Lactococcus lactis subsp. *lactis* LTM 32**, a New Bacteriocin-Producing Strain Isolated from Vietnamese Fermented Milk**

TUYET MAI DO, MILADA PLOCKOVÁ and JANA CHUMCHALOVÁ

Institute of Chemical Technology – Department of Dairy and Fat Technology, Prague, Czech Republic

Abstract

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Screening for bacteriocin production of 60 strains of lactic acid bacteria (LAB) both isolated from various Vietnamese fermented food and obtained from the culture collection resulted in the detection of a bacteriocin-producing strain, *Lactococcus lactis* subsp. *lactis* LTM 32 isolated from Vietnamese fermented milk. This strain was identified and partly characterized. This bacteriocin inhibited not only closely relative strains of LAB but also strains of *Listeria monocytogenes, Bacillus* sp. including *B. cereus, B. stearothermophilus, B. subtilis, B. pumilus* and *Staphylococcus aureus*. It was heat stable at 121°C for 15 min, at 100°C for 120 min and stable during storage at –20°C and 4°C for 3 months. The bacteriocin was inactivated by some proteolytic enzymes, namely by proteinase K and pronase E in concentrations up to 0.5 mg/ml but not by α-amylase and pepsin. The optimal growth and bacteriocin production were achieved in M17L broth at 30°C and initial pH 6.8. The characteristics of bacteriocin-producing strain *Lactococcus lactis* subsp. *lactis* LTM 32 are of interest for food safety.

Keywords: *Lactococcus* sp.; *Listeria monocytogenes; Staphylococcus aureus*; *Bacillus* sp.; fermented milk; bacteriocin; nisin

Lactic acid bacteria (LAB) are traditionally used as starter cultures for dairy, vegetable and meat fermentation because they have a potential to inhibit the growth of pathogenic and spoilage bacteria and to extend the shelf-life of the product. They produce a wide variety of antagonistic factors such as organic acids, hydrogen peroxide, lactoperoxydase and diacetyl (DAESCHEL *et al*. 1987). Some of them produce bacteriocins, which are bacterial proteins or peptides with bactericidal mode of action against closely related species (TAGG *et al*. 1976; KLAENHAMMER 1988). Besides typical bacteriocins with a narrow antibacterial spectrum, a few bacteriocins of LAB with a wider spectrum were also described (KLAENHAMMER 1993).

Among the bacteriocins isolated from LAB, nisin is studied most frequently (BUCHMAN *et al*. 1998; DODD *et al.* 1990; HURST 1981; KALETTA & ENTIAN 1989). Nisin is produced by some strains of *Lactococcus lactis* subsp. *lactis*. It is a polypeptide containing modified amino acids such as lanthionine, 3-methyl-lanthionine and their precursors, dehydroalanine and dehydrobutyrine (GOSS & MORELL 1971). Other bacteriocins produced by lactococci such as lactococcin A, lactostrepcins, diplococcin and lacticin 3147 were described in the past (KOK *et al.* 1993; ROSS *et al*. 1999; RYAN *et al*. 1999). Lactococcin A produced by *Lactococcus lactis* subsp. *cremoris* possesses a narrower inhibitory spectrum than nisin (GEIS *et al.* 1983).

These bacteriocins can be successfully used to inhibit the growth of undesirable microorganisms in foods but only nisin is produced industrially and is licenced for use as a food preservative in a partially purified form (PARENTE & RICCIARDI 1999). New strains of LAB, so-called "wild strains" can be isolated from different milk environments (COGAN *et al*. 1997) and other non-dairy sources such as plants, animals and soil (KLIJN *et al.* 1995). A number of wild bacteriocin-producing lactococci strains were studied with regard to the characteristics important for cheese making (AYAD *et al.* 2000) or possible use for mastitis prevention in nonlactating dairy cows (RYAN *et al.* 1999).

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The present work focuses on further characterization of a novel bacteriocin-producing strain *Lactoccocus lactis* subsp. *lactis* LTM 32 isolated from Vietnamese fermented milk. It was proved in a previously published paper that the strain *Lactoccocus lactis* subsp. *lactis* LTM 32 could grow and produce bacteriocin not only in M17L medium (TERZAGHI & SANDINE 1975) but also in milk and milk with 0.5% w/w yeast extract. The bacteriocin produced was found to be stable at 121°C for 15 min or 100°C for 120 min (DO *et al*. 2001).

MATERIAL AND METHODS

Micro-organisms: The collection of 60 strains of lactic acid bacteria (LAB) both isolated from Vietnamese fermented food (milk, pork and pickles) and from the Culture Collection of Department of Dairy and Fat Technology, Institute of Chemical Technology Prague (CCDMF) were screened for bacteriocin production by use of well agar diffusion assay eliminating the effects of organic acids and hydrogen peroxide (CHUMCHALOVÁ *et al.* 1995). Among the bacteriocin-producing LAB strains a new bacteriocin-producing strain isolated from Vietnamese fermented milk was detected and identified by use of classical morphological and physiological methods (KANDLER & WEISS 1986; HARRIGAN 1998) and by use of API 50 CH fermentation panel as *Lactococcus lactic* subsp. *lactis* and deposited in CCDDF as *Lactococcus lactis* subsp. *lactis,* LTM 32. *Lactobacillus delbrueckii* subsp. *lactis* LTI 30 used as an indicator strain was chosen from the collection of 10 lactobacilli strains on the basis of previous studies (CHUMCHALOVÁ *et al.* 1995). All lactobacilli and/or lactococci strains were maintained in MRS broth and/or in M17L broth (Oxoid, UK) with 10 % v/v glycerol at –20°C and grown in MRS broth (Oxoid, UK) for 20 h at 37°C and/or in M17L broth for 20 h at 30°C prior to use in the assays. Indicator strains other than LAB were obtained from CCDMF and other sources. All strains of bacilli and *Staphylococcus aureus* CCM 2022 were grown in Nutrient agar (Oxoid, UK) for 24 h at 37°C, *Listeria monocytogenes* CCM 5576 in FP medium (Oxoid, UK) for 24 h at 37°C. *B. pumillus* CCM 2218, *Staphylococcus aureus* CCM 2022 and *L. monocytogenes* CCM 5576 were obtained from the Culture Collection of Bacteria, Brno, Czech Republic (CCM). All strains were subcultured minimally three times before use.

Two different genera of LAB were used and for their maintaining and cultivation the different methods were described in one sentence. To make the text more clear it is possible to divide the sentence into two parts.

Preparation of Supernatants Containing Bacteriocins: The bacteriocin-producing LAB strains were grown in MRS broth for 20 h at 37°C (lactobacilli) or in M17L broth for 20 h at 30°C (lactococci). After 20 h cultivation the pH was adjusted to 6.0 by use of 5 mol/l NaOH. The cell-free supernatants were obtained by centrifugation (4000 rpm, 20 min, 4°C) and then boiled at 100°C for 10 min to eliminate the effect of volatile inhibitory substances.

Well Agar Diffusion Assay: The fresh indicator strains (approx. 10^5 CFU) were inoculated into 15 ml of the respective medium (1.5% agar) in the tube: lactobacilli into MRS agar, bacilli and staphylococci into Nutrient agar, *Listeria* sp. strains into FP agar, then mixed and poured onto Petri dish with diameter of 90 mm. Wells 8.5 mm in diameter were cut into the inoculated agars and 50 µl of neutralized and heat treated cell-free supernatant of each potential bacteriocin-producing strain was pipetted into the well (RODRIGUEZ *et al.* 1995). The Petri dishes were incubated at 37°C for 24 h and immediately after the incubation the size of inhibition zone was read as a difference between the diameter of clear zone and the diameter of well.

Sensitivity of Bacteriocin Produced by *Lactococcus lactis subsp. lactis LTM 32* ***to Enzymes***: The neutralized and heat treated cell-free supernatant obtained after 16h incubation of *Lactococcus lactis* subsp. *lactis* LTM 32 in M17L broth at 30°C was treated for 2 h at 37°C with the following enzymes that were obtained from Fluka (Switzerland): proteinase K (No. 82495, 277 U/mg), α-chymotrypsin (No. 27267, 88 U/mg), pronase E (No.81750, 4.9 U/mg), pancreatin (No.76190, 165 U/mg), pepsin (No.77151, 170 U/mg), trypsin (No.93608, 8382 U/mg), α-amylase (No.10065, 26.4 U/mg) and used in the concentrations from 0.5 to 5 mg/ml. After the incubation the cell-free supernatant containing enzymes was heated at 100°C for 5 min to inactivate the enzymes and then the bacteriocin activity was determined by use of well agar diffusion assay.

Effect of Temperature and pH on the Growth and Bacteriocin Production by *Lactococcus lactis* subsp. *lactis* LTM 32: *Lactococcus lactis* subsp. *lactis* LTM 32 was grown in M17L broth (1% v/v inoculum) at 15, 30 and 37°C. After 12h incubation the growth was measured spectrophotometrically (Carry 50) at 660 nm and bacteriocin activity was determined by well agar diffusion assay. Bacteriocin activity was determined in cell-free supernatants obtained after 12h incubation at 30°C in M17L broth with initial pH adjusted to different values between 5.2–8.5 by using the agar diffusion assay as mentioned above.

Stability of Bacteriocin Produced by *Lactococcus lactis* subsp. *lactis* LTM 32 ***during Storage***: The neutralized and heat treated cell-free supernatant after 16h incubation of *Lactococcus lactis* subsp. *lactis* LTM 32 in M17L broth was stored at –20°C, 4°C and 37°C for 15, 30 and 90 days. After this time the bacteriocin activity was determined by well agar diffusion assay.

All results are average values obtained from minimally two (three) independent trials.

RESULTS AND DISCUSSION

Sixty LAB strains from various sources were screened for possible bacteriocin production using well agar diffusion assay. *Staphylococcus aureus* CCM 2022, *Bacillus pumillus* CCM 2006, *Listeria monocytogenes* CCM 5576 and *Lactobacillus delbrueckii* subsp. *lactis* LTI 30 were used as target micro-organisms. Fifteen lactococci strains showed the antimicrobial activity which was not due to organic acids or some volatile compounds such as hydrogen peroxide against one or more target strains (Table 1). Strain LTM 32 isolated from Vietnamese fermented milk inhibited growth of all tested target strains and was selected for further examinations. As an indicator organism *Lactobacillus delbrueckii* subsp. *lactis* LTI 30 was chosen with respect to the screening procedure. This sensitive strain was successfully used in the past as an indicator strain for nisin (PLOCKOVÁ *et al*. 1995) and acidocin CH5 (CHUMCHALOVÁ *et al.* 1995) detection.

Newly isolated strain LTM 32 was preliminarily identified by the used morphological and physiological tests as *Lactococcus* sp. (KANDLER & WEISS 1986; HARRIGAN 1998) and further specified by the API 50 CH test as *Lactococcus lactis* subsp. *lactis*. Strain LTM 32 was proved to be Gram-positive, in the liquid M17 broth it created short chains of cocci 0.7 μm in diameter and did not produce gas from glucose. The pattern of fermented saccharides and the sensitivity to streptomycin and rifampicin were the same as in some nisin-producing lactococci strains (ŠVIRÁKOVÁ 1999).

As it is evident from Table 2, the antimicrobial activity of heat treated cell-free supernatants of *Lactococcus lactis* subsp. *lactis* LTM 32 was abolished when treated with some proteolytic enzymes, namely α-chymotrypsin and proteinase K in the concentrations up to 0.5 mg/ml, less by trypsin, pronase E and pancreatin and not by α-amylase and pepsin. Similar sensitivity to a limited number of proteolytic enzymes, namely to α-chymotrypsin and proteinase K, was found for the nisin-producing strain *Lactococcus lactis* BFE 1500 isolated from a Nigerian milk product wara (OLASUPO *et al.* 1999). These demonstrate that the antimicrobial compound produced by *Lactococcus lactis* subsp. *lactis* LTM 32 is of proteinaceous character and according to the definition (TAGG *et al.* 1976) it could be indicated as a bacteriocin.

The activity spectrum of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* LTM 32 was tested against fifty strains of Gram-positive and Gram-negative bacteria. The positive inhibitory spectrum is shown in Table 3. Among the inhibited Gram-positive strains there were not only nine strains of *Lactobacillus* sp. but also five *Bacillus* sp. strains, one *Listeria monocytogenes* and one *Staphylococcus aureus* strain. It is of great importance for future possible applications of the strain *Lactococcus lactis* subsp. *lactis* LTM 32 to be able to suppress not only closely related strains of LAB but also some strains of

Table 1. Screening of bacteriocin production

Supernatant of tested strain *Lactococcus* sp.	Indication strain			
	Staphylococcus aureus CCM 2022	*Bacillus pumillus* CCM 2218	*Listeria monocytogenes* CCM 5576	*Lactobacillus delbrueckii lactis* subsp. *lactis* LTI 30
Ex 602_1	–	+	+	–
Ex 686_4	–	–	+	–
Ex R_5 1	–	–	+	–
Ex 731_3	–	+	–	+
Lcc 670	–	+	+	+
Lcc 731	+	+	–	+
Lcc 685	–	–	+	+
Lcc 702	–	+	–	+
Lcc 416	–	–	+	+
Lcc 686	–	–	–	+
NIZO R5	–	+	+	+
NCDO 2054	–	–	+	+
NCDO 1748	+	+	+	+
FI 5876	–	–	+	+
LTM 32	**+**	**+**	**+**	**+**

+ inhibited
– no inhibited

Table 2. Effect of enzyme treatment on bacteriocin activity of LTM 32

Enzyme	Inhibition zone (mm) (Φ_{zone}–Φ_{well})				
	enzyme concentration (mg/ml)				control*
	0.5	1	2	5	
Trypsin	13.5	7.0	5.0	0	
Pepsin	13.5	12.0	11.0	10.0	
α-Amylase	13.5	12.0	12.0	12.0	
Pronase E	12.0	9.0	5.0	0	
Proteinase K	5.0	0	nd	nd	
Pancreatin	12.0	9.0	5.0	0	
α-chymotrypsin	3.0	0	nd	nd	13.5

nd – not detected
*supernatant of LTM 32 without enzyme treatment

food-borne pathogens (e.g. *Listeria monocytogenes, Bacillus cereus)* (CARDIANAL *et al.* 1997), causing the dairy cow's mastitis (*Staphylococcus aureus)* or strains responsible for some defects of food, namely dairy products (*Bacillus* sp.). It can be concluded that bacteriocin produced by *Lactococcus lactis* subsp. *lactis* LTM 32 possessed a broader inhibitory spectrum quite similar to the inhibitory spectrum of nisin (KLAENHAMMER 1988).

As it has been published previously (DO *et al.* 2001), *Lactococcus lactis* subsp. *lactis* LTM 32 could grow and produce bacteriocin not only in M17L medium but also in milk and milk with 0.5% w/w yeast extract. As the maximum of bacteriocin production was reached after 12 h incubation in M17L, the effect of initial pH was read at this time. In this paper the effect of initial pH of medium, and incubation temperature in M17L on the growth and bacteriocin production was evaluated. The effect of initial pH is shown in Table 4. *Lactococcus lactis* subsp. *lactis* LTM 32 grew and produced bacteriocin over a pH range 5.2–8.5 with the maximum for initial pH of M17L medium 6.8. Table 5 illustrates the effect of incubation temperature on growth and bacteriocin production by the tested strain; it can be seen that the strain grew and produced bacteriocin over a range from 15 to 37°C with the maximal growth and bacteriocin production at 30°C. The optimal growth conditions were typical of lactococci strains.

High heat stability of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* LTM 32 (121°C for 15 min or 100°C for 120 min) was found in a previous paper (DO *et al*. 2001). Similar heat stability even at an autoclaving temperature was proved for nisin produced by the strain *Lactococcus lactis* BFE1500 (OLASUPO *et al.* 1999). Another important property of bacteriocin is its stability under different storage conditions tested in the present study. As can be seen from Table 6 the bacteriocin was stable during 90 days of storage at –20°C and 4°C.

Table 3. Activity spectrum of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* LTM 32

Organism	Medium	Inhibition by bacteriocin of LTM 32
B. subtilis DMF 2006	Nutrient Agar	+
B. stearothermophilus DMF 2003	Nutrient Agar	++
B. cereus DMF 2007	Nutrient Agar	++
B. cereus DMF2001	Nutrient Agar	++
B. pumillus CCM 2218	Nutrient Agar	+
Lb. acidophilus D 10	MRS	+
Lb. acidophilus A 92	MRS	+
Lb. acidophilus A 82	MRS	+
Lb. casei NDCO 161	MRS	++
Lb. delbrueckii subsp. *lactis* LTI 30	MRS	++
Lb. sp. BK 4	MRS	+
Lb. sp. LBM 4	MRS	+
Lb. sp. LBM 5	MRS	+
Lb. rhamnosus VT 1	MRS	++
Listeria monocytogenes CCM 5576	FP Agar	+
Staphylococcus aureus CCM 2022	Nutrient agar	+

– no inhibition
+ strong inhibition
++ very strong inhibition zone

Table 4. Effect of initial medium pH on the growth of *Lactococcus lactis* subsp. *lactis* LTM 32 and its bacteriocin production (after 12 h of incubation in M17L broth at 30°C)

$pH_{initial}$	OD_{660}	pH_{final}	Inhibition zone (mm) (Φ_{zone}– Φ_{well})
5.2	0.88	4.32	7.0
5.7	1.15	4.41	10.5
6.2	1.49	4.52	12.5
6.8	2.17	5.01	13.5
7.5	1.99	5.48	13.0
8.5	1.89	5.83	11.0

Table 5. Effect of incubation temperature on the growth of *Lactococcus lactis* subsp. *lactis* LTM 32 and its bacteriocin production in M17 broth

$T_{incubation}$ (°C)	Incubation time [h]	OD_{660}	pH_{final}	Inhibition zone (mm) (Φ_{zone}– Φ_{well})
15	48	1.82	5.30	7.0
30	12	2.26	4.98	13.5
37	12	2.12	5.15	10.5

Table 6. Effect of storage conditions on activity of bacteriocin *Lactococcus lactis* subsp. *lactis* LTM 32

Storage condition (°C)	Inhibition zone (mm) (Φ_{zone}–Φ_{well}) storage time (day) 15	30	90	control*
–20	13.5	13.5	13.5	
4	13.5	13.5	12.0	
37	13.5	9.0	7.5	13.5

*no storage

Conclusion

A new bacteriocin-producing strain *Lactococcus lactis* subsp. *lactis* LTM 32 was isolated from Vietnamese fermented milk and identified. According to the results of this study and a previous one (DO *et al*. 2001) this strain is able to grow not only in M17L broth but also in milk and the bacteriocin produced by this strain shows broad activity spectrum, temperature and storage stability, which is of interest for food production applications. As some characteristics of bacteriocin produced by the strain *Lactococcus lactis* subsp. *lactis* LTM 32 are similar to nisin, we are presently working in our laboratory on indicating the presence of some typical genes of the nisin operon in strain LTM 32.

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Souhrn

DO T.M., PLOCKOVÁ M., CHUMCHALOVÁ J. (2001): **Nový kmen** *Lactococcus lactis* subsp. *lactis* LTM 32 **produkující bakteriocin izolovaný z vietnamského fermentovaného mléka**. Czech J. Food Sci., **19**: 171–176.

Na základě testování 60 kmenů bakterií mléčného kvašení (izolovaných z fermentovaných potravin vietnamského původu a získaných ze sbírek) na produkci bakteriocinu byl nalezen kmen *Lactococcus lactis* subsp. *lactis* LTM 32 produkující bakteriocin. Tento kmen byl identifikován a částečně charakterizován. Bakteriocin inhiboval nejen kmeny blízké produkčnímu kmeni, ale také kmeny *Listeria monocytogenes*, *Bacillus* sp. včetně *B. cereus*, *B. stearothermophilus*, *B. subtilis*, *B. pumilus* a *Staphylococcus aureus*. Bakteriocin byl odolný vůči teplu (121 °C po dobu 15 min a 100 °C po dobu 120 min) a stabilní během skladování při teplotě –20 °C a 4 °C po dobu 3 měsíců. Bakteriocin byl inaktivován vybranými proteolytickými enzymy, především proteinasou K a pronasou E. Pepsin a α-amylasa měly na aktivitu bakteriocinu nepatrný vliv. Optimální růst kmene *Lactococcus lactis* subsp. *lactis* LTM 32 a produkce bakteriocinu byla dosažena za podmínek kultivace kmene v M17 bujonu s laktosou při 30 °C a počátečním pH 6,8. Zjištěné charakteristiky kmene produkujícího bakteriocin *Lactococcus lactis* subsp. *lactis* LTM 32 mají význam pro zabezpečení jakosti potravin.

Klíčová slova: *Lactococcus*; *Listeria monocytogenes*; *Staphylococcus aureus*; *Bacillus* sp.; fermentované mléko; bakteriocin; nisin

Corresponding author:

Ing. TUYET MAI DO, Vysoká škola chemicko-technologická, Ústav technologie mléka a tuků, Technická 3, 166 28 Praha 6, Česká republika

tel.: + 420 2 2435 3274, fax: + 420 2 33 33 99 90, e-mail: dotuyetm@vscht.cz