria [25]. No growth (NG) was reported when OD max values were lower than 0.1 absorbance units at 600 nm.

Identification of LAB isolates with antagonistic activity

Cell morphology and gram staining were used for phenotypic identification of LAB with antagonistic activity using an optical microscope (Olympus BX51, Japan) while biochemical identification was done by the oxidase

and catalase tests. Differential fermentation was carried out using sucrose, trehalose, and rhamnose in the culture medium replacing the glucose [26].

The genotypic identification was carried out by sequencing the 16S ribosomal RNA gene. Genomic DNA extraction was performed with the QIA amp DNA mini kit (QIAGEN, Germany). Genomic DNA was used as a template in a polymerase chain reaction (PCR) using the universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') with an expected final product of 1,460 bp. The PCR reaction mixture with a final volume of 50 µl was placed in an MJ Research PTC-150 thermocycler. The PCR conditions were as follows: initial denaturation at 95℃ for 5 min, 30 cycles of denaturation at 95℃ for 1 min each, primer annealing at 55℃ for 1 min and primer extension at 72℃ for 2 min and a final step of primer extension at 72℃ for 10 min. The PCR product was visualized in 1% agarose gel with Gel-Red staining (Biotium, USA). Purification of the PCR products was performed with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE, Healthcare) and the concentration was measured in a Nanodrop 2000 spectrophotometer. Amplicons were sequenced at Macrogen Inc. (Korea) and their identity was assigned after comparison with the sequences found in the GenBank database.

Type of antagonistic metabolite

Antagonistic metabolite produced by LAB was identified through a modified assay proposed by Cruz-Guerrero *et al.* [27]. Sequential steps were carried out in CFS of each isolate using the well diffusion agar test to measure the antagonistic activity against *Salmonella* Typhimurium (gram-negative) and *Listeria monocytogenes* (gram-positive) as follows:

a) Inhibition by organic acids. A CFS aliquot was pH neutralized (pH 6.5–7.5) with NaOH 1 M and bacterial antagonism was measured.

b) Inhibition by hydrogen peroxide. If CFS aliquot remained antagonist after neutralizing pH, catalase (1 mg/ml) was added, incubated at 37℃ for 90 min and antagonistic activity was measured.

c) Inhibition by proteinic compounds. One aliquot of antagonist CFS after neutralizing pH and adding catalase was taken, and a pool of proteases (protease, proteinase K, and trypsin) 1 mg/ml each was added, incubated at 37℃ for 120 min and at 65℃ for 30 min and inhibition zone was measured.

In addition, the CFS with the different treatments was used to perform growth inhibition curves for indicator pathogens.

Identification of bacteriocin structural genes

According to the results obtained through the identification of the CM175 antagonistic strain, it was investigated in the genomic DNA of CM175 the presence of possible structural genes coding for pediocins; bacteriocins produced by the *Pediococcus* genus. The primers Fw 5'-TTA CTT GTG GCA AAC ATT CCT G-3' and Rv 5'-TGA TTA CCT TGA TGT CCA CCA G-3' were used in a PCR reaction for amplification of the *papA* gene, according to Macwana and Muriana [28]. The PCR reactions containing 10 µl of SYBR® Green Supermix (Bio-Rad, USA), 1 µl of each respective primer and 12 ng of gDNA to a total final volume of 20 µl were run on a StepOne Real-Time PCR System (Applied Biosystems, USA). The identity of amplified products was assigned using the BLAST algorithm after sequencing at Macrogen Inc. (Korea) and comparison with the GenBank database.

Statistical analysis

Well diffusion agar tests were done in quadruplicate; data analysis was performed by Student's t-test to compare the antagonistic activity between the CM175 and C15 CFS on each indicator bacteria. The effect of pH neutralization, catalase, and proteases on the activity of CFS was analyzed with descriptive statistics. Microplate turbidimetric growth inhibition assays were performed in duplicates; both the effect of the different CFS volumes tested and the effect of pH neutralization, catalase, and proteases on the CFS antagonistic activities against indicator bacteria were analyzed by the Kruskal-Wallis test and Dunn's test was used to compare medians. In all the experiments, statistical software (NCSS 2007, NCSS LLC, USA) was used and $p < 0.05$ was considered significant.

Results

Antagonistic activity of bacterial strains isolated from vegetables

A total of 43 and 49 LAB isolates were obtained from

http://dx.doi.org/10.4014/mbl.1808.08014

Table 1. Inhibition zone of cell-free supernatants of lactic acid bacteria isolates.

Indicator bacteria	LAB isolate CFS	Inhibition zone (mm)
Listeria monocytogenes	CM175	17 ± 1^a
	C15	5 ± 1^b
Staphylococcus aureus	CM175	7 ± 0^a
	C15	4.33 ± 0.57^b
Salmonella Typhimurium	CM175	6 ± 1^a
	C15	5 ± 1^a
Salmonella Saintpaul	CM175	6 ± 0^a
	C15	5 ± 0^b
Escherichia coli O157:H7	CM175	0
	C15	0

The mean values ± standard deviations are shown. Different literals indicate significant differences for each indicator bacteria according to Student's t-test ($p < 0.05$). LAB = Lactic acid bacteria; CFS = Cell-free supernatant.

cantaloupe melon fruits and cilantro leaves, respectively. All these isolates were gram-positive, oxidase and catalase negative.

The majority of the LAB isolates exhibited antagonistic activity against at least one of the pathogens tested. The inhibition zone diameters of antagonistic isolates were variable depending on the type of antagonistic bacteria and indicator pathogen strain. Particularly, two isolates showed antimicrobial activity against the majority of pathogens tested in this study; one of these strains was isolated from cilantro and one from cantaloupe melon (from now on referred as C15 and CM175, respectively). Both isolates showed antagonistic activity against all tested pathogens except for *E. coli* O157:H7, using well diffusion agar test. Inhibition zones against indicator strains were similar in C15 CFS, while CM175 CFS showed a higher activity against gram-positive compared to gram-negative. In general CM175 CFS presented a more potent antagonistic activity than C15 CFS (Table 1).

Figs. 1 and 2 show the inhibition growth curves performed with the CFS produced by CM175 and C15 isolates, respectively. These curves confirmed that CM175 isolate exhibits a higher antagonistic activity than C15, similar to the observation in the well diffusion agar test. CM175 CFS showed the capacity to stop the growth of pathogenic bacteria (*L. monocytogenes, S. aureus, S.* Saintpaul and *S.* Typhimurium) up to 24 h, but in the particular case of *E. coli* O157:H7, the volumes used delayed its growth by 10 h. C15 CFS delayed bacterial growth but none of the studied volumes was able to completely stop it. Fifteen microliters of CM175 was enough to stop the growth of pathogens compared to the negative control (Fig. 1).

All the analyzed growth kinetic values were affected by the addition of both CFS, where the lag and the doubling times tended to increase, while the growth rate values and O.D. max showed a decrease by the effect of the CFS (Table 2). Particularly, when the C15 CFS was added to the culture of indicator bacteria, the duration of the growth lag phase was increased up to 8 times, and even though it did not stop their growth it did decrease the O.D. max and increased the doubling time. The addition of CM175 CFS completely stopped the growth of indicator pathogens, except for *E.coli* O157:H7, however it increased its lag time up to 10 times and the doubling time by 3 times, while the reached O.D. max decreased.

Identification of selected LAB strains

A sequence of approximately 1500 bp corresponding to the 16S ribosomal RNA gene was obtained from each strain (C15 and CM175) and compared with other sequences deposited in the GenBank using the BLAST algorithm. According to the analyzed molecular sequences, C15 was identified as *Lactobacillus graminis*, while CM175 analysis matched with different species of the *Pediococcus* genus; *P. acidilactici, P. pentosaceus* and *P. lolli* (Table 3). Hence, differential fermentation of specific carbohydrates was used for differentiation between species, where CM175 strain fermented sucrose, and trehalose, but no rhamnose, distinctive feature of *P. pentosaceus*.

Antagonistic metabolite

Table 4 shows the antagonistic activity of *P. pentosaceus* CM175 and *L. graminis* C15 according to the type of metabolite tested. Two indicator bacteria were tested; a gram-positive and a gram-negative. It was observed that *P. pentosaceus* CM175 CFS remained active against the gram-positive indicator pathogen after pH neutralization; however, the antagonistic activity of *L. graminis* C15 CFS was lost after pH neutralization, suggesting that organic acids were the antagonistic metabolites pro-

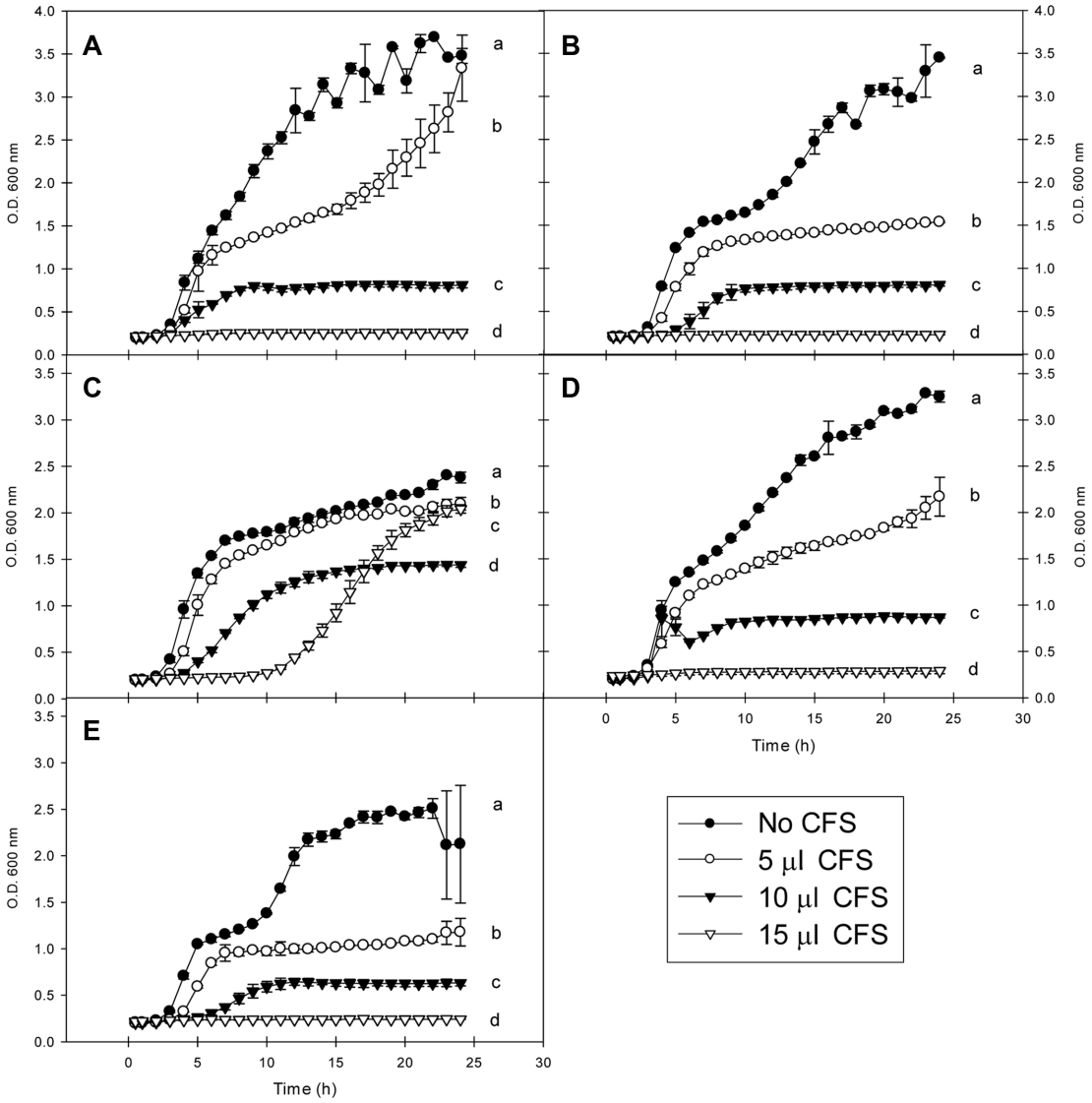

Fig. 1. Effect of CM175 (*Pediococcus pentosaceus*) cell-free supernatants on growth curves of indicators pathogens. *Salmonella* Typhimurium (A); *Salmonella* Saintpaul (B); *Escherichia coli* O157:H7 (C); *Staphylococcus aureus* (D); *Listeria monocytogenes* (E). CFS = Cell-free supernatant. Results were expressed as the means ± standard deviation. Different literals indicate significant differences according to Dunn's Test ($p < 0.05$).

duced by this strain. Therefore, subsequent growth inhibition assays by neutralized CFS were only performed for *P. pentosaceus* CM175 CFS, which lost its activity only after incubation with proteases; suggesting that the antagonistic metabolite is of proteinic origin. The antagonistic effect exerted by organic acids and proteinic components of *P. pentosaceus* CM175 CFS on the growth of pathogens was evidenced in Fig. 3. Significantly important was the antagonistic activity exerted by the components of proteinic nature in *P. pentosaceus* CM175 CFS. Also, it was then interesting to demonstrate if a bacteriocin-coding gene was present in the CM175 strain.

Molecular analysis showed the presence of a bacteriocin structural gene in the genomic DNA of CM175; a PCR product of approximately 116 bp was amplified and sequenced to confirm identity. This identity was up to 98% to the *papA* and *pedA* genes (GenBank accession numbers KC693734.1 and KY038164.1, respectively)

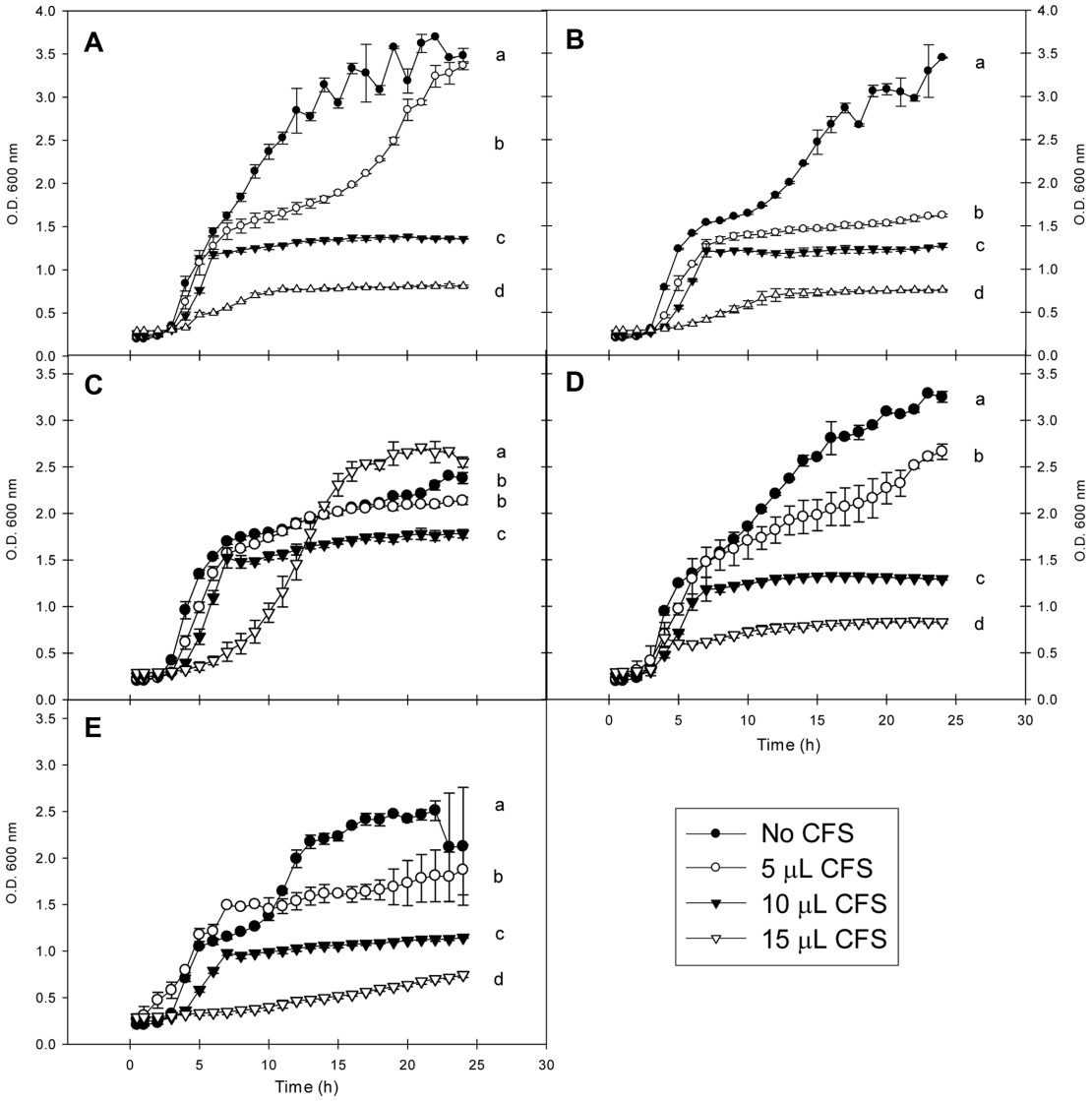

Fig. 2. Effect of C15 (*Lactobacillus graminis*) cell-free supernatants on growth curves of indicators pathogens. *Salmonella* Typhimurium (A); *Salmonella* Saintpaul (B); *Escherichia coli* O157:H7 (C); *Staphylococcus aureus* (D); *Listeria monocytogenes* (E). CFS = Cell-free supernatant. Results were expressed as the means ± standard deviation. Different literals indicate significant differences according to Dunn's Test ($p < 0.05$).

coding for pediocins.

Discussion

Pediococcus pentosaceus CM175 and *Lactobacillus graminis* C15 were isolated from cantaloupe melon fruits and cilantro leaves, respectively, using semi-selective MRS agar medium together with gram staining, catalase and oxidase biochemical tests [29], and specific fermentation tests in the case of *P. pentosaceus* CM175. Doi *et al.* [26] established that one of the differences between *Pediococcus* spp. strains, including *P. pentosaceus*, *P. acidilactici*, and *P. lolli*, is the ability to selectively ferment carbohydrates like sucrose, trehalose and rhamnose. The species *P. pentosaceus* has the ability to ferment sucrose and trehalose, but not rhamnose; *P. acidilactici* can ferment only trehalose, while *P. lolli* can ferment only rhamnose.

Although LAB are widely distributed in nature and fresh vegetables harbor this type of bacteria [30], few

Table 2. Kinetic parameters of growth inhibition of indicator bacteria in the presence of CM175 CFS and C15 CFS.

Sample	Lag (min)	Growth rate (h^{-1})	OD max (λ 600 nm)	Doubling time (min)	R^2
S. Typhimurium					
Control	21.65 ± 20.4	4.92 ± 0.24	3.497 ± 0	8.5 ± 0.4	0.95
CM175 CFS	NG	NG	NG	NG	-
C15 CFS	176.2 ± 10.6	0.53 ± 0.08	0.616 ± 0.02	79.8 ± 12.9	0.92
S. Saintpaul					
Control	61.85 ± 25.9	1.99 ± 0.2	3.284 ± 0.04	21.05 ± 2.2	0.99
CM175 CFS	NG	NG	NG	NG	-
C15 CFS	220.9 ± 32.8	0.27 ± 0.02	0.56 ± 0.007	153.2 ± 10.9	0.99
E. coli					
Control	41.2 ± 19.5	1.97 ± 0.6	2.21 ± 0.02	22.1 ± 6.8	0.99
CM175 CFS	419.6 ± 21.2	0.56 ± 0.01	1.843 ± 0.04	74.3 ± 2.1	0.99
C15 CFS	220.2 ± 59.9	0.35 ± 0.01	2.561 ± 0.003	117.8 ± 3.5	0.99
S. aureus					
Control	51.1 ± 22.7	3.43 ± 2.46	3.194 ± 0.1	16.3 ± 11.7	0.96
CM175 CFS	NG	NG	NG	NG	-
C15 CFS	146.75 ± 3.2	0.77 ± 0.07	0.639 ± 0.03	53.55 ± 4.6	0.93
L. monocytogenes					
Control	58.6 ± 37.2	1.69 ± 0.55	2.438 ± 0.01	25.95 ± 8.4	0.99
CM175 CFS	NG	NG	NG	NG	-
C15 CFS	289 ± 97	0.16 ± 0.02	0.545 ± 0.1	267.7 ± 43.6	0.99

All measures were expressed as the mean ± standard deviation. NG = No growth; CM175 CFS = Cell-free supernatant of strain CM175; C15 CFS = Cell-free supernatant of strain C15.

reports mentioned the antagonistic power of strains isolated from these foods. *P. pentosaceus* and *L. graminis* strains have been respectively isolated from marula trees [20], wheat kernels [31], and grass silage [32], so it would not be uncommon to find them in vegetables. In this context, despite these are commonly found bacteria, the strains characterized in this work, exhibit particular and interesting antagonistic features against bacteria that cause FBD.

The adaptation of bacteria to different niches often confers particular characteristics. Therefore different strains of the same bacterial genus or bacterial species may exert a different antagonistic activity. The antagonistic activity of LAB from different sources has been demonstrated in several studies. Some studies reported LAB with antibacterial activity against gram-positive bacteria, but no activity against negative bacteria [33]. However, recent reports indicate that a strain of *Lactobacillus gasseri* EV1461 is active against both gram-positive and gram-negative bacteria [34], and there are

Table 3. Molecular identification of LAB isolates from cilantro and cantaloupe melon through sequencing of 16S Ribosomal RNA gene.

Isolate	Reference LAB	Strain	GenBank accession number	Coverage (%)	Identity (%)
C15	*Lactobacillus graminis*[a]	G90	NR_042438.1	99	99
CM175	*Pediococcus acidilactici*	DSM 20284	NR_042057.1	98	95
	Pediococcus pentosaceus[a]	DSM 20336	NR_042058.1	98	94
	Pediococcus lolli	CJ66	KJ580428.1	98	96

[a]Identified bacterium.

Table 4. Inhibition zone of metabolite produced by *Pediococcus pentosaceus* and *Lactobacillus graminis*.

Indicator bacteria	LAB	LAB metabolite	Inhibition zone (mm)[A]
Listeria monocytogenes	*Pediococcus pentosaceus* CM175	OA, HP and PC	17.75 ± 0.5
		HP and PC	18.25 ± 0.5
		PC	16.5 ± 1.3
	Lactobacillus graminis C15	OA, HP and PC	4.5 ± 0.58
		HP and PC	0
		PC	0
Salmonella Typhimurium	*Pediococcus pentosaceus* CM175	OA, HP and PC	5 ± 0.82
		HP and PC	0
		PC	0
	Lactobacillus graminis C15	OA, HP and PC	4.75 ± 0.5
		HP and PC	0
		PC	0

[A]Inhibition zone was expressed as the mean ± standard deviation.
OA = Organic Acids; HP = Hydrogen peroxide; PC = Proteinic component.

even reports of a strain of *Lactobacillus coryniformis* XN8 with antagonistic activity against multiresistant bacteria [21]. Kinetic parameters calculated in the present study showed that CFS produced by *P. pentosaceus* CM175 and *L. graminis* C15 either stopped the growth or slowed down the metabolic machinery of both gram-negative and gram-positive pathogens, according to the observed increase in the lag phase and doubling times along with a decrease in the growth rate. This decrease may indicate bacteria are struggling in adapting to the system conditions and the decrease in OD max values may indicate that the efficiency of biomass production from available resources has been compromised.

LAB have the capacity to produce different metabolites that act negatively generally against other related bacteria [35]. Vijayakumar and Muriana [36] performed inhibition growth assays to test the activity of LAB. They found that non-neutralized CFS of some LAB are more effective against *L. monocytogenes* than neutralized CFS, but both delayed pathogen growth by 10 h using 100 µl of CFS, which is 6 times bigger than the volume used in this study. In this point, the antagonistic

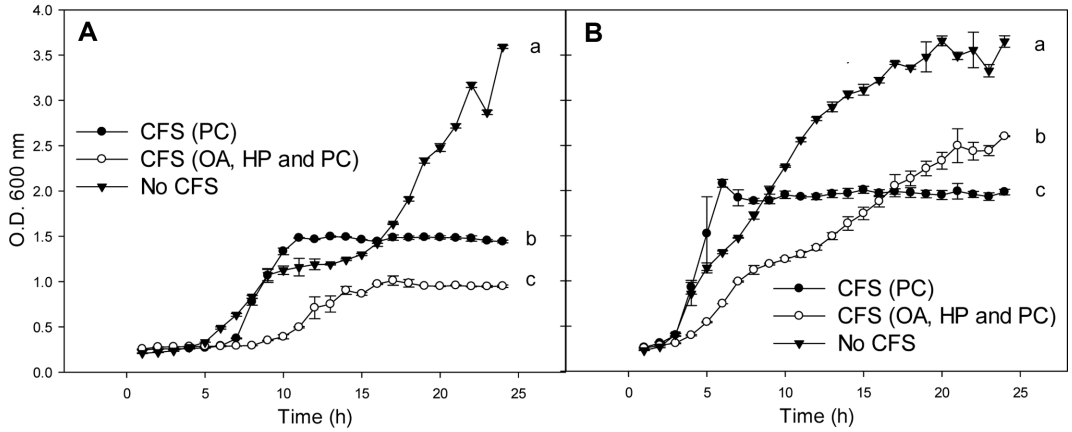

Fig. 3. Inhibition of growth curves of indicator pathogens by effect of metabolites produced by *Pediococcus pentosaceus* CM175. *Listeria monocytogenes* (A); *Salmonella* Typhimurium (B). CFS = Cell-free supernatant; PC = Proteinic Component; OA = Organic Acids; HP = Hydrogen Peroxide. Results were expressed as the mean ± standard deviation. Different literals indicate significant differences according to Dunn's Test ($p < 0.05$).

activity observed in *P. pentosaceus* CM175 and *L. graminis* C15 agree with the production of organic acids and bacteriocin-like compounds as reported for other bacteriocin-producing LAB [37] and acids-producing LAB [19]. The action of the organic acids is clearly shown when the bacterial antagonism is reduced by adjusting the pH to 7, while the proteinic origin of the CM175 CFS antagonistic compound is exposed by the activity disappearance after incubating CM175 CFS with proteases. Additionally, it is possible for this proteinic antagonistic compound produced by *P. pentosaceus* CM175 to be a pediocin-like bacteriocin, as corroborated by amplification of the putative structural genes *papA* and *pedA*, that were formerly reported to code for pediocins in some strains of the *Pediococcus* genus.

Todorov and Dicks [20] found a bacteriocin produced by *P. pentosaceus* ST44AM that was active against some strains of *E. coli, S. aureus* and *L. monocytogenes* but ineffective against *Salmonella* strains, which differs from the antagonism found in this study, where *P. pentosaceus* CM175 inhibited *S. aureus*, *L. monocytogenes,* *S.* Typhimurium and *S.* Saintpaul growth and delayed *E. coli* growth. In the case of *Pediococcus*, the species *P. pentosaceus* is reported to be capable of producing up to 4 bacteriocins, some of which have not been characterized yet. Therefore, in view of the antagonistic profile observed in our study it could be presumed the bacteriocin produced by *P. pentosaceus* CM175 is different from those already reported elsewhere [17]. Therefore, it can be deduced that bacteria from the same genus and species could produce different antagonistic compounds depending on where they grow and the possible adversities they may have to overcome. A similar behavior has been observed for *Lactobacillus* bacteria isolated from a cereal-based beverage, where two *L. plantarum* isolates had different antagonistic capacities, even when they belonged to the same species [38].

The proteinic components in the CFS produced by *P. pentosaceus* CM175 were active against *S.* Typhimurium and *L. monocytogenes* growth, indicating that it can exert antibacterial activity against both gram-positive and gram-negative bacteria; such effect was more potent over gram-positive bacteria than for negative bacteria, however, it was possible to delay their growth in broth cultures. Generally, the bacteriocins produced by antagonistic LAB affect gram-positive bacteria, but not negative bacteria [18, 39]; only in some cases the effect is exerted against both [19, 24]. On the other hand, even when *L. graminis* C15 showed a potent antagonistic activity against indicator pathogens, results suggested that under the experimental conditions carried out in this work, such activity was conferred mainly by the production of acidic compounds. With the results found in this work, it could be proposed that the metabolites produced by *P. pentosaceus* and *L. graminis* (proteinic compounds and organic acids) could be used as biopreservatives for the food industry, with the advantage of being able to act against gram-positive and gram-negative bacteria, which is a characteristic of very few LAB. Therefore, the quality and safety aspects of their direct incorporation in food products should be addressed in future studies.

The use of LAB CFS to avoid food spoilage was addressed by Muhialdin *et al.* [40] who found that the CFS of *L. fermentum* Te007, *P. pentosaceus* Te010, *L. pentosus* G004, and *L. paracasi* D5 delayed the growth of spoilage microorganisms in different food matrices such as tomato puree, cheese and bread. Therefore, these metabolites can potentially be used for developing alternatives to simultaneously retain the quality and improve the safety of fresh fruits and vegetables by reducing the biological contamination from its cultivation to its processing, with the confidence that these LAB are epiphytic bacteria of cantaloupe melon and cilantro and consequently may be naturally present on their surfaces. In this sense, some studies suggest that LAB bacteriocins are produced at levels below the minimum inhibitory concentrations in their ecological niche [41]; hence the production of active molecules found in the CFS at sufficient amounts would be essential to ensure the viability of the above-mentioned applications.

In summary, cantaloupe melon fruit and cilantro leaves are natural reservoirs for lactic acid bacteria with biotechnological potential, related to the production and secretion of antagonistic metabolites against foodborne pathogens. The antagonistic activity of *Lactobacillus graminis* C15 is mainly mediated by the production of extracellular acidic compounds; while, the activity of *Pediococcus pentosaceus* CM175 is mediated by the production of compounds of both acidic and proteinic nature. The proteinic nature of the antagonistic compounds produced by *P. pentosaceus* was verified when

http://dx.doi.org/10.4014/mbl.1808.08014

the activity disappeared after addition of proteases, as well as by the molecular identification of a structural gene coding for a pediocin-like bacteriocin, which may likely be related to the particular and broader antagonistic profile observed in this work for CM175 strain, which is different from that of *P. pentosaceus* strains reported elsewhere. These findings may contribute to the development of future applications in the fields of food safety and agro-food industry by contributing to the mitigation of foodborne diseases related to the consumption of fresh fruits and vegetables, as well as new practical means to avoid food spoilage.

Acknowledgments

This work was supported by FOINS-CONACYT [grant number FON.INST/30/2017]. We are grateful to CONACYT for the scholarships granted to González-Pérez, Aguilar-Gil and Aguirre-Guzmán. We thank Olivia Briceño and Francisco Soto for technical support. Also, to the USDA - National Institute of Food and Agriculture and the Texas A&M Research Foundation for the award 2010-51110-21079, subaward S110080.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. WHO. 2015. Food safety. Available from http://www.who.int/mediacentre/factsheets/fs399/en/. Accessed 8 November, 2017.
2. CDC. 2015. Foodborne germs and illnesses. Available from https://www.cdc.gov/foodsafety/foodborne-germs.html. Accessed 15 December, 2017.
3. Hussain MA, Gooneratne R. 2017. Understanding the fresh produce safety challenges. *Foods* **6**: 23.
4. Wadamori Y, Gooneratne R, Hussain MA. 2017. Outbreaks and factors influencing microbiological contamination of fresh produce. *J. Sci. Food Agr.* **97**: 1396-1403.
5. CDC. 2018. List of selected multistate foodborne outbreak investigations. Available from https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html. Accessed 18 October, 2018.
6. CDC. 2018. Multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce (Final update). Available from https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html. Accessed 14 August, 2018 .
7. CDC. 2012. Multistate outbreak of *Salmonella* Typhimurium and *Salmonella* newport infections linked to Cantaloupe (Final update). Available from https://www.cdc.gov/salmonella/typhimurium-cantaloupe-08-12/index.html. Accessed 17 October, 2018.
8. Tauxe RV. 2002. Emerging foodborne pathogens. *Int. J. Food Microbiol.* **78**: 31-41.
9. Yeni F, Yavaş S, Alpas H, Soyer Y. 2016. Most common foodborne pathogens and mycotoxins on fresh produce: a review of recent outbreaks. *Crit. Rev. Food Sci. Nutr.* **56**: 1532-1544.
10. Janisiewicz WJ, Korsten L. 2002. Biological control of postharvest diseases of fruits. *Annu. Rev. Phytopathol.* **40**: 411-441.
11. Jordan K, Dalmasso M, Zentek J, Mader A, Bruggeman G, Wallace J, *et al.* 2014. Microbes versus microbes: control of pathogens in the food chain. *J. Sci. Food Agr.* **94**: 3079-3089.
12. González-Pérez CJ, Aispuro-Hernández E, Vargas-Arispuro I, Martínez-Téllez MA. 2018. Induction of bacteriocins from lactic acid bacteria; a strategy to improve the safety of fresh fruits and vegetables. *ARTOAJ* **14**: 555927.
13. Karpiński TM, Szkaradkiewicz AK. 2013. Characteristic of bacteriocines and their application. *Pol. J. Microbiol.* **62**: 223-235.
14. Alvarez-Sieiro P, Montalbán-López M, Mu D, Kuipers OP. 2016. Bacteriocins of lactic acid bacteria: extending the family. *Appl. Microbiol. Biotechnol.* **100**: 2939-2951.
15. Barbosa AAT, Mantovani HC, Jain S. 2017. Bacteriocins from lactic acid bacteria and their potential in the preservation of fruit products. *Crit. Rev. Biotechnol.* **37**: 852-864.
16. Lubelski J, Rink R, Khusainov R, Moll G, Kuipers O. 2008. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell. Mol. Life Sci.* **65**: 455-476.
17. Woraprayote W, Malila Y, Sorapukdee S, Swetwiwathana A, Benjakul S, Visessanguan W. 2016. Bacteriocins from lactic acid bacteria and their applications in meat and meat products. *Meat Sci.* **120**: 118-132.
18. Ahmadova A, Todorov SD, Hadji-Sfaxi I, Choiset Y, Rabesona H, Messaoudi S, *et al.* 2013. Antimicrobial and antifungal activities of *Lactobacillus curvatus* strain isolated from homemade Azerbaijani cheese. *Anaerobe* **20**: 42-49.
19. Shokryazdan P, Sieo CC, Kalavathy R, Liang JB, Alitheen NB, Faseleh Jahromi M, *et al.* 2014. Probiotic potential of *Lactobacillus* strains with antimicrobial activity against some human pathogenic strains. *BioMed. Res. Int.* **2014**: 927268.
20. Todorov SD, Dicks LMT. 2009. Bacteriocin production by *Pediococcus pentosaceus* isolated from marula (*Scerocarya birrea*). *Int. J. Food Microbiol.* **132**: 117-126.
21. Yi L, Dang J, Zhang L, Wu Y, Liu B, Lü X. 2016. Purification, characterization and bactericidal mechanism of a broad spectrum bacteriocin with antimicrobial activity against multidrug-resistant strains produced by *Lactobacillus coryniformis* XN8. *Food Control.* **67**: 53-62.
22. Shan B, Cai Y-Z, Brooks JD, Corke H. 2011. Potential application of spice and herb extracts as natural preservatives in cheese. *J. Med. Food.* **14**: 284-290.
23. Hernandez D, Cardell E, Zarate V. 2005. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance pro-

duced by *Lactobacillus plantarum* TF711. *J. Appl. Microbiol.* **99**: 77-84.
24. de Lima Marques J, Funck GD, da Silva Dannenberg G, dos Santos Cruxen CE, El Halal SLM, Dias ARG, *et al.* 2017. Bacteriocin-like substances of *Lactobacillus curvatus* P99: characterization and application in biodegradable films for control of *Listeria monocytogenes* in cheese. *Food Microbiol.* **63**: 159-163.
25. Hall BG, Acar H, Nandipati A, Barlow M. 2014. Growth rates made easy. *Mol. Biol. Evol.* **31**: 232-238.
26. Doi K, Nishizaki Y, Fujino Y, Ohshima T, Ohmomo S, Ogata S. 2009. *Pediococcus lolii* sp. nov., isolated from ryegrass silage. *Int. J. Syst. Evol. Microbiol.* **59**: 1007-1010.
27. Cruz-Guerrero A, Hernández-Sánchez H, Rodríguez-Serrano G, Gómez-Ruiz L, García-Garibay M, Figueroa-González I. 2014. Commercial probiotic bacteria and prebiotic carbohydrates: a fundamental study on prebiotics uptake, antimicrobials production and inhibition of pathogens. *J. Sci. Food Agr.* **94**: 2246-2252.
28. Macwana SJ, Muriana PM. 2012. A 'bacteriocin PCR array' for identification of bacteriocin-related structural genes in lactic acid bacteria. *J. Microbiol. Methods.* **88**: 197-204.
29. Savadogo A, Ouattara AC, Bassole HI, Traore SA. 2006. Bacteriocins and lactic acid bacteria-a minireview. *Afr. J. Biotechnol.* **5**: 678-683.
30. Leff JW, Fierer N. 2013. Bacterial communities associated with the surfaces of fresh fruits and vegetables. *PLoS One.* **8**: e59310.
31. Corsetti A, Settanni L, López CC, Felis GE, Mastrangelo M, Suzzi G. 2007. A taxonomic survey of lactic acid bacteria isolated from wheat (*Triticum durum*) kernels and non-conventional flours. *Syst. Appl. Microbiol.* **30**: 561-571.
32. Beck R, Weiss N, Winter J. 1988. *Lactobacillus graminis* sp. nov., a new species of facultatively heterofermentative lactobacilli surviving at low pH in grass silage. *Syst. Appl. Microbiol.* **10**: 279-283.
33. Albano H, Todorov SD, van Reenen CA, Hogg T, Dicks LM, Teixeira P. 2007. Characterization of two bacteriocins produced by *Pediococcus acidilactic*i isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *Int. J. Food Microbiol.* **116**: 239-247.
34. Maldonado-Barragán A, Caballero-Guerrero B, Martín V, Ruiz-Barba JL, Rodríguez JM. 2016. Purification and genetic characterization of gassericin E, a novel co-culture inducible bacteriocin from *Lactobacillus gasseri* EV1461 isolated from the vagina of a healthy woman. *BMC Microbiol.* **16**: 37.
35. Hayek SA, Ibrahim SA. 2013. Current limitations and challenges with lactic acid bacteria: a review. *Food Nutr. Sci.* **4**: 73-87.
36. Vijayakumar PP, Muriana PM. 2015. A microplate growth inhibition assay for screening bacteriocins against *Listeria monocytogenes* to differentiate their mode-of-action. *Biomolecules* **5**: 1178-1194.
37. Ovchinnikov KV, Chi H, Mehmeti I, Holo H, Nes IF, Diep DB. 2016. Novel group of leaderless multipeptide bacteriocins from gram-positive bacteria. *Appl. Environ. Microbiol.* **82**: 5216-5224.
38. Von Mollendorff J, Todorov S, Dicks L. 2006. Comparison of bacteriocins produced by lactic-acid bacteria isolated from boza, a cereal-based fermented beverage from the Balkan Peninsula. *Curr. Microbiol.* **53**: 209-216.
39. Todorov SD, Perin LM, Carneiro BM, Rahal P, Holzapfel W, Nero LA. 2017. Safety of *Lactobacillus plantarum* ST8Sh and its bacteriocin. *Probiotics Antimicrob. Proteins* **9**: 334-344.
40. Muhialdin BJ, Hassan Z, Sadon SK. 2011. Antifungal activity of *Lactobacillus fermentum* Te007, *Pediococcus pentosaceus* Te010, *Lactobacillus pentosus* G004, and *L. paracasi* D5 on selected foods. *J. Food Sci.* **76**: M493-M499.
41. Chikindas ML, Weeks R, Drider D, Chistyakov VA, Dicks LMT. 2018. Functions and emerging applications of bacteriocins. *Curr. Opin. Biotechnol.* **49**: 23-28.