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Lactic acid Bacteria Isolated from Raw Milk Cheeses: Ribotyping, Antimicrobial Activity against Selected Food Pathogens and Resistance to Common Antibiotics

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Abstract

Fermentation with lactic acid bacteria is one of the oldest and effective methods of food preservation that meets the increasing drive of many consumers for foods perceived as minimally processed or free of unwanted chemical preservatives. The isolation and characterization of new wild LAB strains showing potential for application in food preservation may contribute to the processing of food products with good flavor and increasing safety. In the present work ninety-six LAB isolated from raw milk cheeses were subject to a preliminary characterization via identification (ribotyping) and screened for antimicrobial activity against *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* Newport, while the antibiotic resistance of the isolates against several clinic antibiotics were also studied. Most isolates of interest were identified as *Lactobacillus paracasei* ss. *paracasei*, of which, five were shown to completely inhibit *Listeria monocytogenes*, one inhibited *Escherichia coli*, five had inhibitory activity against *Salmonella* Newport, and five were active against both *Listeria monocytogenes* and *Salmonella* Newport. The *Etest* method determined that the isolates were sensitive to β-lactams, including amoxicillin and ampicillin, but were resistant to glycopeptides and aminoglycosides.

Keywords: *Lb paracasei*; Traditional cheeses; Salmonella; Bacteriocin; Food pathogens; Antibiotic resistance

Introduction

Many consumers avoid foods that have been preserved with chemicals such as sulphites and nitrites, due to their potential toxicity [1,2]. In addition, there are also issues surrounding thermal processing, as it can lead to unwanted changes of the nutritional and organoleptic properties of foods [1]. Therefore, there is an increasing interest in using biopreservation (fermentation) as a way to deliver food products perceived as minimally processed, safe, nutritious, while still having a convenient long shelf-life [2,3]. Fermentation with lactic acid bacteria (LAB), is an old method of food preservation, whereby LAB produce antimicrobial metabolites such as lactic acid, hydrogen peroxide, and eventually bacteriocins, which may be active against many undesirable food microorganisms. In particular, bacteriocins are reported as having a great potential of use in veterinary medicine, crop management and food preservation [1-4], as they exhibit such properties as being active against specific pathogens, have a narrow range of activity, may be thermo resistant, and are easily inactivated in the gastrointestinal tract [1].

However, until a bacteriocin can be fully used, a number of research steps, from identification, isolation, purification, as well as testing for their safety and technological suitability, are required. Today, nisin is one of the few bacteriocins approved by the World Health Organization for use in food [5], thus, discovery of new ones may contribute to increasing the pool of bacteriocins for food preservation. LAB optimal release of said metabolites, may be greatly affected by environmental factors such as temperature, pH and aerobic growth conditions, thus, understanding and eventually control these factors is a key part in characterizing a LAB or its metabolites, namely bacteriocins. Pathogens such as *Listeria monocytogenes*, and *Salmonella spp* are of increased concern in food safety [6], as they have been associated to many foodborne outbreaks, including some associated with cheeses [7]. Therefore, research directed to eliminate the risks associated with these pathogens has been an important activity in Food Safety [8]. The aim of this research was to obtain a preliminary characterization of LAB isolates from local raw milk cheeses, towards undertaking depth studies for those one showing greater potential in food preservation.

Materials and Methods

Production of supernatants

Ninety six lactic acid bacteria isolates from raw-milk cheeses were aseptically streaked on de Man, Rogosa and Sharpe (MRS) agar plates (Sigma- Aldrich, St. Louis, MO, USA). Plates were then incubated at 30°C for 72 hours. A colony was then transferred to 10 mL of MRS broth, and incubated for 48 hours at 25°C under anaerobic conditions (in anaerobic jars with a 2.5 L AnaeroGen pouch (Sigma- Aldrich, St. Louis, MO, USA)), to hinder the formation of hydrogen peroxide. Following incubation, 700 µL of 1 M NaOH were added (giving a final pH of 6.5) to neutralize the pH and prevent the antimicrobial effects of acids in the broth. The suspension was centrifuged at 12000 × g at 4°C for 10 minutes, and filtered using a 0.22 µm pore size filter (EMD Millipore, Billerica, MA, USA). The supernatant was removed and stored at -80°C for later use.

Growth of indicator bacteria

Tryptic Soy Broth (TSB), or Tryptic Soy Agar (TSA) (Difco Laboratories, Detroit, MI, USA) were used to grow *Salmonella* Newport

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(C1158). Brain Heart Infusion (BHI) agar or BHI broth (Difco Laboratories, Detroit, MI, USA) were used to grow *Listeria monocytogenes* (NCTC 7973), and *Escherichia coli* (ATCC 51739). All bacterial cultures were obtained from the culture collection at the Canadian Research Institute for Food Safety (Guelph, ON, Canada).

Screening for supernatants antimicrobial effect

The anti-microbial activity of the LAB supernatants were tested against *Listeria monocytogenes*, *Salmonella* Newport, and *Escherichia coli*. This was determined by measuring the optical density (OD) of these bacteria in the presence of the LAB supernatant using the Bioscreen C Microbiology Plate Reader (Labsystems, Helsinki, Finland). All 96 supernatants were tested against the three indicator micro-organisms, using a sterile 100-well honeycomb plate (Growth Curves USA, NJ, USA). The wells were inoculated with 150 µL of a diluted overnight bacterial culture (10^2 CFU/mL) and 50µL of the LAB supernatant and all samples were tested in triplicate. The overnight culture was produced by transferring one colony into 15 mL BHI or TSB, and put into a C24 incubator shaker (New Brunswick Scientific, NJ, USA) at 37°C with shaking at 200 rpm. The Bioscreen settings were: single, wide band (wb) wavelength; 37°C incubation temperature; 20 minute reheating time; kinetic measurement; 24 hour run time; medium intensity shaking for 10 seconds before reading; and reading time every 10 minutes.

Effect of temperature

LAB supernatants showing inhibitory activity were further tested to establish the effects of temperature on their activity. The LAB supernatants were heated using a digital heat block (VWR, Radnor, PA, USA) to an internal temperature of 45°C or 80°C for 30 minutes, in sterile Eppendorf tubes. In the case of 45°C, the supernatants were heated for a total of 45 minutes, as it took 15 minutes to reach the internal temperature of 45°. For 80°C, it was a total of 75 minutes, as it took 45 minutes to reach the internal temperature. The same Bioscreen procedure as described above was used to assess the antibacterial properties of the heat treated samples.

Effect of pH

The effect of pH on antimicrobial activity was determined at pH 4-5 or 8-9. Three hundred µL of the LAB supernatant were dispensed into sterile eppendorf tubes. To check the pH, pH-indicator strips (non-bleeding) pH 0-14 (colorPHast©, EMD, Germany) were used. To adjust the pH of the LAB supernatant to pH 4-5, 25 µL of 1 M HCl were added; for pH 8-9 15 µL of 1 M NaOH were added. The inhibitory activity of the supernatants against the test organisms was then determined using the Bioscreen as described above.

Proteinaceous nature of the antimicrobial substances

In order to determine if the anti-microbial activity was due to a proteinaceous substance, trypsin was added to the LAB supernatants to achieve final concentrations of 0.5 mg/mL. The resulting mixture was incubated at 37°C for 30 minutes. Following incubation, the antibacterial activity of the enzyme treated samples were determined using the Bioscreen.

Ribotyping

Considering the degree of inhibition they caused to specific indicator microorganism, selected LAB isolates were chosen and identified by automated ribotyping using the Ribo Printer (DuPont-Qualicon, Wilmington, DE, USA).

Nucleic acid was extracted from the cultures by boiling and was digested with the endonuclease EcoRI. Ribotyping was performed according to the manufacturer's instructions. The identity of the culture was determined using the Ribo Printer database.

Antibiotic resistance

The resistance of the isolates to clinically important antibiotics was tested as a way to shed light on the potential risks associated to their concentrated use in tailor-made starter cultures. The antibiotic resistance of LAB that possessed antibacterial activity and that were fully identified as indicated in 2.7, was determined using the Epsilometer (*Etest*) method [4]. Confluent growth of the LAB was produced on Lactic Acid Bacteria Susceptibility Medium agar [9]. Cells of an overnight culture of the respective LAB grown on MRS broth were harvested by centrifugation, as described above, and re-suspended in 1% saline to achieve an OD at 625 nm of 0.16-0.20 (comparable to McFarland standard 1 or 3×10^8 CFU/ml). A sterile cotton swab was immersed into the saline suspensions, and once excess fluid was removed, it was used to streak the entire surface of the agar plate in three directions. Plates were left to dry, and once dried, one E-test strip containing the antibiotics amoxicillin, ampicillin, clindamycin, erythromycin, gentamycin, kanamycin, rifampicin, streptomycin, tetracycline, or vancomycin (Biomerieux, Marcy-l'Etoile, France) was applied onto the agar, with the side displaying the minimum inhibitory concentration (MIC) facing up. Plates were then inverted (agar side facing upwards) and incubated at 37°C in an anaerobic jar containing an Anaero Gen 2.5 L pouch (Sigma- Aldrich, St. Louis, MO, USA), and readings were taken at 24 and 28 hours. An elliptical zone of inhibition is eventually produced, and the point at which the ellipse meets the strip gives a reading for the minimum inhibitory concentration (MIC) of the drug. Plates with an Etest strip were also compared to a control plate without an antibiotic strip, to see if growth occurred.

Results and Discussion

Bioscreen protocols

The Bioscreen data were analysed to determine the detection time (DT; the time taken to reach an optical density (OD) of 0.3) for each sample. The detection time was calculated as an average of triplicate readings. The average DT was then subtracted from the detection time obtained for the test bacterium grown in the absence of the LAB supernatants, which gave a detection time difference (DTD) [10]. Results were then classified as: (N): growth was similar to the control; (D): delay in growth of bacteria, DTD < 5 hours; (D+): delay in growth of bacteria, DTD >5 hours; (C): complete inhibition of bacterial growth [10]. Table 1 details the LAB supernatants exhibiting complete inhibition (C) of the respective bacterium. It can be seen that five isolates of LAB produced extracellular compounds with antibacterial activity against *Listeria monocytogenes* and *Salmonella* Newport. This is of special interest as most bacteriocins have anti-microbial activity only against gram positive bacteria, with gram negative bacteria being less susceptible [2,10]. One LAB supernatant was also found to exhibit activity against *E. coli*, which could be potentially used to control gram negative bacteria.

Effect of temperature

Table 1 describes the effect of different temperatures on the bioactivity of supernatants. The temperatures that were used are similar to those extreme values of temperatures commonly used in dairy processing. As expected, there was a decrease in inhibition activity after samples were heat treated. It is known that the antimicrobial activity

LAB isolate	Indicator microorganism	Condition[a]				
		Untreated	45°C	80°C	pH 4-5	pH 8-9
119	*Listeria monocytogenes*	C	N/A	N/A	N/A	N/A
154	*Listeria monocytogenes*	C	C	C	C	C
162	*Listeria monocytogenes*	C	C	C	C	C
171	*Listeria monocytogenes*	C	D+	C	C	N
173	*Listeria monocytogenes*	C	C	C	C	C
180	*Listeria monocytogenes*	C	D+	C	C	C
185	*Listeria monocytogenes*	C	C	C	C	C
186	*Listeria monocytogenes*	C	C	C	C	C
189	*Listeria monocytogenes*	C	C	C	C	C
220	*Listeria monocytogenes*	C	D+	C	C	C
174	*E.coli*	C	C	C	C	N
119	*Salmonella* Newport	C	N/A	N/A	N/A	N/A
130A	*Salmonella* Newport	C	D+	D+	C	N
149B	*Salmonella* Newport	C	D+	C	C	D
154	*Salmonella* Newport	C	C	C	C	D
172	*Salmonella* Newport	C	C	D	C	C
179	*Salmonella* Newport	C	D	D+	C	C
186	*Salmonella* Newport	C	C	C	C	D
189	*Salmonella* Newport	C	C	C	C	N
211	*Salmonella* Newport	C	C	C	C	N
	Salmonella Newport	C	D	C	C	D

[a]Abbreviations are: C, Complete inhibition; N, growth is similar to control; D, delay in growth of bacterium
log phase by <5 hours; D+, delay in growth of bacterium log phase by≥5 hours; N/A, no activity detected.

Table 1: LAB isolates culture supernatants activity against the indicated pathogen atdifferent temperature and pH conditions.

and spectrum of activity of compounds, such as lysozyme, changes on heating due to perturbations in tertiary structure that improve access to the active moiety [11,12].

Many samples exhibited the same level of activity after treatment at 45°C as well at 80°C, while unexpectedly, a few showed a slightly increased inhibitory activity. A study by Joshi, Sharma and Rana [13] demonstrated that a partially purified bacteriocin from *Lactobacillus* sp. (CA44) retained high antimicrobial activity against *E. coli*, *B. cereus* and *S. aureus* after heating at 68°C for 10 and 20 minutes. Sankar et al. took a purified bacteriocin produced by *Lactobacillus plantarum* and subjected it to a variety of temperatures. For *Listeria* and *E. coli* at 40 and 50°C there was a slight decrease in the inhibition and heating at 80°C resulted in even less antibacterial activity. Sifour, Tayeb, Haddar, Namous, and Aissaoui [14] also studied a bacteriocin from *Lactobacillus plantarum* and found that following heating to 40°C and 80°C for 30 min the residual activity was almost 100%. This agrees with our results, which in general indicate that due to the properties exhibited by LAB from our study, they may be of use as bio-control agents in products that may undergo a heating process in the studied temperature ranges.

Effect of pH

The effect of the supernatant pH on its antibacterial activity is shown also in Table 1. The pH value of 4-5 was chosen as this is the pH range of many dairy products, such as cheeses and yogurts, because of the activity of starter cultures. All supernatants from the chosen LAB isolates in our study showed inhibitory activity against the indicator bacteria at pH 4-5. As a result, this LAB may be of interest to be used as adjuvants cultures to increase product safety during ripening of most cheeses. At pH 8-9, some supernatants produced a >5 hour delay in the growth of the test organism, or no inhibition was observed.

The pH range 8-9 was chosen to provide an indication of the stability of the antimicrobial compound when exposed to an alkaline environment. Sifour et al. [14] described a bacteriocin with highest residual antibacterial activity at pH 6, and between pH 4-5 the residual activity was between 80-90%. At pH 8-9 the residual activity of this bacteriocin also appeared to be approximately 90% [14]. Joshi et al. [13] found the highest antimicrobial activity of a bacteriocin against *S. aureus, B. cereus*, and *E. coli* was observed at a pH of 4 to 5. For all three bacteria, the inhibitory activity of the bacteriocin at a pH of 8-9 was noticeably decreased [13]. Similar results were reported for the effect of bacteriocin on *Listeria* spp and *E. coli* and were confirmed by this study.

Effect of the proteolytic enzyme on antimicrobial activity

Treatment with trypsin lowered the antibacterial activity of the supernatants, indicating that the supernatants' antibacterial activity was caused by a substance of proteic nature (likely a bacteriocin). This is partially in agreement with data reported by Sankar et al., who found complete inhibition of the antibacterial activity in supernatants treated with trypsin. Recall that in our study the acidity and presence of H_2O_2 were unlikely to cause the antibacterial activity exhibited by the supernatants, as they were produced under conditions designed to ensure that H_2O_2 was absent, via using anaerobic conditions for fermentation, followed by a neutralization of the supernatant pH. Sifour et al. [14] studied the effects of five enzymes (lipase, α- amylase, trypsin, pronase E, α-chymotrypsin) on a bacteriocin and found that trypsin, pronase E, and α-chymotrypsin resulted in a major reduction in activity or complete inactivation of the bacteriocin. Also a study by Ivanova, Kabadjova, Pantev, Danova, and Dousset [15] found a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* b14, which activity was significantly reduced by proteinase K, pronase E, and pepsin.

Ribotyping

Ribotyping showed Lactobacillus *paracasei* ss. *paracasei* to be the dominant species among the studied isolates (Figure 1), followed by *Lactobacillus rhamnosus*. Our study agree with results reported by Kongo, Ho, Malcata, and Wiedmann [16] who also found presence of both species and dominance of *Lactobacillus paracasei* in traditional raw milk cheeses.

Antibiotic resistance

Table 2 shows the antibiotic susceptibility of the identified LAB isolates used in this study. The LAB were susceptible to amoxicillin, ampicillin, clindamycin, erythromycin, rifampicin, and tetracycline. They were found to be more resistant to streptomycin, gentamycin, kanamycin and highly resistant to vancomycin. These results are similar to other studies that determined the antimicrobial resistance of LAB [17]. Antibiotic resistance may be associated with an increasing overuse of antibiotics in the treatment of cattle and LAB are often found to be resistant to β-lactam antibiotics. The fact that our isolates were generally sensitive to β-lactams may suggest that they were isolated from products associated to an environment where these antibiotics are rarely used. However, the same isolates showed resistance to vancomycin (an antibiotic used in extreme situations in clinical applications), and this is in agreement with results from Vescovo, Morelli, and Bottazzi and de Fabrizio, Parada, and Ledford [18,19]. Considering that LAB may potentially transfer resistance to more dangerous bacteria, LAB showing multiple antibiotic resistances should be avoided as potential components of starter cultures. While

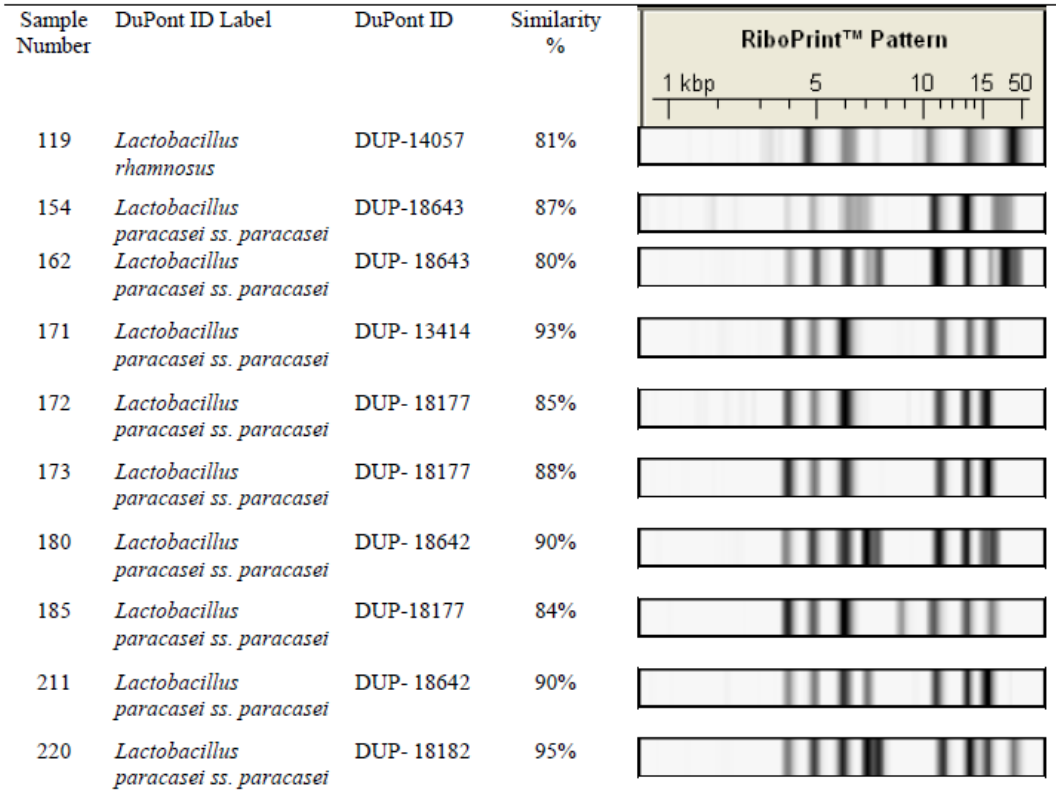

Figure 1: Ribotyping identification of LAB isolates that showed particular interesting inhibition activity.

Sample	Reading				MIC[a] (µg/mL)						
number	time(hours)	Amox	Amp	Clind	Eryth	Gent	Kan	Rif	Strep	Tetra	Van
119	24	0.75	0.5	0.064	0.032	8	48	0.094	16	0.25	>256
119	48	0.5	0.5	0.19	0.094	8	96	0.064	32	0.5	>256
154	24	0.75	0.5	0.064	0.032	8	48	0.094	16	0.25	>256
154	48	0.5	0.5	0.19	0.094	8	96	0.064	32	0.5	>256
162	24	0.5	0.25	0.032	0.032	4	16	0.047	12	0.25	>256
162	48	0.38	0.25	0.094	0.064	8	32	0.047	16	0.5	>256
171	24	0.5	0.5	0.032	0.047	6	64	0.064	16	0.25	>256
171	48	0.5	0.5	0.064	0.064	8	96	0.064	24	0.75	>256
172	24	0.5	0.5	0.064	0.064	4	48	0.047	16	0.38	>256
172	48	0.5	0.5	0.064	0.094	4	64	0.047	16	1	>256
173	24	0.75	0.5	0.047	0.064	6	128	0.094	24	0.25	>256
173	48	0.75	0.5	0.047	0.094	6	>256	0.094	24	0.5	>256
174	24	0.75	0.38	0.047	0.032	6	128	0.032	12	0.25	>256
174	48	0.75	0.25	0.094	0.094	6	>256	0.047	24	0.38	>256
180	24	1	0.38	0.064	0.023	8	>256	0.094	24	0.38	>256
180	48	0.5	0.38	0.125	0.064	8	>256	0.094	24	0.38	>256
185	24	0.5	0.38	0.023	0.064	8	32	0.032	24	0.5	>256
185	48	0.5	0.38	0.047	0.125	8	128	0.032	24	0.5	>256
211	24	0.38	0.19	0.064	0.094	4	64	0.023	16	0.25	>256
211	48	0.38	0.25	0.064	0.064	6	128	0.023	16	0.38	>256
220	24	0.75	0.75	0.064	0.023	6	64	0.094	16	0.125	>256
220	48	0.5	0.5	0.125	0.094	6	96	0.094	24	0.38	>256

[a]Abbreviations are: Amox=Amoxicillin, Amp=Ampicillin, Cind=Clindamycin, Eryth=Erythromycin, Gent=Gentamycin, Kan=Kanamycin, Rif=Rifampicin, Strep=Streptomycin, Tetra=Tetracycline, Van=Vancomycin

Table 2: Antibiotic susceptibility of LAB isolates from this study.

all isolates showed similar resistance to vancomycine, there was a statistically significant difference (not shown) in MICs for most antibiotics.

Conclusion

The preliminary characterization of the 96 isolates included in the present study showed that via identification by ribotyping *Lactobacillus paracasei* ss. *paracasei*, and *Lactobacillus rhamnosus* were the dominant ones in terms of frequency of isolation. Screening of selected isolates for anti-microbial activity against *Listeria monocytogenes*,

Salmonella Newport revealed that five inhibited growth of *Listeria*, five inhibited *Salmonella* Newport, and five inhibited both *Listeria* and *S.* Newport. The antimicrobial compounds they released were thermo stable at both 45°C and 80°C and all isolates inhibited bacterial growth at pH 4-5, while at pH 8-9 the antimicrobial activity of many was decreased. Antibiotic resistance tested via *Etest* found most LAB isolates were sensitive to β-lactam antibiotics, yet they were more resistant to glycopeptides and aminoglycosides. Future studies will be undertaken to fully characterize and identify the antimicrobial substances that showed inhibitory activity against *Listeria* and *Salmonella*.

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