Research Article

Antibacterial Activity of Some Lactic Acid Bacteria Isolated from an Algerian Dairy Product

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In the present study, the antibacterial effect of 20 lactic acid bacteria isolates from a traditional cheese was investigated. 6 isolates showed antibacterial effect against Gram positive bacteria. *Streptococcus thermophilus* T2 strain showed the wide inhibitory spectrum against the Gram positive bacteria. Growth and bacteriocin production profiles showed that the maximal bacteriocin production, by *S. thermophilus* T2 cells, was measured by the end of the late-log phase (90 AU ml⁻¹) with a bacteriocin production rate of 9.3 (AU ml⁻¹) h⁻¹. In addition, our findings showed that the bacteriocin, produced by *S. thermophilus* T2, was stable over a wide pH range (4–8); this indicates that such bacteriocin may be useful in acidic as well as nonacidic food. This preliminarily work shows the potential application of autochthonous lactic acid bacteria to improve safety of traditional fermented food.

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1. Introduction

Lactic Acid Bacteria (LAB) isolated from dairy products have received increased attention as a potential food preservative due to their antagonistic activity against many food borne pathogen such as *Listeria monocytogenes* [1]. LAB are widely distributed in the nature, they are typically involved in a large number of the spontaneous food fermentation, and they have been extensively studied [2]. Some members of LAB produce bacteriocins and bacteriocins-like substances which may inhibit growth of spoilage and pathogenic microorganisms [3]. Bacteriocins from LAB are bioactive peptides or proteins with antimicrobial activity toward Gram positive bacteria, including closely related strains and/or spoilage and pathogenic bacteria [4]. Bacteriocins are ribosomally synthesized and extracellularly released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect [5]. Use of either the bacteriocins or the bacteriocin-producing LAB like starter cultures for food preservation has received a special attention [6]. Moreover, bacteriocins are innocuous due to proteolytic degradation in the gastrointestinal tract [7, 8]. *S. thermophilus* is a lactic acid bacterium of major importance in food industry like the manufacture of yoghourt [9]. Some of *S. thermophilus* strains produce a bacteriocin named thermophilin which is active against several LAB and food spoilage bacteria such as *Clostridium sporogenes*. In view of its technological and biochemical properties the above bacteriocin can be considered as a potential biopreservative [10]. Some of other LAB like *Enterococcus*, *Lactococcus*, and *Pediococcus* are also widely used as natural preservatives, due to the potential production of metabolites with antimicrobial activity such as organic acids, hydrogen peroxide, antimicrobial enzymes and bacteriocins [11].

The aim of the present study is to assess antimicrobial activity of some lactic acid bacteria strains isolated from traditional fermented dairy products prepared from raw milk, Raib, which is obtained after spontaneous curdling of raw milk within 24 to 36 hours at ambient temperature. In addition, preliminary investigations on a bacteriocin produced by *S. thermophilus* strain isolated in this work will be presented.
2. Materials and Methods

2.1. Isolation of Lactic Acid Bacteria. The bacterial strains used in this study were isolated from fermented traditional milk, Raib, manufactured without starter cultures. Samples were collected all over Chlef regions and obtained with collaboration of Bioressources research laboratory. LAB were isolated from Raib, by homogenizing 10 g samples of cheese in 90 mL saline solution and then plating suitable serial dilutions onto different media: BHI, MRS, and M-17 (Biokar Diagnostics, Beauvais, France). The plates were incubated aerobically at 30°C for 48 hours, and then several colonies were picked at random for identification. Cell morphology and Gram-staining reaction were examined by light microscopy, and the catalase activity was carried out. Phenotypic identification was based upon physiological and biochemical characteristics; sugar fermentation profile, in the API-20 Strept CH and API-50 CH fermentation, was carried out according to the manufacturer’s instructions (bioMe`rieux, Marcy l’Etoile, France).

2.2. Detection of Antibacterial Activity. For detection of antagonistic activity, an agar spot test was used. The agar spot test was a modification of that described by Tomé et al. [12]. Overnight cultures, on MRS medium, of the strains to be tested for production of antimicrobial compound were centrifuged (10 minutes at 15000 g, 4°C). Cell-free supernatants were filtered across cellulose acetate filter (0.2 μm) to remove residual cells.

An overnight culture (37°C) of the target strain was diluted in sterile Mueller-Hinton Medium, and 2 mL of ca·10⁶ CFU mL⁻¹ were spread on solid Mueller-Hinton medium. After 5 minutes of contact, the excess was removed and the Petri dishes were dried for 10 minutes. Samples (10 μL) of filtered cell-free supernatants were spotted on the agar plate. The target strains used in this study are Bacillus cereus CIP 6624, Bacillus subtilis ATCC 6633, Escherichia coli CIP 35218, Enterococcus faecalis CIP 29212, Listeria innocua ATCC 51742, Salmonella typhimurium CIP 5858, Staphylococcus aureus CIP 29213, and Staphylococcus epidermidis ATCC 14990.

2.3. Sensitivity of Bacteriocin to Enzymes, pH and Heat Treatment. The biochemical nature of the antibacterial agent was studied on both chloroform extract and cell-free supernatant; all the samples were incubated for 1 hour at 37°C before the antilisterial essay. The pH of cell-free supernatants was adjusted to 6.5 with NaOH (1 N) and then treated with catalase (Sigma; 500 IU mL⁻¹). The cell-free supernatant was also submitted to heat treatment (60–95°C) and to several pH (4–8). The chloroform extract was treated with α-amylase (Sigma; 1 mg mL⁻¹), 100 mM phosphate buffer, pH 6.9), α-chymotrypsin (Sigma, 1 mg mL⁻¹), 0.05 M Tris-HCl buffer (pH 8.0)–0.01 M CaCl₂), Pronase E (Sigma; 1 mg mL⁻¹ in 100 mM Tris–HCl buffer, pH3), Proteinase K (Sigma; 1 mg mL⁻¹ in 100 mM Tris–HCl buffer, pH 7.5), and Trypsin (Sigma, 1 mg mL⁻¹ 50 mM Tris–HCl buffer pH 8.0). Prior to being assayed for bacteriocin activity, preparations containing pronase E were adjusted to pH 6.0. Neutralized cell-free supernatant neutralized cell-free supernatant treated with catalase, heat-treated supernatant, and chloroform extract were spotted against L. innocua. The enzymes were heat inactivated for 3 minutes at 100°C. For each test, untreated bacteriocin plus buffer, bacteriocin plus buffer treated 5 minutes at 100°C, buffer alone and enzymes solutions served as controls [13, 14].

2.4. Growth Kinetic and Bacteriocin Production. Growth experiments were performed in ErleMeyer flask of 500 mL containing 250 mL of MRS broth (pH 6.5) at 37°C without shaking. An overnight pre-culture of S. thermophilus was used for the inoculation of the MRS broth at initial cell density of ca·10⁶ CFU mL⁻¹. At different time intervals, samples were removed from the culture and used for optical density measurement (660 nm), viable and cultivable count (CFU mL⁻¹), extracellular pH measurements, and bacteriocin production. The antibacterial concentration of each sample was conducted with the critical method of dilutions [15]. The bacteriocin concentration Arbitrary Unit mL⁻¹ (AU mL⁻¹) was calculated as the inverse of the strongest dilution which induces the inhibition of L. innocua. All experiments were repeated at least three times. The experiments were repeated three times, and results are expressed as mean ± standard error to the mean.

2.5. Bacteriocin Extraction. The extraction was realized from cell-free culture supernatant of S. thermophilus obtained after centrifugation of overnight culture (20 minutes at 15000 g at 4°C). The extraction was performed according to Burianek and Yousef [16]. The culture supernatant (100 mL) was stirred vigorously for 20 minutes with chloroform (v/v) and transfer in separation funnel, the interface layer between the aqueous and organic phases, which contain bacteriocin, was harvested, and the residual chloroform was eliminated by speed vacuum (50 hours, Unique, Martinsried, Germany). Then bacteriocin activity was measured in the interface layer, aqueous and organic phases.

2.6. Plasmid Extraction. The plasmid extraction was performed from a cell pellet of an overnight culture of S. thermophilus (250 mL) using Miniprep Spin kit together with the corresponding buffers purchased from QIAGEN (Hilden, Germany). S. thermophilus plasmidic DNA analysis was performed by electrophoresis (1 hour, 100 V) using a 0.7% agarose gel dissolved in Tris 45 mM; Borate 45 mM; EDTA 1 mM; pH 8. The electrophoresis gels were analyzed under UV using Molecular Imager Gel Doc System (Bio-Rad, Hercules, USA).

2.7. HPLC Purification of Supernatant Chloroform Extract. The conditions for bacteriocin isolation were realized, through analytical RP-HPLC, on the chloroform extract. The liquid chromatographic system consisted of a Waters 600 E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device, and a Waters 996 photodiode array detector. Spectral and chromatographic data were
Antimicrobial Activity. Twenty LAB strains, isolated from Algerian dairy milk (Table 1), were screened for their antagonistic activity against Listeria innocua, Enterococcus faecalis, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, and Salmonella typhimurium. The results of Table 2 show that six isolates were active against one or more tested strains. In addition, S. thermophilus T2 did not show any inhibitory activity against Gram negative bacteria used in this study: Escherichia coli and Salmonella typhimurium.

Nature of the Inhibitory Agent. Our results showed that the free-cell supernatant remained active, against sensitive target strains, even when the pH was adjusted to pH 7. However, when the cell-free supernatant and the chloroform extract were exposed to the proteolytic enzymes (Table 3) no inhibitory activity was observed against Listeria innocua by contrast to the control tests which showed an inhibitory activity against the target strain (Table 3). In addition, when the cell-free supernatant and the chloroform extract were exposed to the action of α-amylase and catalase similar inhibitory activity was measured when compared with the control test against L. innocua. These results suggest that the biochemical nature of the molecule produced by S. thermophilus is peptidic. Moreover, the antimicrobial activity appeared to be heat resistant. Thus, the inhibitory activity of the chloroform extract was still measured after a heat treatment of 30 minutes at 90°C. Our results showed also that in a range of pH 4–8 similar antibacterial activities of the chloroform extract were obtained against L. innocua.

3.3. Extraction of the Bacteriocin Produced by S. thermophilus. The extraction of the bacteriocin produced by S. thermophilus T2 strain from culture supernatant was realized with chloroform, a water-immiscible solvent. The method used concentrates the bacteriocin at the interface between chloroform and the aqueous culture of the producing bacterium. We demonstrated that no bacteriocin activity was detected in the solvent phase (data not shown). In addition, the precipitate at the interface between the chloroform and culture supernatant fluid contained most of the bacteriocin activity in the mixture. The precipitate at the interface was harvested, and the residual chloroform was eliminated by speed vacuum. After HPLC reversed-phase chromatography, bacteriocin activity was associated with two peaks eluting at 4.6 mm, in MRS broth at 37°C. Under these conditions bacteriocin activity was detected at 4 hours of incubation at the beginning of the exponential phase, at 12 and 14 hours of incubation at 37°C. During the stationary phase both bacteriocin concentration and the cell concentration remained at a steady state (Figure 1). Antibacterial activity decreased after 24 hours of incubation after having reached maximum levels after 14 hours of incubation (data not shown).
Table 2: Antibacterial spectrum of the cell-free supernatant of the six lactic acid bacteria isolated from the traditional dairy product (Raib).

| Strain                   | Strains inhibited                                      |
|--------------------------|--------------------------------------------------------|
| Lactococcus lactis S1    | Listeria innocua, Enterococcus faecalis               |
| Lactococcus lactis S2    | Listeria innocua                                      |
| Lactococcus lactis S7    | Listeria innocua, Enterococcus faecalis Bacillus cereus |
| Lactococcus lactis S9    | Listeria innocua, Bacillus cereus                     |
| S. thermophilus T2       | Bacillus cereus, Bacillus subtilis, Listeria innocua, Enterococcus faecalis, and Staphylococcus epidermidis |
| S. cremoris R3           | Enterococcus faecalis, Bacillus cereus                |
| Lactococcus diacetylactis V1 | Enterococcus faecalis                              |

Table 3: Effect of different treatments on cell-free supernatant and chloroform extract of S. thermophilus T2. Relative activity was measured by an agar diffusion test against Listeria innocua. (-): no inhibition; (+): slight inhibition; (++): moderate inhibition; (+++): strong inhibition.

| Treatments        | Relative activity |
|-------------------|-------------------|
| Proteinase K      | –                 |
| Pronase E         | –                 |
| α-chymotrypsin    | –                 |
| Trypsin           | –                 |
| α-amylase         | ++                |
| Catalase          | ++                |
| Control           | +++               |
| pH treatments     |                   |
| 4                 | +++               |
| 5                 | +++               |
| 6                 | +++               |
| 7                 | +++               |
| 8                 | +++               |
| Control           | +++               |
| Heat treatments   |                   |
| 60°C              | +++               |
| 70°C              | +++               |
| 80°C              | ++                |
| 90°C              | ++                |
| 95°C              | +                 |
| Control           | +++               |

3.5. Plasmid Content. The genes encoding for bacteriocin are either chromosomal or plasmidic [18, 19]. The aim for this preliminary investigation is to assess the presence of plasmid in S. thermophilus cells. The analysis of the plasmidic DNA extraction showed that S. thermophilus seems to have a single plasmid as shown in Figure 2. In addition, Dral fragmentation pattern of the plasmid resulted in three restriction fragments with approximately 2.2 kb, 1.5 kb, and 0.5 kb. Thus the size of the plasmid could be of at least 4.2 kb. This preliminary result is of importance since in many lactic acid bacteria bacteriocins and carbohydrate fermentation exopolysaccharide production and antiphage mechanisms are carried by the same plasmid as reported previously by Martinez-Bueno et al. [20] and Turgeon and Moineau [21].

4. Discussion

Bacteriocins from lactic acid bacteria are of importance in bioconservation of various foods. Moreover, the use of more than one LAB bacteriocin as a combination of biopreservative may have major applications in improving food safety [1]. In the present study, the inhibitory effect of the cell-free filtrates of each of the 20 isolates was evaluated.
Antimicrobial activity was observed for 6 isolates, and only against Gram positive bacteria. The biochemical nature of the antibacterial molecule produced by *S. thermophilus* T2 was studied in both the cell-free supernatant and the chloroform extract. Our results showed that the molecule, produced by *S. thermophilus*, is peptidic since the antibacterial activity of the molecule was lost after digestion with proteolytic enzymes. However, the neutralization (pH 7) and addition of catalase or α-amylase to the cell-free supernatant did not result in the loss of the antilisterial activity. Our results showed also that the bacteriocin produced by *S. thermophilus* is heat stable (up to 30 minutes at 95°C); these results are similar to what has been reported for thoenicin [22]. In addition, the bacteriocin was stable over a wide pH range, this indicates that such bacteriocin may be useful in acidic as well as nonacidic food; similar pH stability results have been reported for propionicin PLG1 [14]. Growth and bacteriocin production profiles showed that the maximal bacteriocin production was measured by the end of the late-log phase. The level of production remained at a steady state during the stationary phase; similar results were obtained by Ivanova et al. [23]. However, bacteriocin production decreases after 24 hours of incubation after having reached maximum levels after 14 hours. This reduction could be a result of the inactivation of bacteriocin by extracellular proteases.

Preliminary characterization of the bacteriocin produced by *S. thermophilus* T2 was realized in the present study. It was found that the bacteriocin inhibits closely related Gram positive strains like *Listeria innocua* and *Enterococcus faecalis*. Activity against Gram negative was rarely reported for bacteriocin [24, 25]. Active substance from culture supernatant of *S. thermophilus* T2 was obtained according to the procedure described by Burianek and Yousef [16]. Chloroform was added to the cell-free supernatant in a separator funnel, the bacteriocin was concentrated at the interface between chloroform and the aqueous phase. This method effectively recovers higher bacteriocin yield and results in relatively clean preparations. Recovery of bacteriocin by the chloroform extraction was 10-fold higher when compared with ammonium sulphate precipitation (data not shown).

In conclusion, the study of autochthonous LAB will help to select the best candidates for improving the microbiological safety of traditional food products such as Raib and may increase their shelf life. Such a collection could be used for construction of specific starter cultures for fermented food products.

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### References

[1] M. Jamuna and K. Jeevaratnam, “Isolation and partial characterization of bacteriocins from *Pedicoccus* species,” *Applied Microbiology and Biotechnology*, vol. 65, no. 4, pp. 433–439, 2004.

[2] W. H. Holzapfel, R. Geisen, and U. Schillinger, “Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes,” *International Journal of Food Microbiology*, vol. 24, no. 3, pp. 343–362, 1995.

[3] T. R. Klaenhammer, “Bacteriocins of lactic acid bacteria,” *Biochimie*, vol. 70, no. 3, pp. 337–349, 1988.

[4] J. R. Tagg, A. S. Dajani, and L. W. Wannamaker, “Bacteriocins of gram positive bacteria,” *Bacteriological Reviews*, vol. 40, no. 3, pp. 722–756, 1976.

[5] S. Garneau, N. I. Martin, and J. C. Vederas, “Two-peptide bacteriocins produced by lactic acid bacteria,” *Biochimie*, vol. 84, no. 5-6, pp. 577–592, 2002.

[6] C. Sabia, G. Manicardi, P. Messi, S. De Niederhaus, and M. Bondi, “Enterocin 416K1, an antilisterial bacteriocin produced by *Enterococcus casseliflavus* IM 416K1 isolated from Italian sausages,” *International Journal of Food Microbiology*, vol. 75, no. 1–2, pp. 163–170, 2002.

[7] L. M. Cintas, J. M. Rodríguez, M. F. Fernandez, et al., “Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum,” *Applied and Environmental Microbiology*, vol. 61, no. 7, pp. 2643–2648, 1995.

[8] L. De Vuyst and E. J. Vandamme, “Nisin, a lanthionine produced by *Lactococcus lactis* subsp. *Lactis*: properties, biosynthesis, fermentation and application,” in *Bacteriocins of Lactic Acid Bacteria*, L. De Vuyst and E. J. Vandamme, Eds., pp. 151–221, Balackis Academic and Professional, Glasgow, UK, 1994.

[9] U. Purwandari, N. P. Shah, and T. Vasiljevic, “Effects of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological and rheological properties of set-type yoghurt,” *International Dairy Journal*, vol. 17, no. 11, pp. 1344–1352, 2007.

[10] A. Aktypis, M. Tychowski, G. Kalantzopoulos, and G. Aggelis, “Studies on bacteriocin (thermophilin T) production by *Streptococcus thermophilus* ACA-DC 0040 in batch and fed-batch fermentation modes,” *Antonie van Leeuwenhoek*, vol. 92, no. 2, pp. 207–220, 2007.
[11] M. Mataragas, E. H. Drosinos, and J. Metaxopoulos, “Antagonistic activity of lactic acid bacteria against Listeria monocytogenes in sliced cooked cured pork shoulder stored under vacuum or modified atmosphere at 4 ± 2°C,” Food Microbiology, vol. 20, no. 2, pp. 259–265, 2003.

[12] E. Tomé, P. Teixeira, and P. A. Gibbs, “Anti-listerial inhibitory lactic acid bacteria isolated from commercial cold smoked salmon,” Food Microbiology, vol. 23, no. 4, pp. 399–405, 2006.

[13] A. Cherif, W. Rezgui, N. Raddadi, D. Daffonchio, and A. Boudabous, “Characterization and partial purification of entomocin 110, a newly identified bacteriocin from Bacillus thuringiensis subsp. Entomocidus HD110,” Microbiological Research, vol. 163, no. 6, pp. 684–692, 2008.

[14] W. J. Lyon and B. A. Glatz, “Partial purification and characterization of a bacteriocin produced by Propionibacterium thoenii,” Applied and Environmental Microbiology, vol. 57, no. 3, pp. 701–706, 1991.

[15] A. Mayr-Harting, A. J. Herdgs, and R. C. W. Berkeley, “Methods for studying bacteriocins,” Methods in Microbiology, vol. 7, pp. 315–422, 1972.

[16] L. L. Burianek and A. E. Yousef, “Solvent extraction of bacteriocins from liquid cultures,” Letters in Applied Microbiology, vol. 31, no. 3, pp. 193–197, 2000.

[17] Q. Zhao, P. Molina, and J. M. Piot, “Peptic peptide mapping by HPLC, on line with photodiode array detection, of a hemoglobin hydrolysate produced at pilot-plant scale from an ultrafiltration process,” Journal of Liquid Chromatography and Related Technologies, vol. 20, no. 11, pp. 1717–1739, 1997.

[18] E. Balla, L. M. T. Dicks, M. Du Toit, M. J. Van Der Merwe, and W. H. Holzapfel, “Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by Enterococcus faecalis BFE 1071,” Applied and Environmental Microbiology, vol. 66, no. 4, pp. 1298–1304, 2000.

[19] H. Abriouel, E. Valdivia, M. Martinez-Boueno, M. Maqueda, and A. Galvez, “A simple method for semi-preparative-scale production and recovery of enterocin AS-48 derived from Enterococcus faecalis subsp. liquefaciens A-48-32,” Journal of Microbiological Methods, vol. 55, no. 3, pp. 599–605, 2003.

[20] M. Martinez-Bueno, A. Galvez, E. Valdivia, and M. Maqueda, “A transferable plasmid associated with AS-48 production in Enterococcus faecalis,” Journal of Bacteriology, vol. 172, no. 5, pp. 2817–2818, 1990.

[21] N. Turgeon and S. Moineau, “Isolation and characterization of a Streptococcus thermophilus plasmid closely related to the pMV158 family,” Plasmid, vol. 45, no. 3, pp. 171–183, 2001.

[22] I. R. Van Der Merwe, R. Bauer, T. J. Britz, and L. M. T. Dicks, “Characterization of thehoincin 447, a bacteriocin isolated from Propionibacterium thoenii strain 447,” International Journal of Food Microbiology, vol. 92, no. 2, pp. 153–160, 2004.

[23] I. Ivanova, V. Miteva, T. Stefanova, et al., “Characterization of a bacteriocin produced by Streptococcus thermophilus 81,” International Journal of Food Microbiology, vol. 42, no. 3, pp. 147–158, 1998.

[24] N.-E. Chihib, L. Monnerat, J. M. Membré, and J.-L. Tholozan, “Nisin, temperature and pH effects on growth and viability of Pectinatus frisingensis, a gram-negative, strictly anaerobic beer-spoilage bacterium,” Journal of Applied Microbiology, vol. 87, no. 3, pp. 438–446, 1999.

[25] G. M. Vignolo, F. Suriani, A. P. de Ruiz Holgado, and G. Oliver, “Antibacterial activity of Lactobacillus strains isolated from dry fermented sausages,” Journal of Applied Bacteriology, vol. 75, no. 4, pp. 344–349, 1993.