Optimization and some characteristics of bacteriocin produced by *Enterococcus* sp. CM9 collected from Mauritanian Camel milk

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ABSTRACT

The use of bacteriocin as a bioconservation is very promising in the agri-food industry to substitute the chemical preservatives. The production of bacteriocin by lactic acid bacteria (LAB) depending on various conditions, the purpose of the present work was to assess the effect of some ingredients of medium on the bacteriocin production. Twenty-four strains of LAB of the genera *Leuconostoc*, *Lactobacillus,* and *Enterococcus*, isolated from camel milk, were tested for antibacterial activity and bacteriocin production. Among these strains, three isolates showed bacteriocinogenic activity, two *Enterococcus* strains (bacteriocin CM9, bacteriocin CM18), and one *Leuconostoc* strain (bacteriocin CHBK310). The bacteriocin produced by *Enterococcus* sp CM9 was subject of optimisation study. The modified MRS with nitrogen sources in media containing tryptone, meat extract and yeast extract and with potassium sources in media containing 2% of K_2HPO_4 allowed higher activity with inhibition zone of 10 mm and this production remained stable at incubation temperatures from 25 to 40°C with an inhibition zone which reached 12 mm at 25°C. The diffusion of this bacteriocin in the neutrality condition, allowed a better evaluation of its inhibitory effect. The activity of the bacteriocin was not affected, neither by acidic or basic pH nor by heat treatments, its activity remaining stable even after 2 weeks at -20 and 4°C. The bacteriocin CM9 belonged to class II, had a molecular weight of 7.6 kDa. These results indicated that the growth and diffusion conditions have a role in the bacteriocin production and its activity.

Keywords: Bacteriocin; Characterization; *Enterococcus* sp CM9; Medium effect; Molecular weight; Stability

INTRODUCTION

The lactic acid bacteria (LAB) are present in a large variety of environments, as milk and dairy products, vegetables and plants, cereals, meat and meat products (Mayo et al., 2010). They produce many antimicrobial agents such as organics acids, hydrogen peroxide, and bacteriocins. The production of bacteriocin by the LAB has received much attention and has been the objective of several studies (Sure et al., 2016). In the last decade, the number of novel bacteriocins isolated from different species of LAB increased (Perez et al., 2014) for use as natural inhibitors in food as bio-preservatives. The use of LAB or their bacteriocins as food bio-preservatives can be prevent the development of undesirable microorganisms in foods. The genus of *Enterococcus* belonging to the LAB are present as

natural flora in the human intestinal tract (Pieniz et al., 2012). Bacteriocins of LAB are ribosome synthesized proteins or protein complexes, usually antagonistic against genetically closely related organisms and bacteria cohabiting with the bacteriocinogenic bacteria (Collins et al., 2010). Generally, they are classified into three classes: lantibiotics, non-lantibiotics peptides, and large heat-labile proteins. Class I bacteriocins or lantibiotics possess a cationic and amphiphilic structure with a low molecular weight, from approximately 3 to 5 kDa. Class II bacteriocins, or the non-lantibiotics, most frequently occurring in nature, they are small (<10 kDa), heat-stable, do not require posttranslational modification (Sacchini et al., 2017). Classes I and II are the main classes of bacteriocins well studied due to their ubiquity and potential applications (Arthur et al., 2004). The bacteriocin production occurs

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from mid-exponential phase to early-stationary phase achieving a maximal level, so considering as growth associated (Todorov et al., 2007). The culture conditions, as the medium composition, incubation atmosphere, pH of medium, incubation temperature, and microorganism growth phase can influence their production (Settani and Corsetti 2008). Their maximum activity depends on diffusion conditions, like pH and salt concentration. Optimizing the production of bacteriocin and improving inhibitory activity against potential food contaminants have great economic importance.

In the present study, bacteriocinogenic bacteria among twenty-four LAB strains collected from camel milk and belonging to the collection of our laboratory, were screened to optimize the production of bacteriocin from conventional laboratory media and to determine some of their characteristics.

MATERIALS AND METHODS

Bacterial strains and culture conditions
The bacteria tested in our study belonged to the Laboratory of Micro-organisms Biology and Biotechnology (LBMB) collection (Table 1). Isolated from camel milk, the strains were stored at -20°C in the presence of 40% glycerol. For testing, the strains were propagated twice in appropriate broth media. The cultures of all strains were realized on de Man Rogosa Sharpe (MRS) agar (Fluka, Steinheim, Germany), broth or on soft agar (De Man et al., 1960). For the well diffusion method, the growth of the strains was achieved on buffered MRS medium (KH_2PO_4-Na_2HPO_4, pH 7.0 at 0.2 M), supplemented with 25% of skim milk (Jozala et al., 2005) and 2.5% of yeast extract (Benkeroum et al., 2000).

Screening of bacteriocinogenic LAB
The agar spot assay
The agar spot assay was done using the method described by Fleming et al. (1975). 5 µL of the strains cultures (to be tested for production of an antimicrobial agent) were spotted onto the surface of MRS agar plates and incubated for 18-20 h at 30°C. Then, 7 mL of soft agar (0.7%) containing 500 µL of an overnight culture of the indicator strains were overlaid on preincubated cultures. After incubation at 30°C for 24-48 h (depending on the indicator strain), the diameters of clear halos appearing around the spotted cultures were measured.

The well diffusion test
The antimicrobial activity of the cell-free supernatant (CFS) was confirmed by the agar well diffusion assay as described by Schillinger and Lücke 1989. The CFS of the strains grown for 18-20 h at 30°C were obtained after centrifugations at 6000 × g for 15 min at 4°C. One-hundred µL of CFS was

Table 1: Lactic acid bacteria strains, isolated from cow and camel milk, used in the present study

Preliminary identification	Strains	Origins
Lactobacillus sp.	LVK11	Raw cow milk
Lactobacillus sp.	LVK30	Raw cow milk
Lactobacillus sp.	LVK31	Raw cow milk
Lactobacillus sp.	LVK32	Raw cow milk
Enterococcus sp.	CM9	Raw camel milk
Enterococcus sp.	CM18	Raw camel milk
Enterococcus sp.	CM19	Raw camel milk
Enterococcus faecium	H3	Fermented wheat
Lactobacillus brevis	CHTD27	Raw camel milk
Lactobacillus cellobiosus	CHTD29	Raw camel milk
Lactobacillus plantarum	BH14	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK 309	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK310	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK311	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK312	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK314	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK315	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK316	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK318	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK319	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK320	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK323	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK325	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK326	Raw camel milk
Leuconostoc mesenteroides subsp. *dextranicum*	CHBK327	Raw camel milk

placed in wells of ~ 5mm in diameter cut into the agar plate using a cork borer, pre-seeded with the indicator strain (1% v/v). After a diffusion at 4°C for overnight, the plates were incubated at 30°C for 24 h and the diameters of the growth inhibition zones were then measured.

Bacteriocins production

To confirm that the inhibitory agent is bacteriocin, which is proteinaceous in nature, the CFS were treated with pepsin, catalase, trypsin, and pronase E (Sigma-Aldrich) at the concentration of 2 mg/mL (Cocolin et al., 2007), respectively. Samples were incubated at 30°C for 2 h and then heated at 100°C for 5 min to inactive enzymes. The catalase was tested for detecting the inhibition caused by the hydrogen peroxide. All the samples and a control sample (supernatants not treated with enzymes) were tested, by the agar well diffusion assay as described above.

Optimization of bacteriocin production and activity produced by *Enterococcus* sp CM9
Nitrogen source effect

The effect of nitrogen source on bacteriocin production was carried as described by Von Mollendorff et al. (2009) with some modification, five milliliters of culture of bacteriocinogenic strain was used to inoculate 100 mL of the medias: MRS broth without organic nutrients (peptone, meat extract, yeast extract), supplemented with 20 g/L tryptone (media 1), 20 g/L meat extract (media 2), 20 g/L yeast extract (media 3), 12.5 g/L tryptone plus 7.5 g/L meat extract (media 4), 12.5 g/L tryptone plus 7.5 g/L yeast extract (media 5), or a combination of 10 g/L tryptone, 5 g/L meat-extract, and 5 g/L yeast extract (media 6), respectively. Incubation for all medias was at 30°C for 20 h and the well diffusion method was used.

Carbon source effect

Glucose, maltose, lactose, saccharose, and glycerol were used separately in MRS medium at the concentration of 2%. The different modified MRS were inoculated at 5% of CM9 strain culture. After incubation, the same procedures of well diffusion method were employed.

Potassium source effect

The 0.2% of K_2HPO_4 was substituted by 1% of K_2HPO_4, 2% of K_2HPO_4, 2% of KH_2PO_4 or by 1% of K_2HPO_4 + 1% KH_2PO_4. The culture media were inoculated at 5% of CM9 strain culture and for testing; the well diffusion method was used.

Incubation temperature effect

The medium that improved the maximal bacteriocin CM9 production was used for this test. The medium named MRSm was inoculated at 5% of CM9 strain culture and incubated at 25, 30, 35, 40, and 45°C. After incubation, the well diffusion method was employed.

Diffusion conditions effect

The comparison of the bacteriocin diffusion through the bilayer (solid and soft agar) buffered (Bu) (KH_2PO_4-Na_2HPO_4, pH 7.0 at 0.2 M) and/or unbuffered has been studied using the method described by Schillinger and Lücke 1989. The supernatants used at this step corresponded to above test.

Characterization of bacteriocin
Effect of pH and temperature

The effect of pH on the bacteriocin activity was tested by adjusting the pH of the CFS from 2.0 to 12.0 with sterile 3 N NaOH or HCl. Duplicates of such samples were re-adjusted to pH 7.0 (initial pH) with the same concentration of NaOH or HCl before tested. All of samples were tested for antimicrobial activity using the well diffusion assay. To evaluate the effect of the temperature incubation on the bacteriocin CM9 activity, the CFS were heated at 60, 80, 100°C for 10, 15, and 20 min and at 120°C for 20 min (autoclaving condition). The samples were tested by the method described by Schillinger and Lücke 1989.

Effect of surfactant

Sodium dodecyl sulfate (SDS), Tween 20, Tween 80, urea, EDTA, Triton X-100 at 1% and the NaCl at 2, 4 and 6% were added in the CFS, for testing their effect on bacteriocin activity. Distilled water treated with the same detergents and untreated bacteriocin were considered as control. All treated and control samples were tested using the agar well diffusion assay.

Stability of bacteriocin during storage

Samples of CFS were stored at - 20, 4, 25, 30, 37 and 45°C for 24 h, 1 week and 2 weeks. The samples treated were tested by the agar well diffusion method.

Molecular weight of bacteriocin

One thousand mL of CFS was precipitated at 50% of ammonium sulfate saturation for 24-48 h at 4°C with magnetic agitation. After precipitation, the pellet was collected by centrifugations ($6000 \times g$ for 30 min at 4°C) and resuspended in 10 mL of buffer KH_2PO_4/Na_2HPO_4 (pH 7.0; 50 mM), the precipitate obtained was desalted by dialysis against distilled water.

The estimation of the molecular weight of the bacteriocin CM9, was determined using tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE: 5%, 10% and 16% for stacking, spacer and separating gels, respectively), as described by Schägger and von Jagow (1987). The samples used for this assay, the CFS, the precipitate at 50% ammonium sulfate saturation and desalted were filed in duplicate. Three markers proteins

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were used: BSA (66 kDa), lysozym (14.4 kDa) and nisine (3.5 kDa). The electrophoresis was performed at 50 V overnight. After electrophoresis, the gel was cut into two parts; the first part with markers proteins was stained with Coomassie brilliant blue R-250 and the second part was treated as described by Jiang et al. (2012), and the antimicrobial peptide was evaluated. This gel was compared with the stained gel to situate the active band.

RESULTS AND DISCUSSION

Detection of antimicrobial activity

The 24 LABs screened for antimicrobial activity by agar spot assay were pre-identified by our laboratory and the *Enterococcus faecium* H3 strain was identified by Lazreg et al. (2015). These 25 strains were used as indicators for bacteriocin production. The antimicrobial activity of bacteria, including the lactic acid, hydrogen peroxide, bacteriocins, and others minor antimicrobial agents produced by LAB. Among 532 interactions cases tested, 476 correspond to inhibitions. All the indicators strains were inhibited by the LAB strains except two ones, *Lactobacillus brevis* CHTD27 and *L. cellobiosus* CHTD29.

No antimicrobial activity was detected in the CFS obtained after culture in the MRS medium against the indicator strains. However, adding milk (25%) and yeast extract (2.5%) in the MRS was followed by a clear inhibitory activity of the CFS of the three strains: *Enterococcus* sp CM9, *Enterococcus* sp CM18, and *Leuconostoc mesenteroides* subsp. *dextranicum* CHBK310 against *Enterococcus faecium* H3. Jozala et al. (2005) indicated that adding 25% of milk in M17 medium showed a good influence in the production of nisin by *Lactococcus lactis* subsp. *lactis* ATCC 11454. Cocolin et al. (2007) observed that the production of bacteriocins by the strains of *Enterococcus faecium* M241 and M249 was higher when the strains were cultured in milk than in MRS and explained that milk contains elements that stimulate the production of bacteriocins, such as growth factors and vitamins.

The presence of bacteriocins was performed in the CFS treated separately with three different proteases. Inhibitory activity of the CFS of CM9, CM18 and CHBK310 strains were affected by trypsin, pronase E, and pepsin action but not by catalase action (Fig. 1). Similar results have been observed by various authors for other bacteriocinogenic strains (Klaenhammer 1993; Floriano et al., 1998), suggesting that the inhibitory activity was not due to H_2O_2, but to inhibitor agents with protein nature (*i.e.* bacteriocins). Our bacteriocins were called as following: bacteriocin CM9, bacteriocin CM18 and bacteriocin CHBK310. Regarding the stability of bacteriocin production by *Enterococcus*
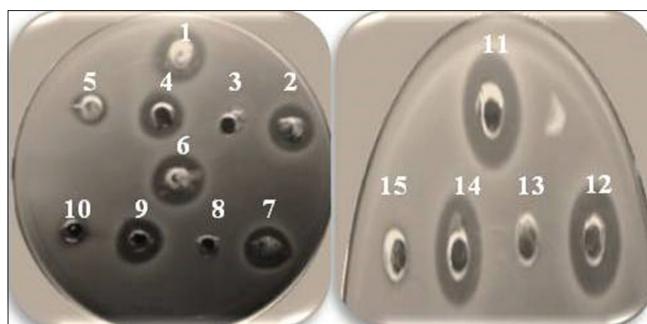

Figure 1. Effect of proteolytic enzymes and catalase on inhibitor agents produced by Enterococcus sp CM9, Enterococcus sp CM18, and Leuconostoc mesenteroides subsp. dextranicum CHBK310. (1-5) 1: CFS of CM9, 2: CFS + catalase, 3: CFS + pronase E, 4: CFS +).

sp CM9, it was selected for further study as bacteriocin producer. *Enterococcus faecium* H3 showed a good evaluation of inhibitor activity of bacteriocin production and was selected as indicator strain.

Optimization of bacteriocin production and its activity

The influence of the composition of the culture medium and incubation temperature on bacterial growth and bacteriocin production were achieved using MRS medium. The effect of diffusion conditions was also studied. Various carbon sources, nitrogen and potassium were tested as components of the culture medium of the bacteriocinogenic strain CM9, for optimizing the production of its bacteriocin.

Some nitrogen sources affected only the growth of the bacterial strain while others affected the production of the bacteriocin (Fig. 2). The growth in the media containing yeast extract and tryptone-yeast extract combination, separately, was better than others nitrogen sources used, but the bacteriocin production is more important on the medium containing tryptone-yeast extract. In other cases, the growth on MRS, medium containing tryptone, meat extract, and tryptone-meat extract combination, separately, is almost identical. However, the medium containing tryptone-yeast extract-meat extract improved the bacteriocin production and bacterial growth. These results involve that the production of bacteriocin is associated to growth but is not apparently proportional to the growth rate. Von Mollendorff et al. (2009) observed a good growth of *L. plantarum* JW3BZ and JW6BZ, *L. fermentum* JW11BZ and JW15BZ in the presence of yeast extract. This source contains growth factors such as vitamins favouring a good growth.

The growth on MRS containing glucose, maltose or lactose was optimum, compared with the MRS with saccharose or glycerol, while the bacteriocin production was more important on MRS with glucose as carbon source (Fig. 2B). Similar result was found by several researchers, who
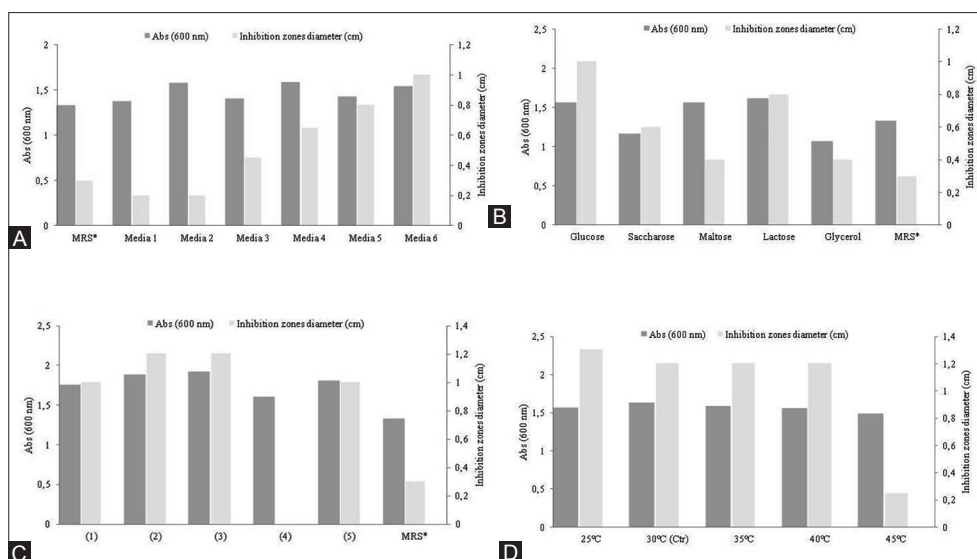
Figure 2. The influence of the composition of the culture media and incubation teperature on bacterial growth and bacteriocin production. A. Effect of nitrogen source on bacterial growth and bacteriocin production. MRS* the absorbance (Abs) of the strain).

observed a maximum production of bacteriocins in the presence of glucose at 20 g/L (Mataragas et al., 2004).

The effect of potassium sources on bacteriocin production was studied by several authors (Von Mollendorff et al., 2009; Lim, 2010). The growth on MRS containing 1%, 2% of K_2HPO_4 and $K_2HPO_4 + KH_2PO_4$ was optimal for the strain CM9, while the bacteriocin production was maximum in the MRS medium with 1% of K_2HPO_4 (Fig. 2C), but the inhibiting activity was conserved for many months at the concentration of 2% of K_2HPO_4. Comparable finding were obtained by Kim et al. (2006) who observed that the highest concentration of bacteriocin was produced on medium containing potassium phosphate at 2-2.5%; the activity was eight-fold highest than that obtained at a phosphate potassium concentration of 0.2%. Bacteriocin production was repressed in MRS with 2% KH_2PO_4. Acidic in nature; this one can reduce the pH of the medium and therefore lead to the suppression of the production of bacteriocins. De Vuyst and Vandamme (1993) noted that 6% of KH_2PO_4 caused cell lysis, consequently a higher culture viscosity which it is due to liberation of the intracellular cell contents.

The bacteriocin production appeared to be similar when the strain was incubated at temperature from 25 to 40°C, the diameters of inhibition zones being observed between 12 and 13 mm (Fig. 2D), while at 45°C the bacteriocin production was affected (inhibition zone diameter was 2 mm). Messens et al. (2003) suggested that high incubation temperature activate proteases in response to heat stress. In addition, Juàrez-Tomas et al. (2002) assumed that temperatures above 44°C inhibit the synthesis or secretion of bacteriocin by *L. salivarius* CRL1328, while

the growth of the strain was not affected by the same temperature.

The increase of inhibition zone was observed in buffered conditions (at pH 7) for all the samples tested compared to the unbuffered conditions (data not shown). This indicates that the diffusion of the bacteriocin CM9 produced by *Enterococcus* sp CM9 was better in buffered agar than in unbuffered one. Blom et al. (1999) noted the same result with the piscicolin. In our case, the buffering agents were KH_2PO_4 and Na_2HPO_4. Indeed, Wolf and Gibbons (1996) observed a high diffusion of the nisin Z by decreasing the amount of agar and adding the sodium phosphate (Na_2HPO_4). On the other hand, Blom et al. (1997) illustrated that the pH can induce matrix ionization and consequently the decrease of diffusion. They recorded a low diffusion zone at pH 7 in the case of nisin and pediocin PA-1.

Characterization of bacteriocin

In general, the activity of the bacteriocin CM9 produced by *Enterococcus* sp CM9 was retained in a broad range pH from 2 to 9 (Fig. 4A). However, a lack of inhibition on the sample readjusted at pH 8 was observed; this was due to a dilution when adding NaOH or HCl to readjust. However, the absence of inhibition zone on the sample at pH 12 not readjusted was due to the sensitivity of the bacteriocin at this pH. Ohmomo et al. (2000) noted that at pH above 7, activity of the bacteriocin produced by *Enterococcus faecium* NIAI 157 decreased. Several bacteriocins remain active at acidic, neutral, and alkaline pH, which may reflect their tolerance to the environmental conditions of bacteriocinogenic bacteria growth.
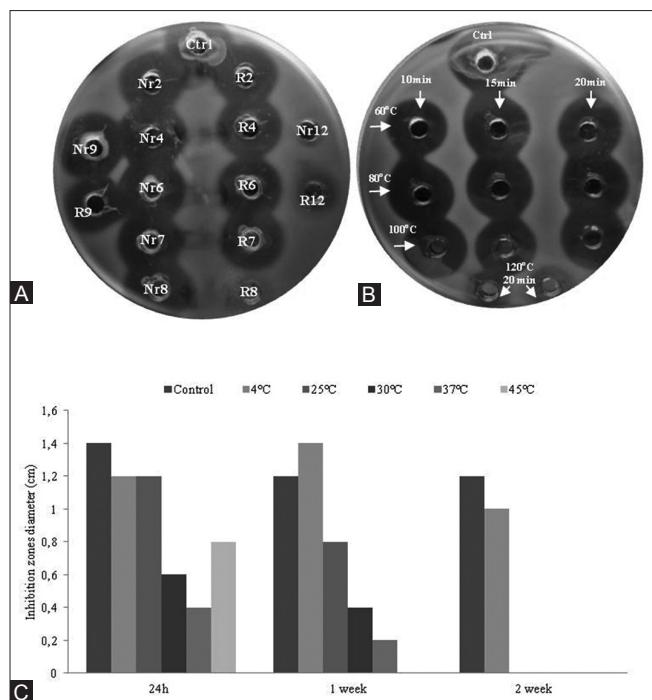
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Figure 3. Influence of physico-chemical factors on bacteriocin activity. A: Effect of acid or basic pH on activity of the bacteriocin. Nr: Not readjusted, R: readjusted at pH7, Ctrl: control. B: Effect of different temperature treatments on bacteriocin ac).

The activity of the bacteriocin CM9 produced by *Enterococcus* sp CM9 was not affected by treatments at 60, 80 and 100 for 10, 15 and 20 min (Fig. 4B). While, the autoclaving (121°C, 20 min) of the crude extract of CM9 strain caused a partial loss of the activity estimated to 30%. Similar results were obtained on the activity of the plantaricin LR/14 which was not affected after treatments at 121°C for 20 min (Tiwari and Srivastava 2008). However, the activity of bacteriocin ST15 was completely abolished at the same conditions (Ivanova et al., 2000). The heat stability and the tolerance at a wide range of pH, indicate that it can be used in pasteurized and sterilized foods. It can be also, exploited in the bio-preservation of acidic and non-acidic foods (Joshi et al., 2006). Moreover, it evidenced a good potential of bacteriocin to survive in the gastrointestinal tract.

The activity of bacteriocin CM9 remained stable after treatment with Tween 20, Tween 80, urea, EDTA and NaCl. A both bacteriocin CM9 and distilled water treated with SDS exhibited inhibition of the H3 strain. In this case, anything about the effect of SDS on bacteriocin structure can be concluded. The treatments with Triton X100 affected the bacteriocin CM9 activity. Ahmadova et al. (2013) reported that the treatments of the supernatants of *E. faecium* AQ71 with these detergents did not affect the inhibition activity, and they indicated that the bacteriocin was resistant to tension-active compounds and salt.
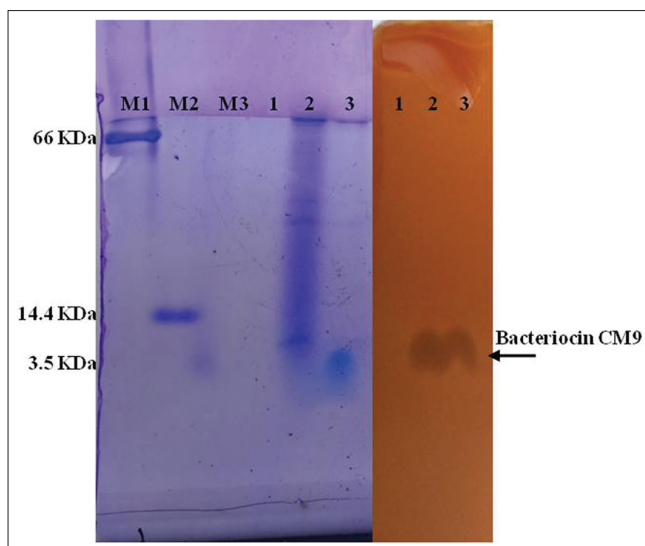

Figure 4. Tricine SDS-PAGE of bacteriocin produced by Enterococcus sp CM9. (A) Molecular mass of bacteriocin. Lane M1: BSA (66 KDa). Lane M2: Lysozym (14.4 KDa). Lane M3: nisine (3.5 KDa). Lane 1 : CFS of CM9 strain. Lane 2: sample obtained after precipitation)

The activity of bacteriocin CM9 produced by *Enterococcus* sp CM9 was maintained after 2 weeks at 4°C. When stored at 25°C, the activity of bacteriocin CM9 was maintained after 24 h, but the activity was partially lost after 1 week of storage at this temperature. In the case of storage at 30°C and 37°C, the activity of bacteriocin decreased after 24 h and completely abolished after 1 week at 45°C (Figure 4C). In a similar study, the activity of bacteriocin PPB KT2W2G produced by *Enterococcus faecalis* KT2W2G was fully maintained after 8 weeks of storage at -20, 4 and 37°C (H-Kittikun et al., 2015). These authors reported that the stability of bacteriocin activity at different temperatures during storage depend on the kind of bacteriocin.

The molecular weight of the bacteriocin CM9 was determined using tricine SDS-PAGE (16%) along with a low molecular weight protein marker with sizes: BSA (66 kDa), lysozym (14.4 kDa) and nisin (3.5 kDa). The migration of precipitate of CM9 showing 3 bands, this indicated that the sample deposited was not purified. However, the migration of dialysate showed a main band with antimicrobial activity, with an estimated molecular weight of 7.6 kDa. However, the presence of single band in this step of purification does not mean that a bacteriocin was pure but the other molecules were present but not detectable. The most enterocins described in the literature are characterized by molecular weights between 4 and 8 kDa (Abriouel et al., 2001). The bacteriocin produced by *Enterococcus* sp CM9 must be subject to other studies in order to classify it.

CONCLUSIONS

In the present study, the assessment of the effect of some MRS ingredients medium on bacteriocin production showed that the production of bacteriocin by *Enterococcus* sp CM9 was influenced by the kind of nitrogen source and potassium source and also by incubator temperature and diffusion condition. The bacteriocin produced by *Enterococcus* sp CM9 was a good candidate for food industrial application regarding their stability in different pH and heat treatment and storage condition. According to these result and the tricine SDS PAGE, the bacteriocin produced by *Enterococcus* sp CM9 belong to the class II of bacteriocin. More studies should be done in the future to know the effect of our LAB and their bacteriocins against the pathogenic and spoilage bacteria and the mode of action of the bacteriocin CM9. However, for their application other experiments proving the safety of the strains and their bacteriocins need to be done.

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