International Journal of

Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 6 (6): 314-322, 2011
ISSN 1811-9743 / DOI: 10.3923/ijds.2011.314.322
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Inhibiting Potential of the Bacteriocine Produced by *Enterococcus faecium* VL47 Strain in the Presence of Prebiotics

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ABSTRACT

The biochemical characterization of the bacteriocine synthesized by *Enterococcus faecium* VL47 was realized, resulting a high thermal stability, resistance to acid pH, organic solvents and to the used enzymes. The production of antimicrobial peptides (bacteriocines) was used against three bacterial strains, *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218, *Escherichia coli* CBAB2. The synthesis of bacteriocines was tested by using the MRS medium, thus, resulting an inhibiting effect against all three sensitive strains, even if the carbon source was replaced by lactose, sucrose, sorbitol, sorbose, arabinose, maltose, galactose and trehalose. The inhibiting effect of the precipitated bacteriocine was obtained when using a concentration of ammonium sulphate, with an average diameter of 1 cm against sensitive strains. To outline the effect of prebiotics on bacteriocine synthesis, MRS was supplemented by 1% inulin from Chichori, inulin from Dahlia, lactulose, raffinose, stachyose, xylose. It resulted that the maximum inhibiting effect was obtained when using lactulose, in a concentration of 1%.

Key words: Lactulose*, Listeria innocua, Bacillus cereus, Escherichia coli,* ammonium sulphate

INTRODUCTION

Numerous lactic bacteria strains produce antimicrobial bacteriocine strains, used against pathogenic strains. In food industry, they are used due to the protection potential exercised in case of pathogenic bacteria. In general, bacteriocines produced by lactic bacteria strains are smaller, temperature stable, hydrophobic. The effect of pH and of temperature is very important for the bacteriocine production (Sharma *et al*., 2010).

A good biochemical characterization supposes appropriate purification. Thus, a rapid, quantitative and precise method of detecting bacteriocines becomes an essential factor in searching new alternatives which may be applied in the industry (Atta *et al*., 2009). *Enterococcus* strains may produce bacteriocines, with the help of which the multiplication of pathogens involved in food poisoning can be controlled; in addition, these bacteriocines may have a great potential as natural preservatives (Alvarez-Cisneros *et al*., 2010).

Among the pathogens which may be inhibited by the bacteriocines of lactic bacteria, *Listeria* sp. and *Bacillus cereus* have exercised a particular interest for food products, due to their capacity of multiplying at small temperatures, due to the produced toxines and the resistance to chemical preservatives (Martinez and De Martinis, 2005; Norwood and Gilmour, 1999). Therefore, the aim of the study was to realize the biochemical characterization of the bacteriocine synthesized by *Enterococcus faecium* VL47.

MATERIALS AND METHODS

Microorganisms and culture media: Within the studies, the *Enterococcus faecium* VL47 strain was used for the bacteriocin synthesis. The following sensitive strains were used: *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218 and *Escherichia coli* CBAB2. All strains are kept at a temperature of -82°C in glycerol 20%. The revival was made in MRS medium for the *Lactobacillus* strain and in LB medium for the three sensitive strains.

The bacteriocin synthesis was marked out by growth at 37°C for a maximum of 72 h in MRS medium (M1). Furthermore, the glucose (carbon source in its composition) was replaced with lactose (M2), sucrose (M3), sorbitol (M4), sorbose (M5), arabinose (M6), maltose (M7), galactose (M8) and trehalose (M9). The minimum inhibitory concentration (AU mL^{-1}) was established (Vamanu *et al.*, 2010; Cabo *et al.*, 1999).

The prebiotics effect on the bacteriocin synthesis was performed by supplementing the MRS with 1% of each of the following: chicory inulin, dahlia inulin, lactulose, raffinose, stachyose and xylose. Once the prebiotic was selected, there was also determined the influence of various concentrations on the bacteriocin's capacity of synthesis (Vamanu *et al.*, 2010).

The partial biochemical characterization of the bacteriocin was performed by determining the resistance to temperature, pH, enzymes and organic solvents. After the cells were removed, the supernatant was submitted for 15 min to temperatures of 60, 80, 100 and 121°C in order to check its resistance to temperature. After incubation, the liquid was cooled in an ice bath and the inhibiting diameter was determined (Karthikeyan and Santhosh, 2009).

In order to determine the pH effect, it was corrected at the values of 2, 5, 7, 9 and 11 using NaOH 1N or HCl 1N sterilized by filtration. The samples were kept at 30°C for an hour. (Karthikeyan and Santhosh, 2009; Khalil *et al.*, 2009).

The effect of the proteolytic enzymes (pepsin, trypsin, chymotrypsin) and nonproteolytic enzymes (lipase, pronase E) was tested by adding them to the supernatant sample, with a concentration of 1 mg mL^{-1}. The samples were kept at 30°C for two hours and afterwards the inactivation was performed by immersing them in a water bath at 95°C, for 3-5 min. The cooling was made by a nice-bath and, thus, the inhibiting effect was determined by measuring the diameter obtained against the sensitive strains (Karthikeyan and Santhosh, 2009; Khalil *et al.*, 2009; Vijayendra *et al.*, 2010).

The effect of organic solvents (methyl alcohol, ethyl alcohol, acetone, ethyl acetate, acetonitrile, benzene, chloroform) with a concentration of 10% was observed by keeping the samples at a temperature of 30°C for an hour. The solvent was removed by evaporation and afterwards the inhibiting diameter was determined (Karthikeyan and Santhosh, 2009; Khalil *et al.*, 2009; Vijayendra *et al.*, 2010; Jagadeeswari *et al.*, 2010).

The partial purification of the bacteriocin was performed by adding ammonium sulphate in concentrations of 10, 20, 30, 40, 50 and 60% to the supernatant. The obtained precipitates were re-dissolved in 10 mL of phosphate buffer with pH 7 and the inhibiting effect was determined (Vijayendra *et al.*, 2010).

RESULTS AND DISCUSSION

The first phase consisted of evaluating the synthesis capacity of the bacteriocine in the presence of various carbon sources. The tests were performed in parallel using all three sensitive strains. The results of the antimicrobial activity of bacteriocine expressed in AU mL^{-1} are provided in Fig. 1-3.

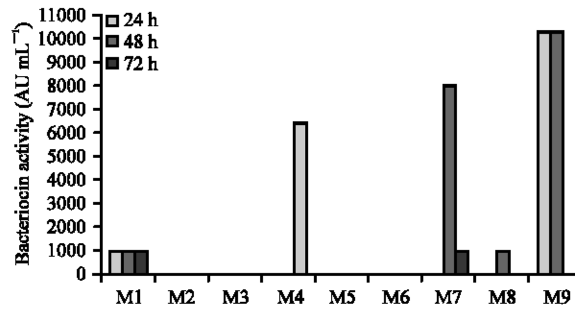

Fig. 1: Inhibiting activity of the *Enterococcus faecium* VL47 strain against *Escherichia coli* CBAB2 in the presence of various carbon sources

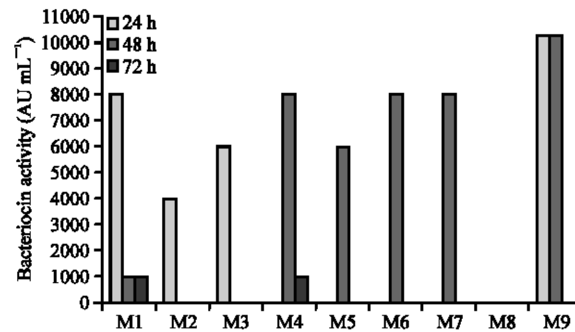

Fig. 2: Inhibiting activity of the *Enterococcus faecium* VL47 strain against *Bacillus cereus* CMGB 215 in the presence of various carbon sources

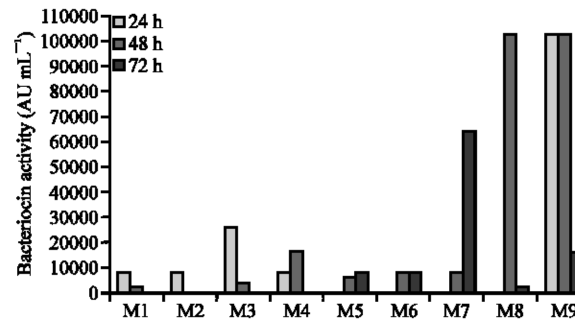

Fig. 3: Inhibiting activity of the *Enterococcus faecium* VL47 strain against *Listeria innocua* CMGB 218 in the presence of various carbon sources

From the submitted data, it resulted that the *Enterococcus faecium* VL47 strain had the most important activity against *Bacillus cereus* CMGB 215 and *Listeria innocua* CMGB 218. The weakest activity was obtained against *Escherichia coli* CBAB2. The maximum inhibiting activity was obtained at the value of 102400 AU mL^{-1} at 24 and 48 h of fermentation using trehalose as a carbon source against the three sensitive strains. From the change of the carbon source with sorbitol, arabinose, maltose and galactose it resulted that the strain was active at a maximum value of 8000 AU mL^{-1} against *Bacillus cereus* CMGB 215. For *Listeria innocua* CMGB 218, the maximum inhibiting activity was present as well when galactose was used as the carbon source.

Note should be made that the strain *Enterococcus faecium* VL47 had inhibiting activity at 72 h mainly when using M1. The exception occurred when using sorbitol against *Bacillus cereus* CMGB 215, without exceeding 1000 AU mL^{-1}. In case of mediums M3, M5, M6, M7, M8 and M9 against *Listeria innocua* CMGB 218 the inhibiting activity had a maximum value of 64000 AU mL^{-1} when using maltose. In general, it resulted that a maximum of 48 h of fermentation was sufficient to obtain the maximum antimicrobial activity against the sensitive strains which were used.

Further on, it was determined which was the prebiotic with maximum inhibiting activity by growing the strain in MRS supplemented by 1% prebiotic. The strain was grown in Duran tubes and the samples were taken through the septum of the autoclavable cap. The diameter of the inhibiting area was determined for each sensitive strain.

The submitted data, regarding the inhibition of the three sensitive strains, proved that a concentration of 1% lactulose determined the maximum inhibiting area. Furthermore, in 48 h as of the fermentation, the maximum inhibition area was obtained relatively constant for all three strains which were used (Fig. 4-6). The maximum inhibiting effect was obtained against *Listeria innocua* CMGB 218, resulting a 2 cm diameter. For the other two strains the diameter of the inhibiting area was of 1.4 cm against *Escherichia coli* CBAB2 and 1.5 cm against *Bacillus cereus* CMGB 215.

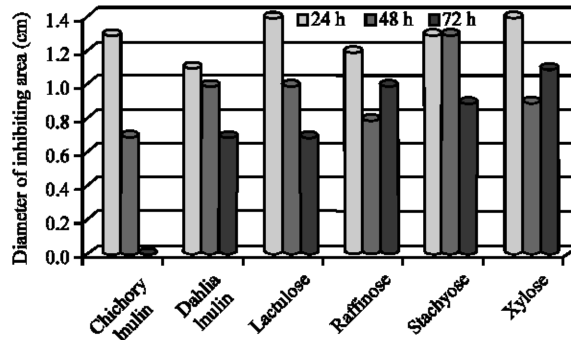

Fig. 4: Inhibition of *Escherichia coli* CBAB2 by *Enterococcus faecium* VL47 cultivation in MRS medium supplemented by 1% prebiotic

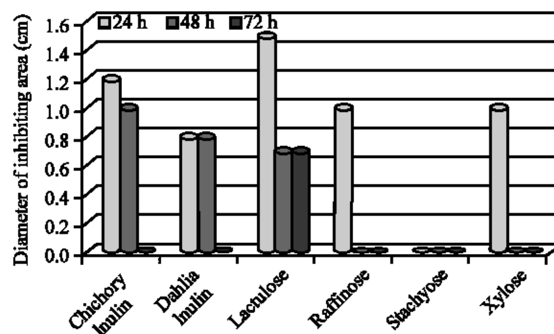

Fig. 5: Inhibition of *Bacillus cereus* CMGB 215 by *Enterococcus faecium* VL47 cultivation in MRS medium supplemented by 1% prebiotic

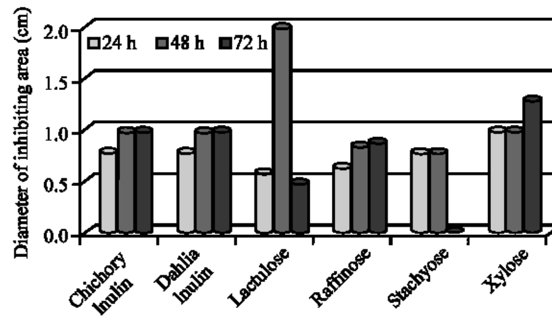

Fig. 6: Inhibition of *Listeria innocua* CMGB 218 by *Enterococcus faecium* VL47 cultivation in MRS medium supplemented by 1% prebiotic

In case of the other prebiotics which were used, high inhibiting activity was noticed against *Escherichia coli* CBAB2, the average diameter being of 0.95 cm in 48 h of fermentation. When using inulin from Dahlia, a constant inhibiting area was maintained, with a 1 cm diameter in 48 h of fermentation. Such finding was valid as well for using raffinose and xylose against *Bacillus cereus* CMGB 215, also in 48 h. In the same situation, it was noticed that the supplementation by 1% stachyose did not cause bacteriocine synthesis because no inhibiting activity was determined.

In case of *Listeria innocua* CMGB 218 inhibition, the used prebiotics were relatively constant in stimulating the bacteriocine synthesis mainly within 48 h of fermentation. The two types of inulin caused the appearance of a constant inhibition area of 1 cm. For raffinose the diameter decreased by only 10%. The exception was the xylose which caused an increase of the inhibition area by 30% in 72 h.

Once the prebiotic causing the maximum inhibiting effect was determined i.e. lactulose the minimun concentration was determined. The studies were made in Duran tubes, provided with septum for sampling purposes, against the same three sensitive strains. It was noticed that the maximum antimicrobial effect was obtained at a concentration of 1% lactulose. For *Escherichia coli* CBAB2 (Fig. 7) it was noticed that an average inhibiting area of 0.9 cm appears, notwithstanding the lactulose concentration from the medium. The doubling of the lactulose concentration to 2% did not cause the increase of the inhibition area, the medium being characterized by acid pH and a significant synthesis of biomass. For *Bacillus cereus* CMGB (Fig. 8) it was noticed an increase of the inhibition area at 0.8% lactulose up to 1.2 cm i.e. 80% of the one obtained for 1% lactulose. The average diameter was of approximately 0.95 cm. For *Listeria innocua* CMGB 218 (Fig. 9) it was noticed an absence of inhibiting activity in 72 h of fermentation, except for the presence of 0.6-1% lactulose in the medium. The strain was only sensitive to large quantities of bacteriocine synthesized in the medium. This finding was confirmed as well by the absence of inhibiting activity, at 2% lactulose, when pH below 4 caused for the cellular metabolism to cease.

For the chemical and physical characterization of the bacteriocine produced against the same three sensitive strains, there were determined the effect of temperature, pH, enzymes, solvents and the effect of precipitation with various concentrations of ammonium sulphate. *Enterococcus faecium* VL47 (Fig. 10) produced a thermal resistant bacteriocine, its effect being constant, even upon incubation from 121°C, against the three sensitive strains. A decrease in the inhibiting diameter occurred against *Escherichia coli* CBAB2 and *Bacillus cereus* CMGB 215, the diameter decrease being of 18 and 8% respectively. However, for *Listeria innocua* CMGB 218 a constant inhibiting

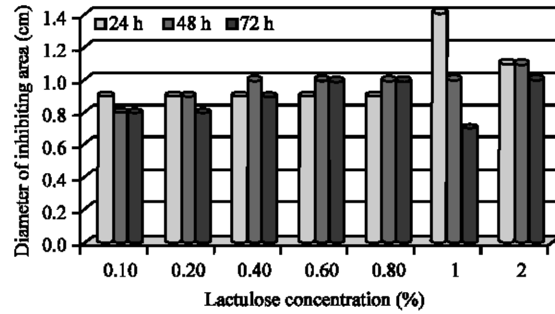

Fig. 7: Inhibition of *Escherichia coli* CBAB2 by *Enterococcus faecium* VL47 cultivation in MRS medium supplemented with lactulose

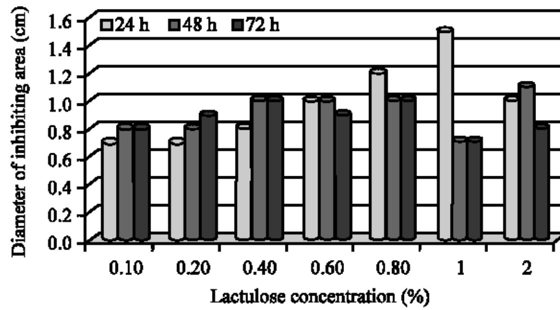

Fig. 8: Inhibition of *Bacillus cereus* CMGB 215 by *Enterococcus faecium* VL47 cultivation in MRS medium supplemented with lactulose

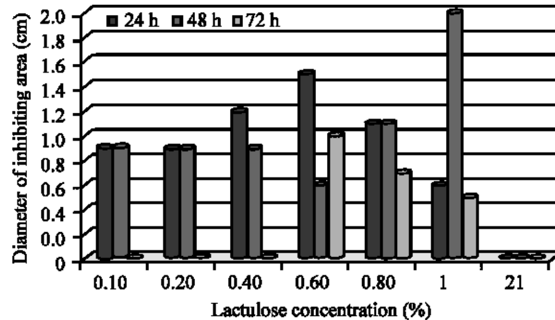

Fig. 9: Inhibition of *Listeria innocua* CMGB 218 by *Enterococcus faecium* VL47 cultivation in MRS medium supplemented with lactulose

diameter was maintained (1.1 cm), the sole exception being the temperature of 60°C which did not affect the inhibition capacity of 1.4 cm present in the non-treated supernatant.

The bacteriocine produced by *Enterococcus faecium* VL47 was resistant to pH included between 2 and 11 (Fig. 11). Once pH value increased it was more active against *Bacillus cereus* CMGB 215. As to *Escherichia coli* CBAB2 the inhibiting diameter was by 15% larger and as to *Listeria innocua* CMGB 218 by 23%.

In case of precipitation with ammonium sulphate (Fig. 12) at a concentration of 60% the maximum inhibiting area was obtained. For *Escherichia coli* CBAB2 and *Bacillus cereus*

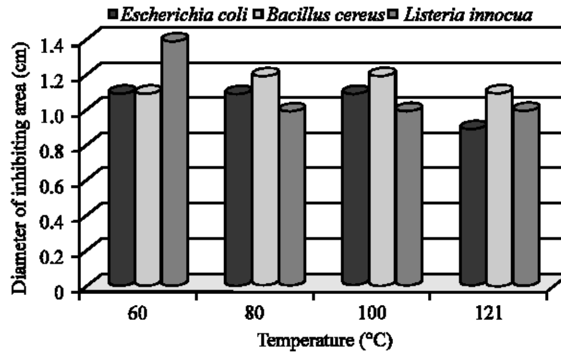

Fig. 10: The effect of temperature on the bacteriocin produced by *Enterococcus faecium* VL47

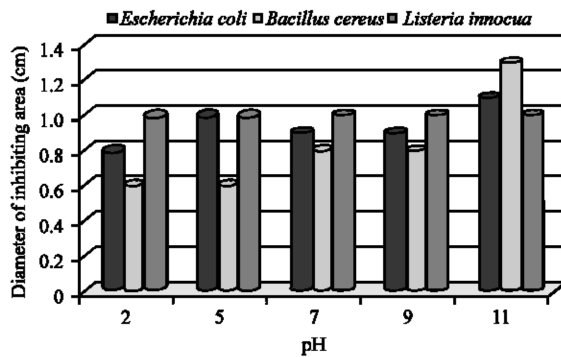

Fig. 11: The effect of pH on the bacteriocin produced by *Enterococcus faecium* VL47

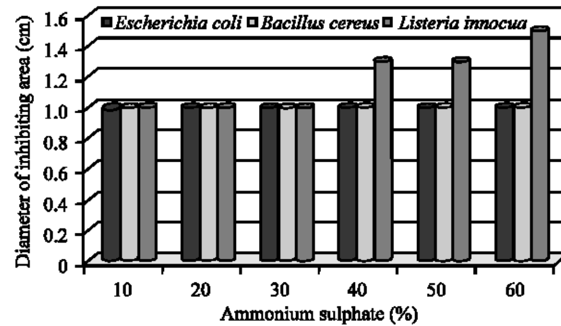

Fig. 12: The effect of precipitation with ammonium sulphate on the bacteriocin produced by *Enterococcus faecium* VL47

CMGB 215, the inhibiting diameter was constant, up to 60% ammonium sulphate, with a 1 cm diameter. For *Listeria innocua* CMGB 218 the precipitate obtained at 40 and 50% ammonium sulphate had an inhibition area of 1.3 cm. The precipitate obtained at 60% ammonium sulphate had a maximum level of the inhibition area of 1.5 cm against *Listeria innocua* CMGB 218. It determined an increase of the inhibiting area included between 13.33 and 33.33%.

Furthermore, the resistance of the bacteriocine produced by *Enterococcus faecium* VL47 was tested in relation to the action of various enzymes, resulting that it was active in the presence of pepsin, α-chemotrypsin, proteinase K and trypsin but not in the presence of catalase and α-amylase. The inhibiting diameter was of maximum 1 cm i.e. approximately 30% smaller than the untreated one. The bacteriocine produced was resistant to the treatment with organic solvents in a concentration of 10%, thus resulting an inhibition area, even if smaller in diameter, against all the three tested sensitive strains.

The synthesis and characterization studies of the bacteriocine produced by the *Enterococcus faecium* VL47 strain have led to important results. By the presented resistance, the bacteriocine may be used for obtaining products acting on the biological control of the human intestinal microflora. The studies prove that the type of culture medium and the used prebiotic influence directly the inhibiting capacity. The lactulose, in a 1% concentration, determines a maximal inhibiting capacity. Therefore, the combination between the lactulose and the prebiotic strain is beneficial for obtaining synbiotic products, with modulating effect on human intestinal microflora.

ACKNOWLEDGMENT

The researches were financed through a project PNCDI II 62-050/2008 (http://proiectprobac.emanuelvamanu.ro/).

REFERENCES

Alvarez-Cisneros, Y.M., F.J. Fernandez, C. Wacher-Rodarte, M.B. Aguilar, T.D.R.S. Espunes and E. Ponce-Alquicira, 2010. Biochemical characterization of a bacteriocin-like inhibitory substance produced by *Enterococcus faecium* MXVK29, isolated from Mexican traditional sausage. J. Sci. Food Agric., 90: 2475-2481.

Atta, H.M., B.M. Refaat and A.A. El-Waseif, 2009. Application of biotechnology for production, purification and characterization of peptide antibiotic produced by probiotic *Lactobacillus plantarum*, NRRL B-227. Global J. Biotechnol. Biochem., 4: 115-125.

Cabo, M.L., M.A. Murado, M.P. Gonzalez and L. Pastoriza, 1999. A method for bacteriocin quantification. J. Applied Microbiol., 87: 907-914.

Jagadeeswari, S., P. Vidya, D.J.M. Kumar and M.D. Balakumaran, 2010. Isolation and characterization of bacteriocin producing *Lactobacillus* sp. from traditional fermented foods. Electron. J. Environ. Agric. Food Chem., 9: 575-581.

Karthikeyan, V. and S.W. Santhosh, 2009. Study of bacteriocin as a food preservative and the *L. acidophilus* strain as probiotic. Pak. J. Nutr., 8: 335-340.

Khalil, R., Y. El-Bahloul, F. Djadouni and S. Omar, 2009. Isolation and partial characterization of a Bacteriocin produced by a newly isolated *Bacillus megaterium* strain 19. Pak. J. Nutr., 8: 242-250.

Martinez, R.C.R. and E.C.P. De Martinis, 2005. Evaluation of bacteriocin-producing *Lactobacillus sakei* 1 against *Listeria monocytogenes* 1/2a growth and haemolytic activity. Braz. J. Microbiol., 36: 83-87.

Norwood, D.E. and A. Gilmour, 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. J. Applied Microbiol., 86: 576-582-10212403.

Sharma, S., A.P. Garg and G. Singh, 2010. Optimization of fermentation conditions for bacteriocin production by *Lactococcus lactis* CCSULAC1 on modified MRS medium. Int. J. Dairy Sci., 5: 1-9.

Vamanu, E., A. Vamanu and D. Pelinescu, 2010. Synthesis and partial biochemical characterization of bacteriocin produced by *Lactobacillus paracasei* YR strain. Rev. Chim., 61: 502-505.

Vijayendra, S.V.N., K. Rajashree and P.M. Halami, 2010. Characterization of a heat stable anti-listerial bacteriocin produced by vancomycin sensitive *Enterococcus faecium* isolated from *idli* batter. Indian J. Microbiol., 50: 243-246.