Turkish Journal of Agricultural and Natural Sciences
Special Issue: 2, 2014

TÜRK
TARIM ve DOĞA BİLİMLERİ
DERGİSİ

TURKISH
JOURNAL of AGRICULTURAL
and NATURAL SCIENCES

www.turkjans.com

Characterization of bacteriocin produced by Lactococcus lactis ssp. lactis strains isolated from marine fish caught in the Algerian west coast.

SAHNOUNI F*[a]., BOUTIBA-MAATALLAH A[a]., BOUHADI D[b]., BOUTIBA Z[a].
[a]Environmental Monitoring Network, Faculty of Science, University of Oran, Algeria
[b]Bioconversion Laboratory, Microbiology engineering and health Safety. Faculty of Sciences, University of Sidi Bel-Abbès, Algeria
*Corresponding author: Sahnouni_fatima@yahoo.fr

Abstract

A total of 38 strains of *Lactococcus lactis ssp. lactis*, were isolated from gastrointestinal tract of coastal fish: sardine (*Sardina pilchardus)* and bug (*Boops boops)*. These isolates were tested for their ability to produce bacteriocins against *Listeria innocua, Brochothrix thermophacta, Salmonella sp., Staphylococcus aureus, Bacillus cereus*, *Aeromonas hydrophila, Pseudomonas aeuroginosa Escherichia coli* and Methycilin resistant *Staphyococcus aureus*. However characterization of the antimicrobial substances showed that only 04 of isolates produced antimicrobial activity in the neutralized cell-free supernatant treated with catalase against indicator strains. The compounds produced by the selected strains were fully or partially inactivated by some of the proteolytic enzymes (trypsine, α-chymotrypsin and proteinase K), which indicates their proteinaceous nature. The antimicrobial activity of crude supernatant fluid was stable after heating at 100 °C for 30 min and declined thereafter. It was also active over a wide pH range (4–8). Selected strains in this study might be valuable for practical application as source of bacteriocin, providing future scope for the biopreservation of seafood products.

Key words: Marine fish, *Sardina pilchardus, Boops boop, Lactococcus lactis ssp. lactis,* Antimicrobial activity, bacterioci

Introduction

Owing to some of their metabolic properties, lactic acid bacteria (LAB) play an important role in the food industry, because they significantly contribute to the flavour, texture, and in many cases to the nutritional value of the food products. The lactic acid bacteria (LAB) produce an array of antimicrobial substances (such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides, and bacteriocins

(Holtzel et al. 2000, Magnusson and Schnürer, 2001). However, the leading role in the explanation of the antagonism of lactic acid bacteria is assigned to specific antimicrobial substances of a protein nature, bacteriocins. Many bacteriocins produced by LAB exert strong antagonistic activity against many related and unrelated microorganisms, including food spoilage organisms and pathogenic bacteria such as *Listeria, Clostridium, Staphylococcus, Bacillus spp. Brochothrix, Aeromonas, and Vibrio spp.* (Gonzalez-Rodriguez et al. 2002, Budde et al. 2003, Brillet et al. 2005). Bacteriocins produced by LAB are small, ribosomally synthesized, antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocin . Activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or chelating agent, or after pulsed electric field or high-pressure treatment (Stevens et al.1991).

Hence, the last two decades have seen intensive investigation on LAB and their antimicrobial products to discover new bacteriocinogenic LAB strains that can be used in food preservation. Indeed it has been demonstrated that lactic acid bacteria isolated from marine environments such as fish and fish products, are expected to show additional capabilities compared with milk- and plant-derived.

In spite of overwhelming information about biopreservatives properties of dairy lactic

acid bacteria (LAB), only few publications are dedicated to LAB from marine bacteria. Indira K. et al. (2011) have isolated *Lactobacillus fermentum* from fish gut (*Mugil cephalus*) and prawn muscle (*Peneaus monodon*) and they revealed that *Lactobacillus* strains of marine origin are having the potential to use as biopreservatives especially in seafoods and the production of bacteriocin from *L. fermentum*, seems to be ideal for industrial scale production and commercial utilization. A bacteriocin produced from *Lactobacillus brevis* was isolated from fresh water fish mrigala (*Cirrhinus mrigala*) by Banerjee et al. (2011). Also Campos et al. (2006) isolated three bacteriocin-producing LAB from the muscle of turbot (*Psetta maxima*). All strains were identified as *Lactococcus lactis subsp. lactis* subsp. *lactis*, *Enterococcus faecium* and *E. mundtii*.

Thus, the intent of present study was therefore to screen a number of *Lactococcus lactis ssp. lactis*, isolated from gastrointestinal tract of coastal fish for their ability to produce bacteriocins. Stability at different pH values and temperature conditions of bacteriocin were also studied.

Materials and methods

Bacterial strains and growth media

38 strains of *Lactococcus lactis ssp. lactis*, used in this study, were isolated from gastrointestinal tract of coastal fish: sardine (*Sardina pilchardus)* and bug (*Boops boops)*, characterized by physiological and biochemical tests (Stiles and Holzapfel, 1997 and Collins et al. 1989). Carbohydrate fermentation reactions were recorded by using (API System Biomerieux SA, France). They were maintained at -20°C in MRS broth with 10% glycerol and enriched twice in MRS broth before use. The organisms used for testing antagonistic activity were all either spoilage bacteria or pathogens (Table 1).

Screening of antagonistic activity

The spectrum of antimicrobial activity of isolated LAB was detected by agar-spot test (Schillinger and Lücke, 1987). In this case, colonies of the strains were grown on the surface of MRS containing 1.5% agar for 24 h at 30 °C. The indicator strains, as described in Table 1, were inoculated (1% v/v) into 7 ml of soft agar medium (containing 0.7% agar) specific for each strain, at a final concentration of 10^5CFU/ml. The soft media were poured on the plate where growth of the producers occurred and the plates were incubated at the optimal growth temperature for the indicator strains, for 24 h. Inhibition was recorded positive in presence of a detectable clearing zone around the colony of the producer strain.

Bcateriocin preparation

Strains that showed antimicrobial activity were investigated for their potential to produce bacteriocins as described by Ammor et al. (2005). The assay of bacteriocin was carried out as follow; the cell-free supernatants (CFS) of LAB were adjusted to pH 6.5 using 1M NaOH to exclude the antimicrobial effect of organic acids. Inhibitory activity of hydrogen peroxide was eliminated by the addition of catalase at a final concentration of 1mg/ml. Untreated and treated (neutralized and neutralized + catalase) CFS placed in the wells were allowed to diffuse into the agar for 1 h at room temperature. The plates were then incubated at 37°C in microaerophilic conditions for 24 h. The diameter of inhibition zone formed around the wells was calculated as the difference between the diameter of the total inhibition zone and the diameter of the well. The inhibition is noted positive if the diameter is superior to 2 mm (Thompson et al. 1996).

Effect of enzymes, pH, and temperature on bacteriocin activity

Sterile cell-free supernatants at pH 6.5 were treated with the following enzymes (0.2 mg ml^{-1}): proteinase K (pH 7.0 Sigma USA); trypsin (pH 8.2 Merck, Germany) ; α chymotrypsin (pH 8.0, Sigma, USA) ; lipase (pH6 Sigma, USA), α-amylase (pH 7, Sigma, USA). All these solutions were filter-sterilized and then added to supernatants (v/v, 1/1). Untreated bacteriocin and enzyme solutions were used as controls. All samples and controls were incubated at 37°C for 2h.

The pH and temperature ranges tested were chosen based on their usual levels in foods and in their processing operations. To determine the activity of bacteriocins at different pH levels, sterile cell-free supernatants were adjusted with sterile 2N NaOH or HCl to different pH values (2.0, 4.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 12.0) and was incubated at 37°C for 2 h. The pH-treated samples were neutralized to pH 6.0 before measuring the residual bacteriocin. Buffer solutions (100mM) without supernatants were used as control

To evaluate thermostability, aliquots of sterile cell-free supernatants were heated to 60°C, 80°C, 100°C at 15min /30 min, and 121°C/15 min, immediately cooled in ice and tested for antibacterial activity. The effect of Refrigeration temperature (4 °C) and freezing temperature (-20 °C) on bacteriocin stability was also evaluated. At different time interval (every 30 days) the bacteriocin were taken from the stored materials for detection of antimicrobial activity using well diffusion assay.

In all the cases the residual activity of treated and untreated samples was determined against the

indicator strain against *S.aureus* (ATCC25923) by using agar well diffusion method. All experiments were performed in triplicate.

Results and discussion

Results

Screening of antagonistic activity

38 strains of *Lactococcus lactis ssp. lactis*, were isolated from gastrointestinal tract of coastal fish: sardine (*Sardina pilchardus)* and bug (*Boops boops).* Firstly, these isolates were investigated for their antibacterial activity against both Gram positive and Gram negative bacteria as shown in Table 1. Thirty two strains showed antimicrobial activity against at least one of the target strain (Figure 1). Six strains showed no antibacterial activity.

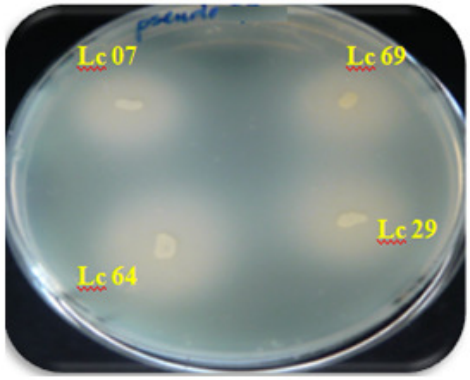

Figure 1. Inhibition of *P.aeruginosa* (ATCC27853) by *L.lactis subsp lactis* Lc 07, Lc 64, Lc29and Lc64

Characterization of the inhibitory agent

Selected stains were analyzed for their antibacterial compounds. The results showed that among the isolates that exhibited antagonistic properties against target microorganisms, twenty six kept their antimicrobial activity in neutralized culture supernatants (pH 6.5). From this group, twenty two lost their antimicrobial activity when samples were treated with catalase. This explains that the inhibition was due to the production of hydrogen peroxide. Only four strains of *Lactococcus lactis ssp. lactis* (Lc 07, Lc 64, Lc 29, Lc 69) kept their antibacterial activity against *S. aureus* in neutralized and catalase-treated culture supernatants. Also, Addition of proteinase K; trypsin and α-chymotrypsin stopped their antibacterial activity.

The other enzymes tested in our study (amylase and lipase) did not cause inactivation. This confirmed that carbohydrate and lipid moieties if existing were not required for the inhibitory activity thereby providing further evidence of the proteinaceous nature of the substance responsible.

Effect of temperature and pH on the antibacterial compound

The effect of enzymes, pH and heat treatments on the activity of the bacteriocin produced by Lc 07, Lc 64, Lc 29 and Lc 69 are presented in Table 2. Sterile cell-free supernatants of Lc 64 remained stable at pH values between 4.0 and 10. However sterile cell-free supernatants of other strains remained stable at pH values between 4.0 and 8. In all cases, the antimicrobial activity was unaffected by temperature at 60, 80, and 100^{o}C for 15 and 30 min. Only activity of Lc 29 was detected even after autoclaving (Table 2). The results suggest that partial purified bacteriocin is strongly heat resistant. Furthermore, the stability of bacteriocin were also determined at various storage temperature of -20^{0}C and 4^{0}C.It doesn't show any considerable changes in their activity after 120 days.

Discussion

It is well known that LAB species are parts of the gut microbiota of finfish (e.g. Ringø and Gatesoupe 1998, Campos et al. 2006; Balcázar et al. 2008, Itoi 2008, Valenzuela 2010, Indira et al.2011 Merrifield et al. 2014). For this purpose, is interesting to note that the presence of these bacteria in the intestinal tract of marine fish may reflect the high adaptability to various environments.

As cited above, thirty eight allochthonous strains of *Lactococcus lactis ssp. lactis* were isolated from gastrointestinal tract of coastal fish: sardine (*Sardina pilchardus)* and bug (*Boops boops)*. They were screened for their antagonistic activities against indicators microorganisms such *Listeria innocua, Brochothrix thermophacta, Salmonella sp., Staphylococcus aureus, Bacillus cereus*, *Aeromonas hydrophila, Pseudomonas aeuroginosa Escherichia coli* and Methycilin resistant *Staphyococcus aureus* (MRSA). Thirty two strains showed antimicrobial activity against at least one of the target strain. The inhibitory effect, which was observed by the formation of clear and distinct zones around the wells, may be due to the production of several antimicrobial compounds; organic acids, H_2O_2 or bacteriocins (Corsetti et al. 2004).

Table1. Inhibitory spectra *Lactococcus lactis ssp. lactis strains* exhibiting antimicrobial activity

	Lc 07	Lc 64	Lc 29	Lc 69
***Listeria innocua* (LRSE 12)**	+++	+	++	++
***Brochothrix thermophacta* (LRSE58)**	++	++	++	++
***Salmonella* sp (LRSE 05)**	++	++	-	+
Staphylococcus aureus	++	++	++	+++
***Bacillus cereus* (LRSE 01)**	-	+++	++	++
***Aeromonas hydrophila (*LRSE 04)**	++	++	_	++
***Pseudomonas aeuroginosa (*ATCC27853)**	_	+++	++	+
***Escherichia coli* (ATCC25922)**	_	++	+	++
***Staphyococcus aureus* (MRSA) (LRSE41)**	++	++	+++	+++

– =no antimicrobial activity; + = inhibition zone < 10 mm (the smallest inhibition zone was 4mm); ++ = inhibition zone > 11 mm; +++ = inhibition zone > 20 mm.

Table 2. Effect of enzymes, pH and temperature on antibacterial activity

	Treatment	Lc07	Lc64	Lc29	Lc69
Enzyme	α -Chymotrypsin	-	-	-	-
	proteinase K	-	-	-	-
	trypsin	-	-	-	-
	Lipase	+	+	+	+
	amylase	+	+	+	+
	Catalase	+	+	+	+
pH		4-8	**4-10**	4-8	4-8
Heat	60,80,100°C /15min	+	+	+	+
treatment	60,80,100°C /30min	+	+	+	+
	121°C/15min	-	-	+	-

Four strains (Lc 07, Lc 64, Lc 29, Lc 69) have exhibited inhibition zones for neutralized culture supernatants and catalase treated supernatants. Results from enzyme inactivation studies confirmed that antimicrobial activity was lost after treatment with the proteolytic enzymes like proteinase K; trypsin and α-chymotrypsin, whereas treatment with lipase and α-amylase , did not affect the activity of antimicrobial substance produced by the test isolates. The destruction of the antimicrobial activity by proteases suggested that this compound could be a peptide or bacteriocin-like inhibitory substances (BLIS). These results were comparable to those obtained by Ayad et al. (2002), Campos et al. (2006), Vescovo et al. (2006) and Díaz-Ruiz et al. (2012).

bacteriocins from LAB usually are ineffective against Gram-negative bacteria and rather relate to a narrow antimicrobial spectrum (Cleveland et al. 2001), both presumptive bacteriocins Lc 64 and Lc 69 showed broad antimicrobial activity against several genera of Gram-positive and Gram-negative bacteria. These results were comparable to those obtained by Ghanbari et al. (2013). Recent studies have shown that several marine bacteria may produce inhibitory substances against bacterial pathogens in aquaculture systems (Indira et al. 2011). Hence the use of such bacteria releasing antimicrobial substances in now gaining importance in fish farming as probiotics or as antibiotics in the seafood industry (Verschuere et al. 2000, Galvez et al.2008; García et al. 2012; Pilet and Leroi 2011). All supernatants were stable to heat treatment and to a wide range of pH values. These results are in agreement with (Bromberg et al. 2005, Tuncer and Ozden 2010). This stability could be exploited in biopreservation of acid and non-acid foods. Also, they were stable at low temperatures. this property could be used as a potential barrier to inhibit the growth of psychrotrophic or mesophilic spoilage and foodborne pathogens, such as *Brochothrix thermosphacta, L. monocytogenes, S. aureus, B.*

cereus, and *Clostridium perfringens* frequently found in foods stored under refrigeration.

Conclusion

The results obtained in this study revealed that LAB strains derived from marine fish possess potentially important properties. Selected strains in this study , might be valuable for practical application as starter, adjunct and protective cultures or as source of bacteriocin, providing future scope for the biopreservation of food products.

Acknowledgment

Authors gratefully acknowledge the financial support of Laboratory: Environmental Monitoring Network, Department of Biology, Faculty of Science, University of Oran- Algeria and Ministry of Higher Education and Scientific Research, Government of Algeria.

References

Ammor, S., Rachman, C., Chaillou, S., Prévost, H., Dousset, X., Zagorec, M., 2005. Phenotypic and genotypic identification of lactic acid bacteria isolated from a small-scale producing traditional dry sausages. Food Microbiol 23(5):373- 382.

Ayad, E.H.E., Nashat, S., El-Sadek N., Metwaly H., El-Soda M., (2004) Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. Food Microbiol 21:715–725

Balcázar, J.L., Vendrell, D., De Blas, I., Ruiz-Zarzuela, I., Muzquiz, J.L., Girones, O., 2008. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*. 278: 188-191.

Banerjee, S.P, Chandra Dora, K., Chowdhury, S., 2011.Detection, partial purification and characterization of bacteriocin produced by Lactobacillus brevis FPTLB3 isolated from freshwater fish Bacteriocin from Lb. brevis FPTLB3. *J Food Sci Technol*. 50 (1):17-25.

Budde, B.B., Hornbaek, T., Jacobsen, T., Barkholt, V, Koch, A.G., 2003. *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuumpacked meats: culture isolation, bacteriocin identification, and meat application experiments. Int J Food Microbiol 83:171-184.

Brillet, A., Pilet, M.F., Prevost, H., Cardinal, M., Leroi, F., 2005. Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, and sensory quality of cold-smoked salmon. Int J Food Microbiol 104:309-324.

Bromberg, R., Moreno, I., Delboni, R., Cintra, H., Oliveira, P., 2005.Characteristics of the bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* CTC 204 and the effect of this compound on the mesophilic bacteria associated with raw beef. J Microbiol Biotechnol. 21: 351-358.

Campos, C. A., Rodriguez, O., Calo-Mata, P., Prado, M. et Barros-Velazquez, J., 2006).Preliminary characterization of bacteriocins from *Lactococcus lactis, Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (*Psetta maxima*). *Food Res Int.* 39 : 356–364.

Cleveland, J., T. J., Montville, I. F. Nes, Chikindas,M.L., 2001. Bacteriocins: safe natural antimicrobials for food preservation. International Journal of Food Microbiology. 71: 1-20.

Collins, M.D., Phillips, B.A., Zanoni, P., 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei, Lactobacillus paracasei* sp nov, subsp *paracasei* and subsp *to/erans,* and *Lactobacillus rhamnosus* sp nov, comb nov. */nt J Syst Bacterio.*39*:* 105-108.

Corsetti, A., Settanni, L., Van Sinderen D., 2004. Characterisation of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their in vitro and in situ activity. J Appl Microbiol 96:521–534.

Díaz-Ruiz, G. Ben Omar, N., Abriouel, Martínez Cañamero, M. et Gálvez, A., 2012. Inhibition of *Listeria monocytogenes* and *Escherichia coli* by bacteriocin-producing *Lactobacillus plantarum* EC52 in a meat sausage model system. *African Journal of Microbiology Research* Vol. 6(6), pp. 1103-1108.

Galvez, A., Lopez, R.L., Abriouel, H., Valdivia, E., Omar, N.B., 2008. Application of bacteriocins in the control of food borne pathogenic and spoilage bacteria. Crit. Rev. Biotechnol., 28: 125-152.García et al., 2012;

Gonzalez-Rodriguez, M.N., Sanz, J.J., Santos, J.A., Otero, A., Garcia-Lopez, M.L. 2002. Numbers and types of microorganisms in vacuum-packed cold-smoked freshwater fish at the retail level. *Int. J. Food Microbiol.* 77: 161-168.

Holtzel, A., Ganzle, M.G., Nicholson, G.J., Hammes, W.P., Jung, G., 2000. The first low-molecular-weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid.

Angewandte Chemie. International Edition 39, 2766–2768

Indira, K., Jayalakshmi, S., Gopalakrishnan, A., Srinivasan, M., 2011. Biopreservative potential of marine *lactobacillus* spp. India. *African Journal of Microbiology Research*. 5(16): 2287-2296.

Itoi S, Abe T., Washio, S., Ikuno, E., Kanomata, Y., Sugita, H., 2008. Isolation of halotolerant Lactococcus lactis subsp. lactis from intestinal tract of coastal fish. *Int. J. Food Microbiol.* 121: 116–121.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., Schnürer, J., 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters.* 219: 129-135.

Merrifield, D.L., Balcázar, J.L., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y.Z., Hoseinifar, S.H., Ringø, E., 2014. Indigenous lactic acid bacteria in fish and crustaceans. In: Merrifield DL,Ringø (eds) Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. Wiley Blackwell, pp 128-168.

Pilet, M.F. et Leroi, F., 2011. Applications of protective cultures, bacteriocins, and bacteriophages in fresh seafood and seafood products*, In: Protective cultures, antimicrobial metabolites and bacteriophages for food and bevarage biopreservation*, pp. 1- 21.

Ringø, E., Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160:177-203

Schillinger, U., et Lücke, F.K., 1987. Identification of Lactobacilli from meat and meat products. *J. Food Microbiol*. 4: 199-208.

Stiles, M., Holzapfel, W., 1997. Lactic acid bacteria of foods and their current taxonomy. *Areview J. Food Microbiol*. 36: 1-29.

Stevens, K.A., Sheldon, B.W., Klapes, N.A., Klaenhammer, T.R., 1991. Nisin treatment for inactivation of Salmonella species and other gram-negative bacteria. Applied and Environmental Microbiology 57, 3613–3615.

Thompson, J. and Gentry-Weeks, C.R., 1994. Métabolisme des sucres par les bacteries lactiqiues. *in « Bacterie lactique », de Roissard et Luquet, Tech.Doc., Lavoisie*r, Paris.

Tuncer Y., Ozden B., 2010. Partial Biochemical Characterization of Nisin-Like Bacteriocin Produced by *Lactococcus lactis* subsp. *lactis* YBD11 Isolated from Boza, A Traditional Fermented Turkish Beverage. Romanian Biotechnol Letters. *Vol. 15, No.1.* 4940- 4948.

Valenzuela, A.S., Ben Omar, N., Abriouel H., Martinez Canamero, M., Gálvez, A., 2010. Isolation and identification of Enterococcus faecium from seafoods: Antimicrobial resistance and production of bacteriocin-like substances. *Int. Food Microbiol.* 27: 955-961.

Verschuere,L.,G. Rombaut, P. Sorgeloos, Verstraete, W., 2000.. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews. 64: 655-671.

Vescovo, M., Gianluigi, S. and Zacconi, C., 2006. Inhibition of *Listeria innocua* growth by antimicrobial-producing lactic acid cultures in vacuum-packed cold-smoked salmon. Food Microbiol. 23, 689-693.