Antimicrobial susceptibilities and random amplified polymorphic DNA-PCR fingerprint characterization of *Lactococcus lactis* ssp. *lactis* and *Lactococcus garvieae* isolated from bovine intramammary infections

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ABSTRACT

In total, 181 streptococci-like bacteria isolated from intramammary infections (IMI) were submitted by a veterinary clinic to Quality Milk Production Services (QMPS, Cornell University, Ithaca, NY). The isolates were characterized by sequence analysis, and 46 *Lactococcus lactis* ssp. *lactis* and 47 *Lactococcus garvieae* were tested for susceptibility to 17 antibiotics. No resistant strains were found for β-lactam antibiotics widely used in clinical practice (penicillin, ampicillin, and amoxicillin), and all minimum inhibitory concentrations (MIC) were far from the resistance breakpoints. Eight strains had MIC intermediate to cefazolin. The random amplification of polymorphic DNA (RAPD)-PCR fingerprint patterns showed a slightly higher heterogeneity for *Lc. lactis* ssp. *lactis* isolates than for *Lc. garvieae* isolates.
Key words: *Lactococcus lactis* ssp. *lactis*, *Lactococcus garvieae*, intramammary infection, minimum inhibitory concentration, random amplification of polymorphic DNA (RAPD)-PCR fingerprint

INTRODUCTION

Lactococcus lactis is a member of the lactic acid bacteria (**LAB**), a functional group of gram-positive, microaerophilic cocci that produce lactic acid as the main product of hexose fermentation (Makarova et al., 2006). They also colonize the gastrointestinal and urogenital tracts of humans and animals and are present in foods such as dairy products, fermented meats, fruits, and vegetables. The LAB are intentionally added to several

probiotic products because of their potential health benefits (von Wright and Axelsson, 2012).

Lactococcus lactis was thought to be a nonpathogenic bacterium and is used as starter culture for the production of dairy products. In the past, reported opportunistic infections associated with the genus *Lactococcus* were rare (Collins et al., 1983; Teixeira et al., 1996). Now, *Lc. garvieae* is classified as an emerging pathogen (Morita et al., 2011), causing infections in fish (Vendrell et al., 2006) and mastitis in the bovine (Collins et al., 1983; Teixeira et al., 1996), and has been associated with human clinical cases (Reimundo et al., 2011). Recently, *Lc. lactis* has been isolated from diseased fish (Wang et al., 2008; Chen et al., 2012; Pérez et al., 2011), bovine mastitis cases (Wyder et al., 2011; Romero et al., 2011, Plumed-Ferrer et al., 2013, Werner et al., 2014), bird infections (Goyache et al., 2001), and many human clinical infections (Mofredj et al., 2007; Uchida et al., 2011). Isolates from the genus *Lactococcus* are often misidentified as enterococci or streptococci, and the difficulties in correctly identifying them have probably hindered elucidation of their clinical significance (Goyache et al., 2001). *Lactococcus lactis* ssp. *lactis* and *Lc. garvieae* are now recognized as species with clinical significance for human and veterinary medicine, including bovine IMI associated with mastitis (Werner et al., 2014). *Lactococcus lactis* has been included in the Qualified Presumption of Safety list of the European Food Safety Authority. It is increasingly found as the cause of human or animal infections, probably due to better identification of infective microorganisms. Under selective pressure, resistance to antibiotics is a common characteristic of bacteria. In the interaction among bacteria, genetic material may be transferred from one bacterium to another, and genes coding for resistance to several antibiotics may be passed on to other bacterial species. *Lactococcus* may acquire antibiotic resistance

and survive antimicrobial treatments, and can subsequently act as a reservoir for antibiotic resistance genes for other bacteria (Walther et al., 2008). Because LAB are natural and beneficial inhabitants in many environments (gastrointestinal tract, several foods), strains with antibiotic resistance would not be detrimental to the wellbeing of humans or animals. However, there is some concern that antibiotic resistance in LAB could be transferred to potentially pathogenic bacterial species, complicating the treatment of a disease or infection and leading to the spread of antibiotic-resistant bacteria. Most LAB strains were found to be susceptible to all antibiotics used in past studies. The most frequently found resistance was against tetracycline, followed by resistance to aminoglycosides (Korhonen, 2010). Elliott and Facklam (1996) noted differences in antimicrobial susceptibility between *Lc. lactis* and *Lc. garvieae*.

The aim of this study was to examine the MIC of 17 antimicrobials in 46 *Lc. lactis* ssp. *lactis* and 47 *Lc. garvieae* isolated from bovine IMI. Moreover, the genotypic characteristics of these bacterial isolates were examined to assess their diversity.

MATERIALS AND METHODS

Herds

The isolates examined in this study were from 2 herds (farms A and B) with problems of chronic mastitis, repeat clinical mastitis cases, and individual cows with high SCC due to streptococci-like organisms.

Farm A, a Holstein Friesian herd with 1,693 lactating cows housed in freestalls, had an average daily milk production of 45 kg/cow and a bulk tank SCC average of 268,000 cells/mL. From May 1, 2013, through March 31, 2014, the herd exhibited a high prevalence of chronic infections (13%) as determined by 2 or more consecutive monthly DHIA test-day linear scores >4.0 (SCC ≥200,000 cells/mL). The prevalence of new infections (new infection risk) was 8.7%. The new infection risk was determined by the number of cows each test day with a current test-day linear score >4.0 and a previous test-day linear score <4.0. Bedding in all lactating groups was mechanically recycled sand except in the fresh cow pen, where fresh sand was used. Lame cows were housed on a composted pack (wood shavings). Dry cows were housed on recycled sand and pre-fresh heifers were bedded with straw. Feed consisted of a TMR of corn silage, alfalfa haylage, wheat straw, corn grain, various commodities, and protein mix. Cows were milked in a 50-cow rotary parlor.

Farm B, a Holstein Friesian herd with 914 lactating cows housed in freestalls, had an average daily milk production of 39 kg/cow and a bulk tank SCC average of 365,000 cells/mL. From May 1, 2013, through March 31, 2014, the herd exhibited a high prevalence of chronic infections (21.7%), the new infection risk was 8.8%, as described for farm A, and the prevalence of new infections was 8.8% of the total lactating cows. Bedding was recycled sand (sand lanes) in all lactating groups, corn stalk pack in the prefresh group, and sand for dry cows. Feed was TMR of corn silage, alfalfa haylage, grass hay, corn grain, various commodities, and protein mix. Cows were milked in a double-20 parallel parlor.

Milk Sampling

Before sampling, teat ends were carefully cleaned and disinfected with iodine followed by alcohol. The first streams of foremilk were discarded, and then approximately 10 mL of milk was collected aseptically from each teat into sterile vials. Samples were stored at 4°C until bacteriological assays and SCC tests were initiated, immediately after arrival back to the veterinary clinic.

Bacteriological Analysis and Isolates

The veterinary clinic submitted milk samples from May 2013 through November 2013 to Quality Milk Production Services (QMPS, Cornell University, Ithaca, NY), from which 181 isolates were found and identified as environmental "other" streptococci (meaning not *Enterococcus* spp., *Streptococcus uberis*, or *Streptococcus dysgalactiae*).

Bacteriological cultures were performed according to standards of the National Mastitis Council (NMC, 1999). Ten microliters of each milk sample was spread on blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and examined after 24 h. Colonies were provisionally identified based on Gram stain, morphology, and hemolysis patterns, and the numbers of each colony type were recorded. Representative colonies were then subcultured on blood agar plates and incubated again at 37°C for 24 h to obtain pure cultures. Gram-positive, catalase-negative cocci were further evaluated using esculin hydrolysis, hemolysis, and Christie, Atkins, Munch-Petersen (CAMP) reaction.

Molecular Identification of Isolates

Polymerase chain reaction and sequence analysis of 16S rDNA and *rpoB* were used for molecular identification of all isolates. For these analyses, a crude cell DNA lysate was first prepared for each isolate (Furrer et al., 1991). Briefly, pure culture streaks were made

on tryptic soy agar with 5% sheep blood plate (Becton Dickinson, Sparks, MD) and incubated for 18 to 24 h at 37°C. One isolated colony was transferred to a 1.5-mL tube of Todd Hewitt broth (Becton Dickinson) and incubated for 18 to 24 h at 37°C. Then, 250 µL of each broth culture was transferred to 1.5-mL microcentrifuge tubes and centrifuged to pellet for 10 min at 6,000 × g. The supernatant was removed and discarded, and the pellet was resuspended in 95 µL of 1× PCR buffer (Roche Applied Science, Indianapolis, IN). A 4-µL aliquot of freshly prepared 50 mg/mL lysozyme (Promega, Madison, WI) solution was added to this suspension and mixed well by pipetting to result in a final concentration of 2 mg/mL lysozyme. After a 15-min incubation at room temperature, 1 µL of a 20 mg/mL proteinase K (Promega) solution was added to the suspension and mixed, resulting in a final concentration of 200 µg/mL proteinase K. The tubes were incubated up to 2 h in a heat block set at 58°C until the lysates cleared. The enzymes were inactivated for 8 min at 95°C. After equilibrating to room temperature, tubes were centrifuged for 3 s at 6,000 × g to remove condensate from the sides and lids. The crude cell lysates containing the DNA were stored at −20°C and used directly for further analysis. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine DNA quantity.

Two PCR were performed to amplify 2 different gene targets: rpoB (Drancourt et al., 2004) and 16S rDNA (Greisen et al., 1994). A region of the rpoB gene was amplified using the primer pair rpoBF (5′-AARYTIGGMCCTGAAGAAAT-3′) and rpoBR (5′-GCCTTTAACTTCAGACTTATCA-3′). The 16S rDNA gene target was amplified using the primer pair DG74 (5′-AGGAGGTGATCCAACCGCA-3′) and P5SH (5′-TGAAGAGTTTGATCMTGGCTCAG-3′). All primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The PCR amplification mixtures for both assays were carried out in a total volume of 50 µL, consisting of 0.25 µL of 100 µM forward and reverse primer, 25.00 µL of GoTaqGreen (Promega), 27.50 µL of H_2O, and 2.0 µL of cell DNA lysate suspension. All PCR amplifications were performed using the MyCycler Thermal Cycler (Bio-Rad, Hercules, CA). The rpoB gene target was amplified using the following thermal conditions: initial denaturation step of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; and a final 7-min extension at 72°C. The 16S rDNA gene target was amplified using the following thermal conditions: initial denaturation step of 4 min at 94°C; 25 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C; and a final 5-min extension at 72°C. The PCR products were analyzed by agarose gel electrophoresis. The expected amplicon size was 740 bp for rpoB and 1,040 bp for 16S rDNA.

Clustering

In preparation for sequencing, excess primers and nucleotides were removed from each sample by treatment with ExoSAP-IT (USB Corporation, Cleveland, OH). Sequencing of each sample was performed in 2 directions using Big Dye Terminator chemistry on an ABI Prism 3700 DNA analyzer (Applied BioSystems, Foster City, CA). Sequences were proofread in SeqMan (version 5.08, Lasergene; DNAStar Inc., Madison, WI), compared with publicly available sequence data using the National Center for Biotechnology Information (NCBI) BLAST programs (Altschul et al., 1997) and identified using interpretive criteria provided by the Clinical and Laboratory Standards Institute (CLSI, 2008).

Randomly Amplified Polymorphic DNA-PCR Analysis

To further characterize the mastitis isolates, randomly amplified polymorphic DNA (RAPD)-PCR analysis was carried out using 3 primers: P16 (5′-TC-GCCAGCCA-3′), P17 (5′-CAGACAAGCC-3′), and P2 (5′-GATCGGACGG-3′). The PCR reaction was performed in a total volume of 25 mL, containing 1 unit of GoTaq DNA polymerase (Promega), 10 pmol of primer, 200 mM of each dNTP, 3 mM $MgCl_2$, and 50 ng of genomic DNA. Amplifications were performed as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 24°C for 2 min, and 72°C for 2 min; final extension at 72°C for 5 min. Amplification products were electrophoretically separated in 1% agarose gels containing SYBR safe DNA gel stain (Life Technologies Ltd., Paisley, UK). Gels were visualized and photographed using the Gel Doc UV transilluminator 2000 (Bio-Rad Laboratories Inc., Hercules, CA). Two replications of each sample and primer were performed to increase the reproducibility of the technique. The DNA fingerprint patterns obtained with the 3 primers were analyzed using GelCompar II (Applied Maths NV, Sint-Martens-Latem, Belgium). Similarity among isolates was determined by means of the Dice comparison and the clustering determined by the unweighted average pair group algorithm.

Antimicrobial Susceptibility Testing

The MIC of 17 antimicrobials were determined for 46 Lc. lactis ssp. lactis and from 47 Lc. garvieae (1 isolate from farm A did not grow when recultured) isolates

using the broth dilution test, according to the procedure described in Clinical and Laboratory Standards Institute (**CLSI**) guidelines VET01-A4 (CLSI, 2013a). A commercially available microdilution MIC system, the Sensititer Compan2F Plate Format veterinary panel and Sensititer ARIS system (Trek Diagnostics Systems, Cleveland, OH), was used. Results were interpreted using CLSI resistance breakpoints according to VET01-S2 guidelines (CLSI, 2013b). When present, specific resistance breakpoints for enterococci or streptococci species were used. For cefpodoxime, the only resistance breakpoint cited was the one for canine wounds. The resistance breakpoint used for ceftiofur was for cattle mastitis organisms. For fluoroquinolones (enrofloxacin and marbofloxacin) and trimethoprim-sulfamethoxazole, we used the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical resistance breakpoints respectively for ciprofloxacin and trimethoprim-sulfamethoxazole (clinical breakpoints tables, version 4.0; EUCAST, 2014), because of the lack of specific breakpoints for streptococci in the CLSI veterinary guidelines. For clindamycin, the only resistance breakpoints available were those established by the European Food Safety Authority (EFSA, 2012) for the assessment of microorganism used as feed additives. The Sensititer plate reading was performed manually, recording the last concentration of antimicrobial that did not show turbidity or deposit of cells at the bottom of the well.

RESULTS

On farm A, 130 streptococci were isolated. After DNA sequencing, specific identification included 40 (31%) *Lc. lactis* ssp. *lactis*, 27 (21%) *Lc. garvieae*, 5 (4%) *Lc. lactis*, 6 (5%) *Lactococcus* spp., 37 (28%) *Enterococcus saccharolyticus*, 5 (4%) *Streptococcus uberis*, 4 (3%) *Streptococcus dysgalactiae* ssp. *dysgalactiae*, and 6 (5%) others (Table 1).

On farm B, 51 streptococcal organisms were isolated. After DNA sequencing, specific identification included 6 (12%) *Lc. lactis* ssp. *lactis*, 21 (41%) *Lc. garvieae*, 1 (2%) *Lc. lactis*, 1 (2%) *Lactococcus* spp., 18 (35%) *Enterococcus saccharolyticus*, 1 (2%) *Streptococcus uberis*, 1 (2%) *Streptococcus dysgalactiae* ssp. *dysgalactiae*, and 2 (4%) others.

Genotypic Characterization

Three primers were used to create fingerprint patterns from the total genome of 46 *Lc. lactis* ssp. *lactis* and 48 *Lc. garvieae* isolates collected from farms A and B. Similarity and clustering results were based on 2 rep-

licates. The results showed slightly higher heterogeneity for *Lc. lactis* ssp. *lactis* isolates than for *Lc. garvieae* (Figure 1 and Figure 2), even though most of the *Lc. lactis* ssp. *lactis* isolated came from only one farm (farm A). At 75% similarity, *Lc. garvieae* isolates separated in one large cluster that included 81% of the isolates, with 54% of those being identical at the conditions chosen for the comparison. *Lactococcus lactis* ssp. *lactis* isolates, at 75% similarity, separated in 2 large clusters, the larger one comprising 50% of the isolates (cluster A) and the slightly smaller cluster including 38% of the isolates (cluster B). In cluster A, 33% were identical. For both species, only a few isolates did not cluster together with any other isolate and thus can probably be considered different strains (Figure 3 and Figure 4). Thus, from 48 *Lc. garvieae* isolates, genotyping analysis showed 4 strains or isolates with low similarity, whereas 6 strains from *Lc. lactis* ssp. *lactis* were found from the 46 original isolates. Nine isolates were excluded due to unreproducible fingerprints.

Antimicrobial Susceptibility Testing

The results of the antimicrobial resistance patterns of *Lc. lactis* ssp. *lactis* are given in Table 2. For the β-lactam antibiotics widely used in clinical practice (penicillin, ampicillin, and amoxicillin), no resistant isolates were found and all MIC were far from the resistance breakpoints. Eight strains had a MIC intermediate to cefazolin, a first-generation cephalosporin. Among the isolates that showed resistance to one or more antimicrobials, 44 were resistant to rifampin, 6 to trimethoprim-sulfamethoxazole, 1 to erythromycin and clindamycin, and 1 to amikacin. The number of isolates displaying multidrug resistance patterns was very low: only 1 isolate was resistant to 3 antibiotics (erythro-

**Table 1.** Number (% in parentheses) of mastitis pathogens after DNA sequencing

Species	Farm A	Farm B
Lactococcus spp.	6 (5)	1 (2)
Lactococcus lactis	5 (4)	1 (2)
Lactococcus lactis ssp. *lactis*	40 (31)	6 (12)
Lactococcus garvieae	27 (21)	21 (41)
Aerococcus viridans	2	0
Enterococcus aquimarinus	1	0
Enterococcus faecalis	1	1
Enterococcus saccharolyticus	37 (28)	18 (35)
Streptococcus uberis	5 (4)	1 (2)
Streptococcus dysgalactiae ssp. *dysgalactiae*	4 (3)	1 (2)
Streptococcus suis	0	1
Streptococcus acidominimus	1	0
Pseudochrobactrum spp.	1	0
Total	130	51
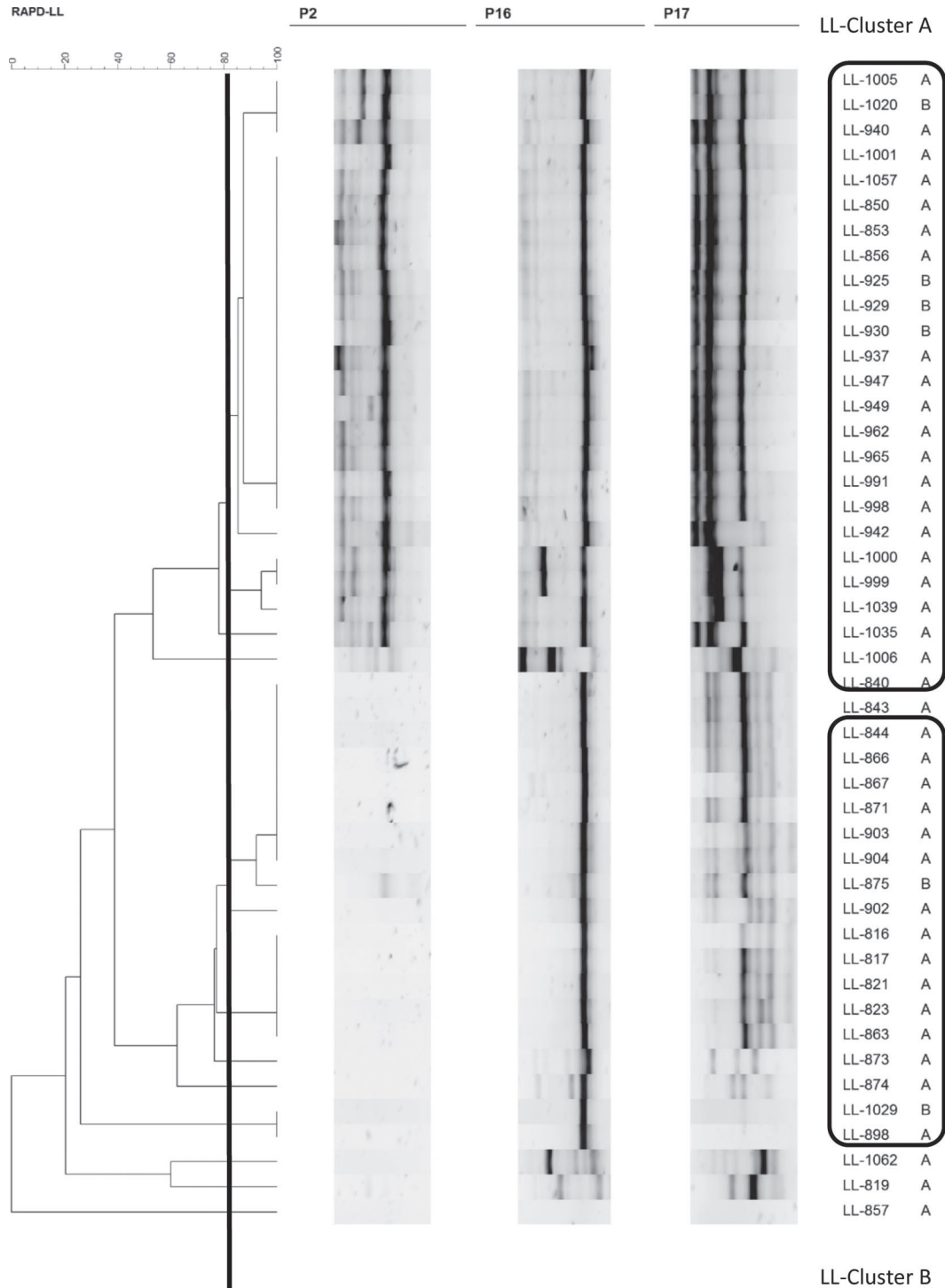
Figure 1. Dendrogram analysis of 46 *Lactococcus lactis* (LL) ssp. *lactis* isolates based on their genotypic patterns, which were clustered by the unweighted average pair group algorithm. P2, P16, and P17 indicate different primers used. RAPD = random amplified polymorphic DNA.

mycin, rifampin, and trimethoprim-sulfamethoxazole), 4 isolates were resistant both to rifampin and trimethoprim-sulfamethoxazole, and 1 isolate was resistant to amikacin and trimethoprim-sulfamethoxazole.

The antimicrobial resistance patterns of *Lc. garvieae* are displayed in Table 3. The major differences with *Lc. lactis* ssp. *lactis* were the presence of a high number of strains (44) resistant to trimethoprim-sulfamethoxazole
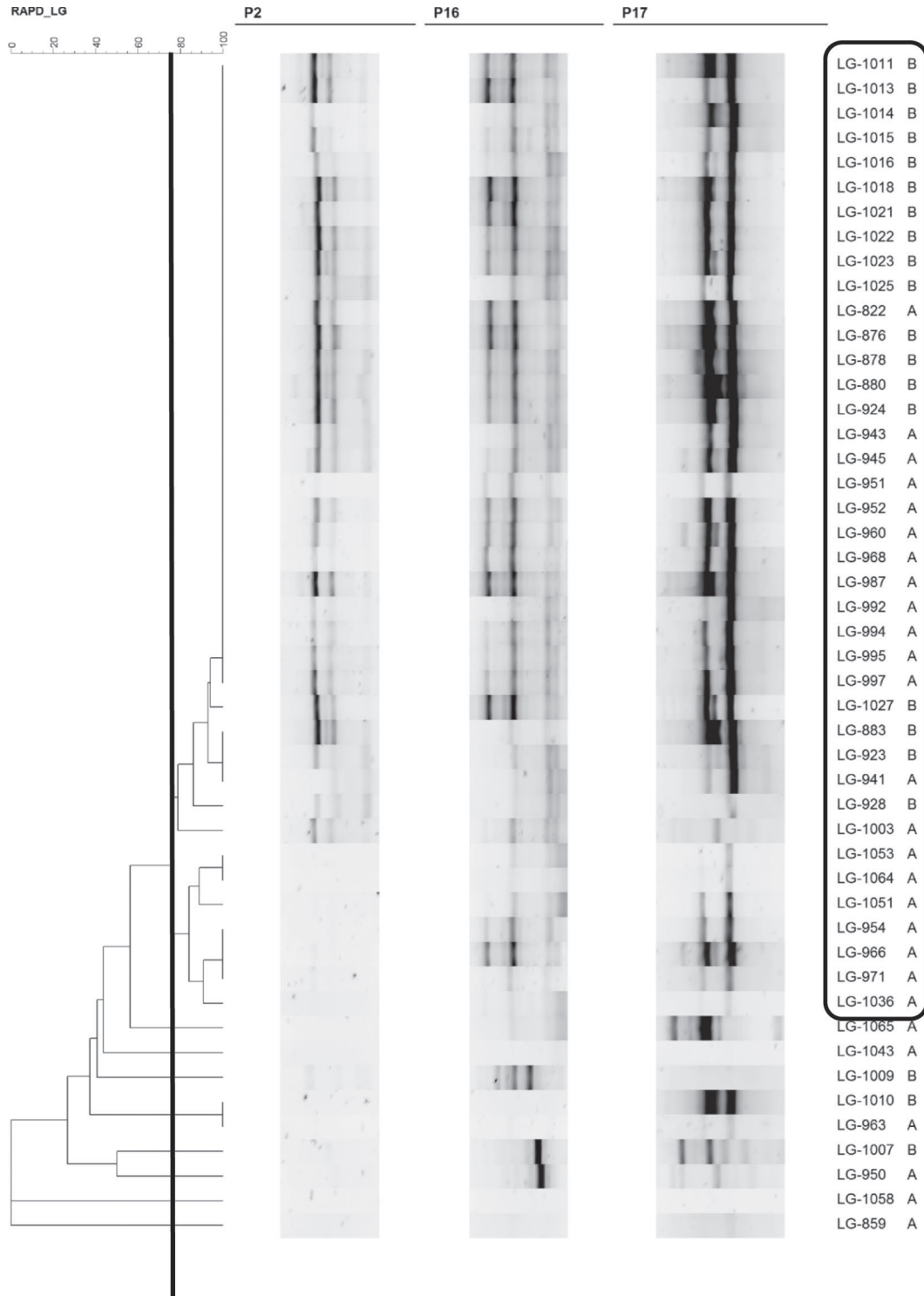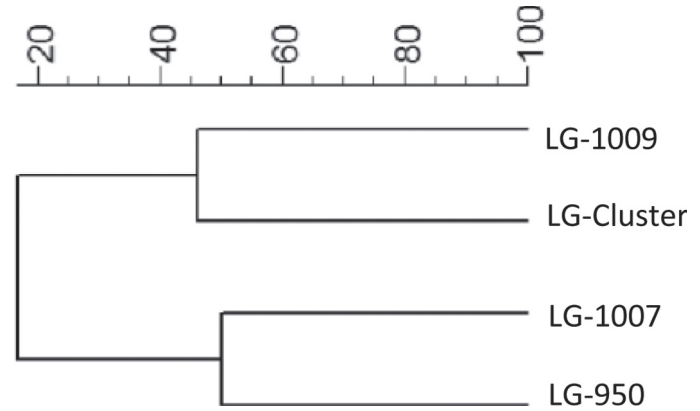
Figure 2. Dendrogram analysis of 48 *Lactococcus garvieae* (LG) isolates based on their genotypic patterns, which were clustered by the unweighted average pair group algorithm. P2, P16, and P17 indicate different primers used. RAPD = random amplified polymorphic DNA.

and the presence of 2 isolates resistant to cefazolin and 1 to chloramphenicol. *Lactococcus garvieae* also differed greatly in susceptibility to clindamycin. Most of the *Lc.* *garvieae* isolates (43 out of 47) were resistant to this antibiotic and all were susceptible to erythromycin, the other macrolide antibiotic tested. The great majority
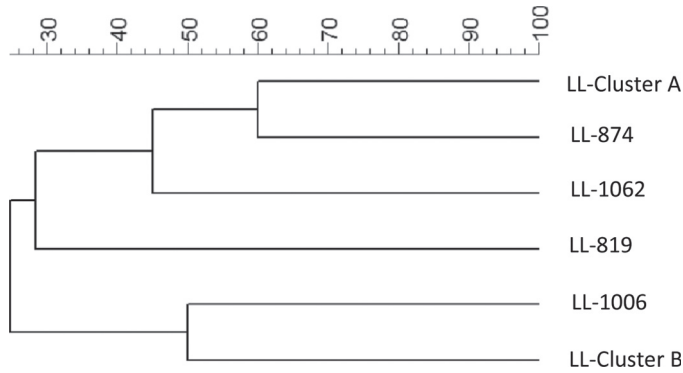
Figure 3. Dendrogram analysis of *Lactococcus lactis* (LL) strains. The LL clusters contain the following isolates: (A) 1005, 1020, 940, 1001, 1057, 850, 853, 856, 925, 929, 930, 937, 947, 949, 962, 965, 991, 998, 942, 1000, 999, 1039, 1035; (B) 840, 843, 844, 866, 867, 871, 903, 904, 875, 902, 816, 817, 821, 823, 863, 873 (some isolates were excluded due to low reproducibility).

Figure 4. Dendrogram analysis of the *Lactococcus garvieae* (LG) strains. The LG cluster contains the following isolates: 1011, 1013, 1014, 1015, 1016, 1018, 1021, 1022, 1023, 1025, 822, 876, 878, 880, 924, 943, 945, 951, 952, 960, 968, 987, 992, 994, 995, 997, 1027, 883, 923, 941, 928, 1003, 1053, 1064, 1051, 954, 966, 971, 1036, 1010, 963 (some isolates were excluded due to low reproducibility).

of *Lc. garvieae* isolates were resistant to rifampin as the isolates of *Lc. lactis* ssp. *lactis*. Regarding multidrug resistance patterns, 39 strains were resistant to both rifampin and trimethoprim-sulfamethoxazole, 2 isolates were resistant to 3 antimicrobials (rifampin, trimethoprim-sulfamethoxazole, cefazolin), and 2 isolates coupled resistance to rifampin and trimethoprim-sulfamethoxazole, respectively, with resistance to chloramphenicol and amikacin.

Table 2. Minimum inhibitory concentrations of the 17 antimicrobial agents tested for 46 *Lactococcus lactis* ssp. *lactis* isolates[1]

Antimicrobial	Breakpoint[2]	MIC (µg/mL)										
		0.06	0.12	0.25	0.50	1	2	4	8	16	32	64
Penicillin	a	2	1	17	26							
Ampicillin	a		8	38								
Amoxicillin/clavulanic acid (2:1 ratio)	b		44	2								
Imipenem	c					45	1					
Cephalothin	c						45	1				
Cefazolin	c					3	35	8				
Cefpodoxime	d						46					
Ceftiofur	e				46							
Trimethoprim/sulfamethoxazole	f					40	2	2	2			
Amikacin	c							38	5	1	1	1
Gentamicin	c					42	3	1				
Erythromycin	a					45		1				
Clindamycin	g					45		1				
Enrofloxacin	f			2	34	8	2					
Marbofloxacin	f			1	1	39	4	1				
Chloramphenicol	a								38	8		
Rifampin	a						2	44				

[1]Resistance breakpoints are highlighted by dark gray shading; intermediate breakpoints are highlighted by light gray shading; cells without shading indicate that no breakpoints were available in the literature.

[2]Where a = Clinical and Laboratory Standards Institute (CLSI) resistance breakpoint for enterococci; b = CLSI resistance breakpoint for other organisms; c = CLSI resistance breakpoint for all microorganisms; d = CLSI resistance breakpoint for dog wounds; e = CLSI resistance breakpoint for cattle mastitis; f = European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for enterococci; g = European Food Safety Authority (EFSA) resistance breakpoint (EFSA, 2012).

Table 3. Minimum inhibitory concentrations of the 17 antimicrobial agents tested for the 47 *Lactococcus garvieae* isolates[1]

Antimicrobial	Breakpoint[2]	MIC (µg/mL)									
		0.12	0.25	0.50	1	2	4	8	16	32	64
Penicillin	a	1	3	20	23						
Ampicillin	a	2	12	31	2						
Amoxicillin/clavulanic acid (2:1 ratio)	b	5	12	27	3						
Imipenem	c				46	1					
Cephalothin	c					4	22	21			
Cefazolin	c				5	23	17	2			
Cefpodoxime	d					47					
Ceftiofur	e		47								
Trimethoprim/sulfamethoxazole	f			3		12	32				
Amikacin	c						32	12	2		1
Gentamicin	c				41	5	1				
Erythromycin	a			47							
Clindamycin	g			2		2	10	33			
Enrofloxacin	f		3	10	31	3					
Marbofloxacin	f		1	4	32	10					
Chloramphenicol	a						44	2	1		
Rifampin	a					2	45				

[1]Resistance breakpoints are highlighted by dark gray shading; intermediate breakpoints are highlighted by light gray shading; cells without shading indicate that no breakpoints were available in the literature.

[2]Where a = Clinical and Laboratory Standards Institute (CLSI) resistance breakpoint for enterococci; b = CLSI resistance breakpoint for other organisms; c = CLSI resistance breakpoint for all microorganisms; d = CLSI resistance breakpoint for dog wounds; e = CLSI resistance breakpoint for cattle mastitis; f = European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for enterococci; g = European Food Safety Authority (EFSA) resistance breakpoint (EFSA, 2012).

DISCUSSION

In recent years, several herds have experienced problems with mastitis cases that did not respond well to treatment and with high bulk tank SCC. Although *Lactococcus* had previously been isolated, it was unclear whether the number of cases observed indicated an increase in disease incidence and growing emergence as a pathogen or whether newer molecular testing allowed identification of lactococcal infections that would previously have been grouped in a simpler classification system of *Streptococcus* species. The latter was certainly possible, because lactococci are often misidentified as other streptococci-like bacteria based on traditional culture and biochemical techniques (Barrett, 2013). Molecular testing such as PCR and sequencing-based methods provide unequivocal identification of pure cultures of *Lc. lactis* ssp. *lactis* and *Lc. garvieae* originating from bovine IMI cases (Werner et al., 2014).

Establishing and interpreting the MIC of these pathogens could be helpful when designing effective treatment protocols to control and eliminate infections. The results of the MIC tests performed in this study agree with those of other studies that found both *Lc.*

lactis ssp. *lactis* and *Lc. garvieae* to be susceptible to β-lactam antibiotics (De Fabrizio et al., 1994; Flórez et al., 2007; Walther et al., 2008). The resistance displayed by almost all the isolates of *Lc. garvieae* has been described as intrinsic to this species (Walther et al., 2008) and has been proposed as a selective criterion for differentiation between *Lc. lactis* ssp. *lactis* and *Lc. garvieae* (Elliott and Facklam, 1996). High resistance of *Lc. lactis* ssp. *lactis* against rifamycins was recorded in previous studies (Orberg and Sandine, 1985; Mathur and Singh, 2005) and confirmed in our study. Furthermore, our results indicated that *Lc. garvieae* displayed a high resistance toward rifamycins, with the 96% of isolates being resistant to rifampin. A greater level of resistance toward trimethoprim plus sulfonamides was found in *Lc. garvieae* isolates compared with the results of a previous investigation (Walther et al., 2008) performed in Switzerland on 31 isolates from mastitis cases: 27 had a MIC below 0.5/9.5 µg/mL, whereas 32 isolates in our study had a MIC above 2/38 µg/mL.

The application of molecular fingerprint methods such as RAPD-PCR allowed us to study genetic differences in isolates and identify isolates at the strain-specific level. The results showed that the isolates

originated from 6 distinct strains of *Lc. lactis* and 4 distinct strains of *Lc. garvieae.* Most of the *Lc. lactis* were rifampin resistant. Isolate LL-1006, a distinctive strain, was resistant to amikacin and trimethoprim-sulfamethoxazole, and strain LL-1062 was resistant to trimethoprim-sulfamethoxazole. On the other hand, most of the *Lc. garvieae* isolates were resistant to rifampin and trimethoprim-sulfamethoxazole. It is not uncommon to find horizontal gene transfer of a plasmid encoding trimethoprim-sulfamethoxazole resistance (Kateete et al., 2013). However, Devirgiliis et al. (2013) suggested that most lactococcal species display intrinsic resistance to trimethoprim. Whether resistance to this particular antibiotic in these isolates was due to horizontal gene transfer or is intrinsic is unknown and remains to be seen in future investigations.

The goal of this study was to lay a foundation of knowledge to support future investigations in the management and treatment strategies of mastitis caused by *Lc. lactis* and *Lc. garvieae.* The fact that these organisms did not display in vitro resistance to antibiotics commonly used to treat dairy cattle IMI implies that they might be susceptible and thus successfully treated on-farm in vivo; however, this remains to be tested. Antimicrobial resistance profiling along with accurate identification of these pathogens will enable targeted and early intervention for reduction of chronic IMI.

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