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Application of colony BOXA2R-PCR for the differentiation and identification of lactic acid COCCI

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ABSTRACT

Repetitive-PCR (rep-PCR) is a well-established genetic method for bacterial strain fingerprinting that is used mostly with REP, ERIC, $(GTG)_5$, BOXA1R and occasionally BOXA2R repetitive primers. In this study, it was demonstrated that BOXA2R-PCR could effectively discriminate between *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus thermophilus*; differentiate *Lactococcus lactis* strains and subspeciate them into *lactis* and *cremoris* in a single reaction; generate unique strain fingerprints of various lactic acid bacteria (LAB species) commonly isolated from fermented dairy products, including occasional spoilage bacteria and yeasts. Furthermore, using direct colony PCR a reproducible and rapid method was developed for the differentiation and identification of lactic acid cocci. The simplicity and speed of this microbial identification method has potential practical value for dairy microbiologists, which was demonstrated through a microbiota investigation of select Australian retail dairy products.

1. Background

Various fermented food and feed products and probiotics contain diverse species and strains of LAB, which contribute to their nutritional, organoleptic, preservation or health-promoting properties. Industrially manufactured dairy products typically use defined starter cultures, while artisanal products, such as the traditional raw milk cheeses (Callon et al., 2004), (Terzic-Vidojevic et al., 2007) represent a rich source of complex natural microbiota. To genetically analyze the composition of complex bacterial communities, a variety of culture-independent techniques have been developed, these include PCR-DGGE, SSCP, LH-PCR (Ndoye et al., 2011).

Lactococcus lactis subsp. *lactis* and subsp. *cremoris* are used as starter cultures for fermented dairy products, in particular cheese. Species identification, subspecies determination and strain differentiation of *Lactococcus lactis,* the main starter culture used in cheese production, usually involve two stages and two sets of methods (Odamaki et al., 2011), (Cavanagh et al., 2015). Presumptive lactococci are initially identified by 16S rRNA analysis (Cavanagh et al., 2015). The amplification of the 16S rRNA genes followed by partial DNA restriction analysis (partial ARDRA) of the amplicons has previously been used to identify isolates as belonging to *Lactococcus lactis* (Fernandez et al., 2011). PCR-based methods with subsequent restriction endonuclease digestion of the products has been used to detect species- and subspecies-specific sequence polymorphisms in *gadB* (glutamate decarboxylase) gene (Nomura et al., 2002), the histidine biosynthesis operon (Cavanagh et al., 2015) or 16S rRNA gene (Pu et al., 2002). If the strain DNA is resistant to digestion with a particular restriction enzyme or if digestion profiles of amplicons with one enzyme are identical, then more than one enzyme will be required. However, these methods do not provide any differential information at the strain level. Conversely, application of a typing technique that is highly discriminatory at the strain level does not infer any genus or species information (Delgado and Mayo, 2004). Restriction fragment length polymorphism (RFLP) typing by PFGE has been used to evaluate strain diversity and relatedness (Fernandez et al., 2011). The use of multiple enzymes in RFLP-PFGE increases the technique's discriminatory power but renders the method technically complicated and time-consuming (Fernandez et al., 2011). MLST analysis of the genetic diversity of *Lactococcus lactis* based on analysis of partial nucleotide sequences of seven house-keeping protein encoding genes (Cavanagh et al., 2015), (Fernandez et al., 2011), (Rademaker et al., 2007) has consistently clustered strains into *lactis* and *cremoris* genotypes. However, the disadvantage of this method is “the substantial costs for reagents, equipment and labour to complete the requisite amplification, sequencing, editing and the concatenating of the multiple housekeeping genes” (LiPuma, 2015).

RAPD and repetitive-PCR are two genetic methods that enable fast and inexpensive fingerprinting. The RAPD method using the 10-mer

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arbitrary primer has been assessed as generally not appropriate for species identification (Psoni et al., 2007) with the major issue being that it is not sufficiently discriminatory for *Lc. lactis* ssp *cremoris* (Corroler et al., 1998). It also requires the use of more than one primer (Psoni et al., 2007), (Prodelalova et al., 2005) and displays a limited capability to distinguish between genetically related strains due to the low number of amplicons obtained with most RAPD primers (Delgado and Mayo, 2004).

Repetitive PCR (rep-PCR) fingerprinting technique enables the amplification of DNA fragments of different sizes located between interspersed repetitive sequences and was originally developed to distinguish strains of diverse Gram-negative species (Versalovic et al., 1994). The BOX family of repetitive DNA elements identified in Gram-positive *Streptococcus pneumonia*e consists of different combinations of three subunits, boxA, boxB, and boxC (Martin et al., 1992), (Koeuth et al., 1995). Only the BOXA-like subunit sequences appear to be highly evolutionary conserved among both Gram-positive and Gram-negative bacteria (Koeuth et al., 1995). BOXA1R and BOXA2R primers are based on the *boxA* sequence and have been used in rep-PCR amplification of DNA from many bacterial species (Koeuth et al., 1995).

This work aimed to investigate the applicability of the repetitive-PCR method using the single oligonucleotide primer BOXA2R for the genetic identification of species, sub-species and strains of *Lactococcus lactis*. Our interest in exploring the use of BOXA2R primer was based on an initial evaluation of several repetitive primers, which indicated that BOXA2R primer produced the most informative fingerprints for lactic acid cocci. Cultures from various Australian dairy products were then isolated to test its usefulness for fingerprinting of diverse dairy microbial isolates.

2. Materials and Methods

2.1. Strains

Thirteen dairy products purchased from retail stores in Sydney, NSW, Australia in the first half of 2016 (see Table 1) were analyzed. Out of one-hundred and six strains isolated from these products and initially screened, forty-four unique isolates were included in this study: thirty-four LAB-, four non-LAB bacteria and six yeasts. Additionally, nine *Lactococcus lactis* ssp *lactis* biovar *diacetylactis* and six *Leuconostoc mesenteroides* strains were isolated from the mesophilic starter type culture Flora Danica (FD). Reference strains and *Lc. lactis* laboratory strains were sourced from our internal culture collection (see Table 2). The total number of tested strains was eighty-one.

2.2. Methods

Strain isolation. Dairy samples (10 g or 10 ml) were homogenized in 90 ml sterile 2% (w/v) trisodium citrate pre-warmed to 45 °C (cheese and sour cream samples) or 0.9% (w/v) sodium chloride (buttermilk and yoghurt samples). Serial 10-fold dilutions of the suspensions in 0.9% (w/v) sodium chloride were thoroughly mixed by vortexing and 0.1 ml of the appropriate successive dilutions were spread plated onto the following media: M17 agar (Oxoid) with 0.5% (w/v) lactose (LM17), MRS agar (Oxoid) and KMK citrate agar (Kempler and McKay, 1980). LM17 plates were incubated anaerobically for 24–48 h at 30 °C and 37 °C, and aerobically at 45 °C; MRS plates were incubated anaerobically at 37 °C and KMK plates at 30 °C. Within two days following the incubation, the plates were inspected for the presence of microbial growth. Morphologically distinct colonies were purified by re-streaking, Gram-stained and stocked as broth cultures (M17 with 0.5% (w/v) glucose or MRS broth) with the addition of 15% (v/v) glycerol or in autoclaved 9.5% (w/v) milk. Yeast colonies, which were occasionally observed and picked from LM17 or MRS agar media, were plated on Sabouraud dextrose agar (Oxoid) and stocked in 30% (v/v) glycerol. All stocks were stored at −80 °C.

Table 1
The list of dairy products used for strain isolation.

Products	Type of product	Made in
Bega Farmers' Tasty Cheese	cheese	Australia
Lemnos Cheese Fetta	cheese	Australia
Riverina Full Cream Australian Fetta cheese	cheese	Australia
Danish Fetta Cheese	cheese	Australia
Henry Willig's Cow Cheese with Herbs	cheese	Holland
Bulla Lite'n Healthy Plain Yoghurt	yoghurt	Australia
CHTAURA Natural Set Yoghurt	yoghurt	Australia
Bekaa Natural Set Yoghurt	yoghurt	Australia
Green Valley Dairy Goat Yoghurt	yoghurt	Australia
"Paprika u pavlaci" (Bell peppers stuffed with sour cream)	sour cream	Serbia
Weight Watchers Extralight Sour Cream	sour cream	Australia
Dairy Farmers Buttermilk	buttermilk	Australia
Sharma's kitchen Mango Lassi	yoghurt smoothie	Australia

Colony-PCR with BOXA2-R primer. PCR reactions were performed in puReTaq™Ready-To-Go™ Polymerase Chain Reaction (PCR) Beads (GE Healthcare). This PCR premix contained a final concentration of ~2.5 units puReTaq DNA polymerase, 200 µM of each dNTP in 10 mM Tris-HCl (pH 9), 50 mM potassium chloride and 1.5 mM magnesium chloride. To each reaction, DNAse RNAse free water (MP Biomedicals) was added to re-suspend the bead followed by addition of 50 pmol of the single BOXA2R primer (Sigma Oligos), which has the oligonucleotide sequence, 5′-ACGTGGTTTGAAGAGATTTTCG-3' (Koeuth et al., 1995). Colonies of representative morphologies were picked directly from a plate with a sterile inoculation loop (blunt side) and mixed into the final 25 µl volume of the PCR mixture. Care was taken to avoid colony overloading in order to generate optimally resolved fragments and clear fingerprints. The finding that vegetative cells can be substituted for genomic DNA as a template for PCR has been reported earlier (Nomura et al., 2002), (Versalovic et al., 1994). PCR amplifications were performed in an automated thermal cycler (Eppendorf), with the lid preheated to 105 °C before the start of the reaction. The negative control reaction had water substituted for the template DNA. The PCR cycling program included an initial denaturation step (95 °C, 7 min) and then 35 cycles of denaturation (90 °C, 30 s), annealing (40 °C, 1 min), and extension (65 °C, 8 min), followed by one final extension step at 65 °C for 16 min (Malathum et al., 1998).

Following amplification, 2.5 µl loading buffer was added to each PCR tube. The amplification products (5 µl) were electrophoresed on 15 × 20 cm 1.5% (w/v) molecular grade agarose (Bio-Rad) gels in 1 × TAE (Tris-acetate, EDTA, pH8.0) at a constant 100 V for 3 h. The amplicons was assessed against the molecular size marker HyperLadder I (M I) (Bioline) or HypperLadder II (Bioline) (M II). After staining with GelRed 3 × staining solution in water (Biotium) for 30 min, the gels were photographed on a UV-transilluminator.

BOXA2R-PCR profiles were analyzed by visual inspection and by using the gel image analysis software PyElph 1.4 (Pavel and Vasile, 2012). Visual assessment of relatedness between individual isolates was carried out by firstly looking for the strongest and most distinctive bands of each isolate, followed by the observation of the presence (or absence) of the less intensive bands, their position and size relative to the marker. A phylogenetic tree generated by PyElph software was based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis applied on the computed distance matrix (Pavel and Vasile, 2012).

Reproducibility testing. Reproducibility of the BOXA2R-PCR method was tested on a total of 20 strains of target and non-target species, including ATCC type strains and wild isolates (see Table 3). Bacterial DNA used in this experiment was isolated using the two commercial products: Illustra Bacteria GenomicPrep Mini Spin Kit (GE Healthcare) (referred to as DNA1) and DNeasy Ultraclean Microbial Kit (Qiagen) (referred to as DNA2). The colonies were randomly picked

Table 2
The list of strains used in this study.

STRAIN NAME	Species	Synonym	Source	Identified by
ATCC19435^T	*Lactococcus lactis* ssp. *lactis*	DSMZ 20481; NCDO 604	Dr Wallace Bridge	
ATCC19257^T	*Lactococcus lactis* ssp. *cremoris*	DSMZ 20069; NCDO 607; HP	Dr Wallace Bridge	
CSIRO4202^T	*Leuconostoc mesenteroides* ssp. *cremoris*	ATCC19254; DSMZ20346; NCDO543	Dr Wallace Bridge	
CSIRO4301^T	*Leuconostoc mesenteroides* ssp. *dextranicum*	ATCC19255; DSMZ20484; NCDO529	Dr Wallace Bridge	
ATCC8293^T	*Leuconostoc mesen*teroides ssp. *mesenteroides*	DSMZ20343; NCDO523	Dr Wallace Bridge	
UK712	*Lc. lactis* ssp *cremoris*	NCDO712	Dr Wallace Bridge	
MG1363	*Lc. lactis* ssp *cremoris*		Dr Wallace Bridge	
E8(NZ)	*Lc. lactis* ssp *cremoris*		Dr Wallace Bridge	
ML8	*Lc. lactis* ssp *lactis*		Dr Wallace Bridge	
C2	*Lc. lactis* ssp *cremoris*		Dr Wallace Bridge	
SL894	*Lc. lactis* ssp *lactis*		Dr Wallace Bridge	
AM1	*Lc. lactis* ssp *cremoris*		Dr Wallace Bridge	
AM2	*Lc. lactis* ssp *cremoris*		Dr Wallace Bridge	
FG2	*Lc. lactis* ssp *cremoris*		Dr Wallace Bridge	
18	*Lc. lactis* ssp *lactis*		Dr Wallace Bridge	
19	*Lc. lactis* ssp *lactis*		Dr Wallace Bridge	
ET2	*Enterococcus faecium*		Dr Wallace Bridge	16S rRNA sequencing
ET5	*Enterococcus durans/faecium*		Dr Wallace Bridge	16S rRNA sequencing
ET4	*Enterococcus faecalis*		Dr Wallace Bridge	16S rRNA sequencing
BA1	*Lactobacillus paracasei*		isolated in this study	16S rRNA sequencing
BA2	*Ochrobactrum anthropi*		isolated in this study	16S rRNA sequencing
BA3	*Lactobacillus casei*		isolated in this study	16S rRNA sequencing
BA4	*Staphylococcus sp.*		isolated in this study	16S rRNA sequencing
BA5	*Lc. lactis* ssp *cremoris*		isolated in this study	16S rRNA sequencing
BA6	*Lc. lactis ssp lactis*		isolated in this study	BOXA2R-PCR/KMK
BA7	*Strep. thermophilus*		isolated in this study	BOXA2R-PCR
BA8	*Lactobacillus paracasei*		isolated in this study	16S rRNA sequencing
BA9	*Staphylococcus warnerii*		isolated in this study	16S rRNA sequencing
BA10	*Staphylococcus epidermidis*		isolated in this study	16S rRNA sequencing
BA11	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA12	*Streptococcus thermophilus*		isolated in this study	16S rRNA sequencing
BA13	*Lactobacillus fermentum*		isolated in this study	16S rRNA sequencing
BA14	*Lactobacillus paracasei*		isolated in this study	16S rRNA sequencing
BA15	*Lactobacillus casei*		isolated in this study	16S rRNA sequencing
BA16	*Lactobacillus plantarum*		isolated in this study	16S rRNA sequencing
BA17	*Lactobacillus fermentum*		isolated in this study	16S rRNA sequencing
BA18	*Streptococcus thermophilus*		isolated in this study	16S rRNA sequencing
BA19	*Lactobacillus delbrueckii* ssp. *bulgaricus*		isolated in this study	16S rRNA sequencing
BA20	*Lactobacillus delbrueckii* ssp. *bulgaricus*		isolated in this study	16S rRNA sequencing
BA21	*Enterococcus faecalis*		isolated in this study	16S rRNA sequencing
BA22	*Enterococcus* sp.		isolated in this study	16S rRNA sequencing
BA23	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA24	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA25	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA27	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA28	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA29	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA30	*Leuconostoc mesenteroides*		isolated in this study	BOXA2R-PCR
BA31	*Enterococcus faecalis*		isolated in this study	16S rRNA sequencing
BA32	*Lc. lactis* ssp. *cremoris*		isolated in this study	BOXA2R-PCR
BA33	*Lc. lactis* ssp. *cremoris*		isolated in this study	BOXA2R-PCR
BA34	*Lc. lactis ssp lactis*		isolated in this study	BOXA2R-PCR/KMK
BA35	*Lactobacillus plantarum*		isolated in this study	16S rRNA sequencing
BA36	*Lactococcus lactis* ssp. *lactis*		isolated in this study	16S rRNA sequencing
BA37	*Lc. lactis ssp lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
BA38	*Streptococcus thermophilus*		isolated in this study	BOXA2R-PCR
BA39	*Enterococcus faecalis*		isolated in this study	16S rRNA sequencing
KV1	*Kluyveromyces lactis*		isolated in this study	ITS1 sequencing
KV2	*Candida parapsilosis*		isolated in this study	ITS1 sequencing
KV3	*Candida zeylanoides*		isolated in this study	ITS1 sequencing
KV4	*Kluyveromyces marxianus*		isolated in this study	ITS1 sequencing
KV5	*Pichia cactophila*		isolated in this study	ITS1 sequencing
KV6	*Kluyveromyces lactis*		isolated in this study	ITS1 sequencing
FD1	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD2	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD3	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD5	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD6	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD9	*Lc. lactis* ssp. *lactis*		isolated in this study	BOXA2R-PCR/KMK
FD8	*Leuconostoc mesenteroides*		isolated in this study	BOXA2R-PCR
FDL4(=L5)	*Leuconostoc mesenteroides*		isolated in this study	BOXA2R-PCR
FDL5	*Leuconostoc mesenteroides*		isolated in this study	BOXA2R-PCR
FDL10	*Leuconostoc mesenteroides*		isolated in this study	BOXA2R-PCR
FDL12	*Leuconostoc mesenteroides*		isolated in this study	16S rRNA sequencing

(*continued on next page*)

Table 2 (*continued*)

STRAIN NAME	Species	Synonym	Source	Identified by
FD11	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD13	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK
FD15	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*		isolated in this study	BOXA2R-PCR/KMK

Table 3
The list of strains used for the reproducibility study.

Strain name	Species	Synonym
ATCC19435^T	*Lactococcus lactis* ssp. *lactis*	DSMZ 20481; NCDO 604
ATCC19257^T	*Lactococcus lactis* ssp. *cremoris*	DSMZ 20069; NCDO 607; HP
ATCC19258^T	*Streptococcus salivarius* ssp *thermophilus*	DSMZ 20617; NCDO 573
ATCC19254^T	*Leuconostoc mesenteroides* ssp. *cremoris*	DSMZ20346; NCDO543
ATCC8293^T	*Leuconostoc mesen*teroides ssp. *mesenteroides*	DSMZ20343; NCDO523
ATCC25302^T	*Lactobacillus paracasei* subsp. *paracasei*	DSMZ5622; NCDO 151
ATCC393^T	*Lactobacillus casei*	DSMZ20011; NCDO 161
ATCC43921^T	*Lactococcus garvieae* ssp *garvieae*	DSMZ20684; ATCC 49156; NCDO 2155
C2	*Lactococcus lactis* ssp. *cremoris*	LMG8523; CECT916
Mo9	*Lc. lactis* ssp *cremoris*	
BA6	*Lc. lactis* ssp *lactis*	
BA37	*Lc. lactis* ssp *lactis*	
FD15	*Lc. lactis* ssp. *lactis* biovar *diacetylactis*	
BA18	*Streptococcus thermophilus*	
BA40	*Streptococcus thermophilus*	
BA21	*Enterococcus faecalis*	
BA31	*Enterococcus* sp.	
BA9	*Staphylococcus warnerii*	
KV2	*Candida parapsilosis*	
KV4	*Kluyveromyces marxianus*	

from the agar plates on which the strains were cultivated. *Leuconostoc mesenteroides* strains ATCC19254, ATCC19255 and ATCC8293 were grown on two agar media: MRS and MRS with 20 μg/ml of vancomycin (MRSV). Three replicates per template for each strain were used for PCR amplifications and were run on individual gels. Yeast DNA was isolated according to the protocol described by Hoffman (1987). The quantity of DNA used in the reproducibility investigation was 50–100 ng.

Statistical analysis. Gel images of the BOXA2R-PCR fingerprints were processed with the PyElph software (Pavel and Vasile, 2012). The generated dendrograms were based on a clustering method applied on the distance matrix computed with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm (Pavel and Vasile, 2012).

High reproducibility of the BOXA2R-PCR method resulted in difficulties when choosing the correct statistical model. The replicates were either very similar or identical (zero variability in the outcome), and this had a significant impact on the intraclass correlation coefficient calculation. Due to the results type and distribution-free data, non-parametric Mann-Whitney test was used for the statistical analysis in IBM SPSS Statistics Version 25. The assumptions of this test; random sampling from the population, independence within the samples and mutual independence, and ordinal measurement scale, were judged as satisfied. The effects of the two templates used, i.e. the isolated DNA and the intact colony, on the reproducibility of the fingerprint profiles were compared. Data points were tested at $p < 0.05$.

16S rRNA sequencing. 16S rRNA sequencing was applied on selected isolates from dairy samples (see Table 2) to firstly corroborate the BOXA2R-PCR speciation inferred from co-migrating fingerprint profiles, and secondly to identify the species of strains with unfamiliar profiles. The sample preparation method involved PCR amplification of a gene coding for 16S rRNA with 16-1A (5′-GTCGGAATCGCTAGTAA TCG -3′) and 23-1B (5′-GGGTTCCCCCATTCGGA -3′) primer pairs following the recommended PCR cycling profile (Tilsala-Timisjärvi and Alatossava, 1997. The 16S rRNA PCR of bacterial strains was performed using either the isolated DNA or a single colony, as a template for the reaction. Gel electrophoresis of the PCR products was carried on in 1% (w/v) mini-agarose gel (7.5 × 10 cm) run in 0.5 × TBE (Tris-base, boric acid, EDTA, pH8.0) buffer for at 80 V for 1 h. The size of the amplified fragment was measured against the molecular size marker, and the concentration and purity were determined by Nanodrop. 16S rRNA gene target Sanger sequencing of the amplicons was performed in the Ramaciotti Center for Genomics using the 16-1A primer. Bacterial identification was achieved by the use of BLAST to compare the obtained sequences with sequences in the GenBank database.

ITS sequencing. The isolated yeasts were identified by the molecular method based on amplification of the Internal Transcribed Spacer (ITS) region employing the primer pair ITS1 (5′-TCCGTAGGTGAACCT GCGG-3′)/ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) following the recommended PCR cycling conditions (White et al., 1990). ITS-PCR was applied directly to a yeast colony. PCR amplicons were sequenced with the ITS1 primer in the Ramaciotti Center for Genomics. The identification of isolates was performed using the best BLAST hits from the NCBI database.

3. Results

3.1. Application of rep-PCR fingerprinting using BOXA2R primer (BOXA2R-PCR) to dairy bacteria

To investigate if BOXA2R-PCR is applicable for fingerprinting of dairy bacteria, a set of cultures including type strains and wild isolates was tested (Fig. 1) and resulted in well resolved, distinguishable fingerprint patterns. Phylogenetic analysis showed that the isolates belonging to the species *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus thermophilus* grouped together into separate clusters (Fig. 1A and B), which indicated the method could be a useful tool for their delineation. *Lb. casei* and *Lb. paracasei* ssp. *paracasei* were all positioned within the same cluster. *Enterococcus faecalis* was placed outside the *Lactococcus* species cluster and *Staphylococcus warnerii* appeared as an outlier to the *Strep. thermophilus* species.

It was observed that *Lc. garviae* ssp *garvieae* ATCC43921 was placed in the same group with *Lc. lactis*. The most pronounced difference in the profiles of *Lc. lactis* ssp *lactis* isolates and *Lc. garvieae* ssp *garvieae* ATCC43921, as inferred from BOXA2R-PCR fingerprints, was the presence of an obvious band of ~300 bp in *Lc. garvieae* (see Fig. 1, A), which was not present in any other *Lc. lactis* ssp *lactis* strains tested in the course of this study.

3.2. Reproducibility of the BOXA2R-PCR method

The reproducibility of the method was explored using both isolated DNA and colony templates from 20 strains of target and non-target species, including ATCC type strains and wild isolates (see Table 3). It was observed that replicates from the same DNA source produced stable fingerprints exclusive to each different strain (data not shown). Profiles obtained from individual colonies were slightly more variable than when sub-sampled from DNA purified using commercial kits; the observed reproducibility for the DNA templates was 98% and for colonies was 97% (see Fig. 2).

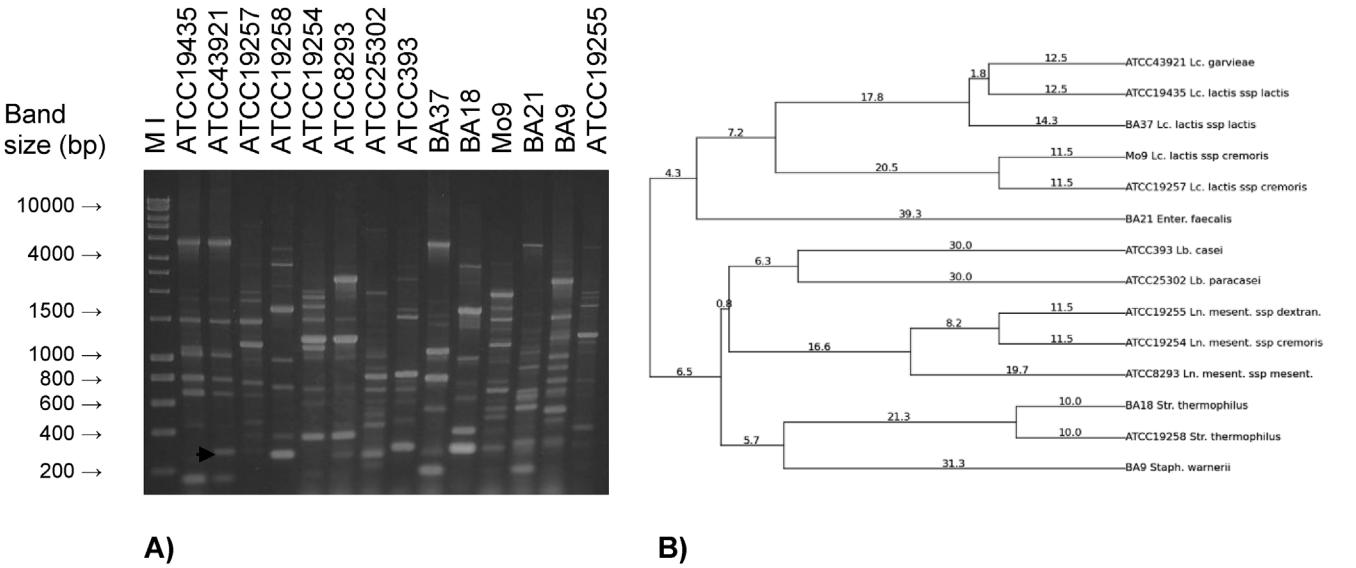

Fig. 1. (A) BOXA2R-PCR fingerprint profiles of some of the strains used for reproducibility testing. HypperLadder I (M I); *Lactococcus lactis* ssp. *lactis* ATCC19435; *Lactococcus garvieae* ssp *garvieae* ATCC43921; *Lactococcus lactis* ssp. *cremoris* ATCC19257; *Streptococcus salivarius* ssp *thermophilus* ATCC19258; *Leuconostoc mesenteroides* ssp. *cremoris* ATCC19254; *Leuconostoc mesen*teroides ssp. *mesenteroides* ATCC8293; *Lactobacillus paracasei* subsp. *paracasei* ATCC25302; *Lactobacillus casei* ATCC393; *Lc. lactis* ssp *lactis* BA37; *Strep. thermophilus* BA18; *Lc. lactis* ssp. *cremoris* Mo9; *Enterococcus faecalis* BA21; *Staphylococcus warnerii* BA9; *Leuconostoc mesenteroides* ssp. *dextranicum* ATCC19255. (B) The corresponding dendrogram of the BOXA2R-PCR patterns was generated using the UPGMA cluster analysis according to the Pearson product moment correlation coefficient (expressed as a percentage value, 0–100%).

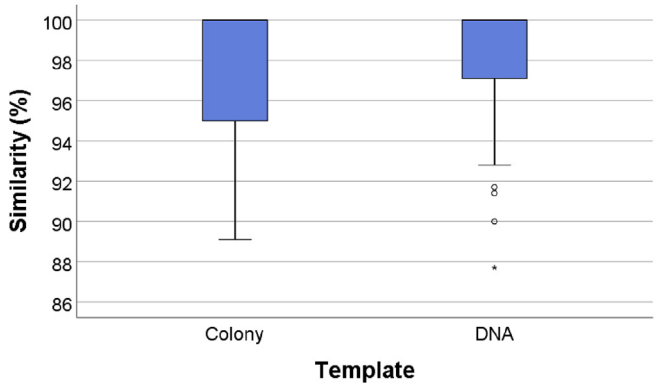

Fig. 2. The plot illustrates 95% Confidence Intervals (CI) of dispersion estimates for colonies and DNA templates.

Table 4
SPSS output of the reproducibility analysis of the BOXA2R-PCR fingerprints.

Test Statistics[a]	Values
Mann-Whitney U	3565.000
Wilcoxon W	6050.000
Z	−1.566
Asymp. Sig. (2-tailed)	.117
Exact Sig. (2-tailed)	.118
Exact Sig. (1-tailed)	.060
Point Probability	.000

[a] Grouping Variable: Source of DNA.

Statistical analysis was performed in SPSS using non-parametric Mann-Whitney test. There was no significant difference ($p = 0.118$) in dispersion (variability) between the replicates generated by application of the BOXA2R-PCR to the DNA vs intact colonies (see Table 4).

3.3. *Lactococcus lactis* subspecies differentiation by BOXA2R-PCR

BOXA2R-PCR fingerprinting of *Lactococcus lactis* strains revealed an evident distinction between the subspecies *lactis* and *cremoris* based on the presence of subspecies-specific fragments. The number of *Lc. lactis* strains and isolates from various sources used for defining the common band pattern of the two subspecies was approximately 100, and included those preliminary screened (data not shown) in addition to those presented in this work. *Lc. lactis* ssp *lactis* type strain ATCC19435 and the strains 18 and 19 displayed a characteristic profile with three amplified fragments of ~200 bp, ~800 bp and ~3800 bp, while two other ssp *lactis* strains, ML8 and SL894, lacked the ~3800 bp band (see Fig. 3, A). The most pronounced band of *cremoris* subspecies was the ~1.1 Kb, and the only other band common to all cremoris strains was ~450 bp. While some bands were unique to individual isolates and others were shared between the subspecies or within the subspecies, the key difference between them was the presence or the absence of the ~200 bp band. This defining band is present in a *lactis* genotype including biovar *diacetylactis*, and absent in a *cremoris* genotype (Fig. 3A). Two pairs of strains, 18 and 19, and AM1 and AM2, displayed identical patterns (Fig. 3A).

Two main clades were identified: group *lactis* included all *Lc. lactis* ssp *lactis* isolates, and group *cremoris* included all *Lc. lactis* ssp *cremoris* isolates (Fig. 3B). Within the *cremoris* cluster, the three differentiated separate sub-clusters were AM1 and AM2; NCDO712-related group of strains (NCDO712, C2, MG1363) and the type strain ATCC19257(HP) and FG2, with the strain E8 being an outlier.

3.3.1. Investigation of the potential of BOXA2R-PCR to detect biovar diacetylactis

The potential of BOXA2R-PCR to distinguish biovar *diacetylactis* strains from *Lc. lactis* strains that are unable to metabolize citrate was assessed on selected citrate-positive and citrate-negative isolates (see Fig. 4).

Based on their BOXA2R-PCR profile, citrate negatives were identified as leuconostocs (FDL4 and FDL10) or lactococci (either *lactis* or *cremoris*).

The correlation between the citrate fermenting phenotype of the *Lc.*

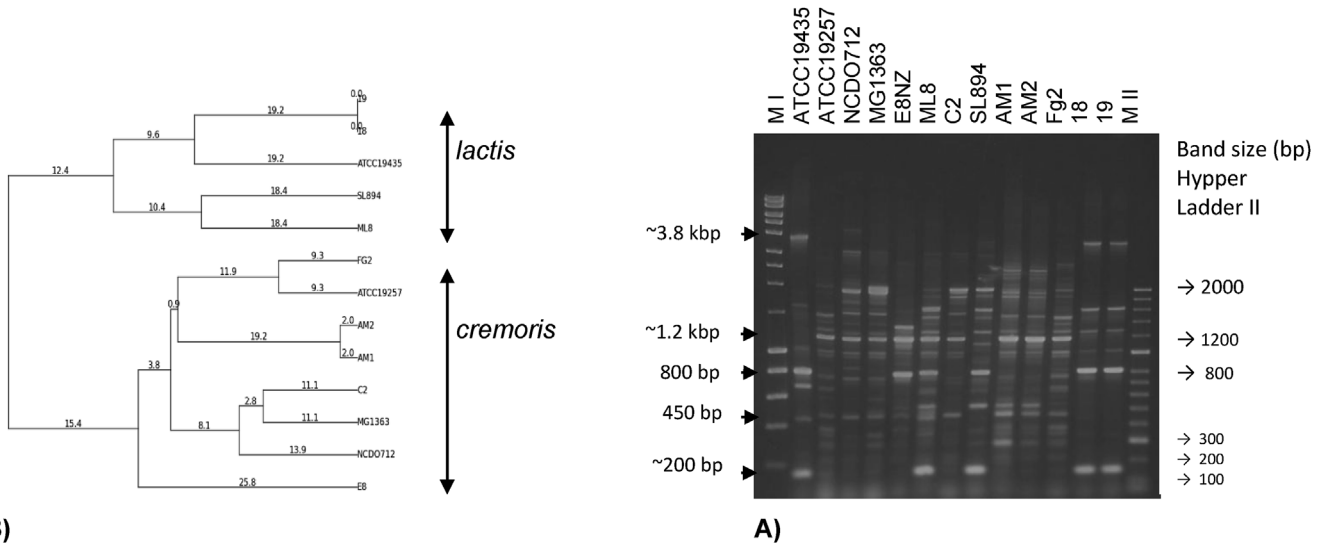

Fig. 3. A) Genetic identification of Lc. *lactis* ssp *lactis* and ssp *cremoris* type strains by BOXA2R-PCR. 1. HyperLadder I; 2. *Lactococcus lactis* ssp *lactis* ATCC19435^{T}; 3. *Lactococcus lactis* ssp *cremoris* ATCC19257^{T}; 4. *Lc. lactis* ssp *cremoris* NCDO712; 5. *Lc. lactis* ssp *cremoris* MG1363; 6. *Lc. lactis* ssp *cremoris* E8NZ; 7. *Lc. lactis* ssp *lactis* ML8; 8. *Lc. lactis* ssp *cremoris* C2; 9. *Lc. lactis* ssp *lactis* SL894; 10. *Lc. lactis* ssp *cremoris* AM1; 11. *Lc. lactis* ssp *cremoris* AM2; 12. *Lc. lactis* ssp *cremoris* FG2; 13. *Lc. lactis* ssp *lactis* 18; 14. *Lc. lactis* ssp *lactis* 19; 15. HyperLadder II. B) The corresponding dendrogram based on the UPGMA method displaying genetic distances above the branches illustrates the genetic relatedness of *Lc. lactis* isolates.

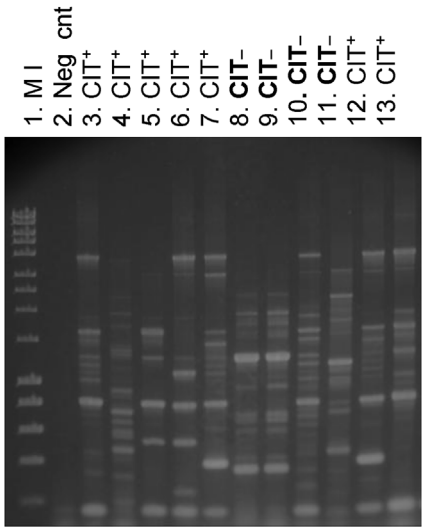

Fig. 4. Genetic comparison of the citrate-positive and citrate-negative isolates from the selective agar medium by BOXA2R-PCR. 1. HyperLadder I (M I); 2. Negative control; 3. *Lc. lactis* ssp *lactis* biovar *diacetylactis* FD3; 4. *Lactobacillus plantarum* BA35; 5. *Lc. lactis* ssp *lactis* BA36; 6. *Lc. lactis* ssp *lactis* biovar *diacetylactis* BA37; 7. *Lc. lactis* ssp *lactis* biovar *diacetylactis* FD11; 8. *Leuconostoc* sp. FDL4; 9. *Leuconostoc* sp. FDL10; 10. *Lc. lactis* ssp *lactis* FD9; 11. *Lc. lactis* ssp *cremoris* BA32; 12. *Lc. lactis* ssp *lactis* biovar *diacetylactis* FD2; 13. *Lc. lactis* ssp *lactis* biovar *diacetylactis* FD1.

lactis ssp *lactis* biovar *diacetylactis* and a specific genetic pattern could not be observed. There were no typical PCR fragments detected which could unambiguously differentiate CIT + and CIT-isolates among all the tested strains and could therefore exclusively point to the biovar *diacetylactis*.

One citrate-positive isolate, BA35, that displayed a profile uncharacteristic for *Lc. lactis* was identified by 16s rRNA sequencing as *Lactobacillus plantarum*. This suggests that BOXA2R-PCR fingerprinting can be useful to discern a citrate fermenting bacterium that does not belong to Lactococci and Leuconostoc. This was further corroborated when other citrate-positive organisms, such as *Lb. plantarum* BA16, *Ent. faecalis* ET4 and *Staph. epidermidis* BA10 (data not shown), were subjected to the BOXA2R-PCR and could be readily separated from *Lc. lactis* ssp *lactis* biovar *diacetylactis* based on their fingerprint patterns.

3.4. Application of BOXA2R-PCR for screening isolates from Australian dairy samples

3.4.1. Strain isolation

The purpose of the strain isolation process was to collect isolates for testing of the BOXA2R-PCR method, hence the origin of the isolated bacterial and yeast strains was intentionally unspecified, and their codes were anonymized.

To test the potential of BOXA2R-PCR as a screening method, fingerprints of the new isolates were first inspected for common patterns. The identity of single isolates and their speciation was confirmed by 16S rRNA gene sequencing. The majority of isolated bacterial strains belonged to common LAB species (see Table 2). *Enterococcus* sp. isolates were identified in two of the analyzed dairy products with one containing *Ec. faecalis* BA21 (10^{4} CFU/g) and other had *Enterococcus* sp. BA22 (10^{8} CFU/g) as a predominant strain. The 16S rRNA sequencing returned an ambiguous identification result showing sequence homology for both *Ec. faecalis* and *Streptococcus salivarius*.

Three dairy products contained non-LAB species including *Staphylococcus epidermidis*, *Staphylococcus warneri*, *Staphylococcus* sp. and *Ochrobactrum anthropi*. Additionally, the following six yeast strains were isolated from five different dairy products; *Kluyveromyces marxianus, Kluyveromyces lactis, Pichia cactophila, Candida zeynaloides* and *Candida parapsilosis*.

3.4.2. BOXA2R-PCR strain fingerprinting

To investigate its value as a fingerprinting tool BOXA2R-PCR was tested on the isolates from dairy products. The distinguishing fingerprints of all isolates tested in this study were generated (see Figs. 5–6). The size of the obtained PCR products ranged from approximately 200 to 5000 bp. The smallest number of bands (Terzic-Vidojevic et al., 2007; Ndoye et al., 2011) was detected in *Staphylococcus* sp. BA4 and *Staph. epidermidis* BA10 (refer Fig. 5), while the LAB genera usually displayed from 5 to 15 bands. The individual bacterial isolates were easily differentiated by visual comparison with each strain displaying a unique and distinctive BOXA2R-PCR profile. Interestingly, BOXA2R-PCR fingerprinting of yeast genomes also yielded different fingerprint

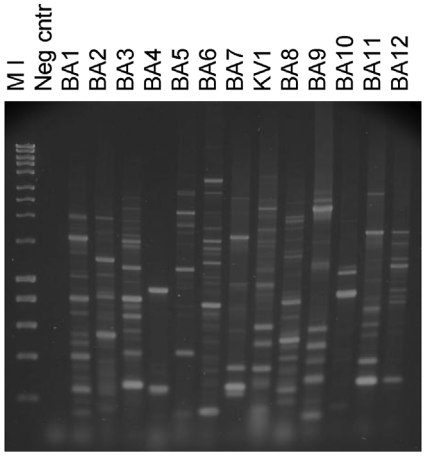

Fig. 5. HypperLadder I (M I); Negative control; *Lb. paracasei* BA1; *Ochrobactrum anthropi* BA2; *Lb. casei* BA3; *Staphylococcus sp.* BA4; *Lc. lactis* ssp *cremoris* BA5; *Lc. lactis ssp lactis* BA6; *Strep. thermophilus* BA7; *Kluyveromyces lactis* KV1; *Staph. warnerii* BA9; *Staph. epidermidis* BA10; *Strep. thermophilus* BA11; *Strep. thermophilus* BA12.

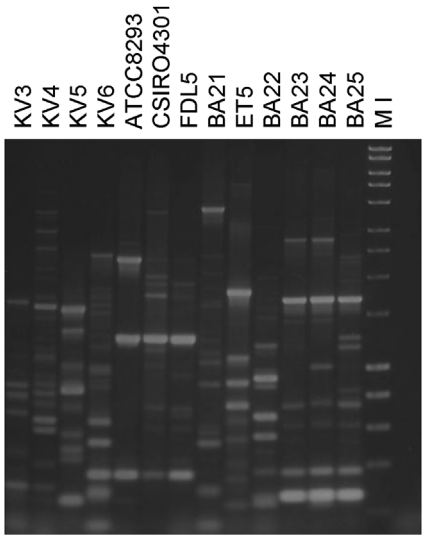

Fig. 6. *Candida zeylanoides* KV3; *Kluyveromyces marxianus* KV4; *Pichia cactophila* KV5; *Kluyveromyces lactis* KV6; *Leucon. mesen*teroides ssp *mesenteroides* ATCC8293; *Leucon. mesen.* ssp. *cremoris* CSIRO4301; *Leucon* sp. FDL5; *Ent. faecalis* BA21; *Ent. durans/faecium* ET5;*Enterococcus* sp. BA22; *Strep. thermophilus* BA23; *Strep. thermophilus* BA24; *Strep. thermophilus* BA25; 14. HypperLadder I (M I) 15. Negative control.

patterns (Fig. 5, isolate KV1; Fig. 6, isolates KV3-KV6).

Figs. 5 and 6. BOXA2R-PCR fingerprint profiles of the strains isolated in this study from retail dairy products. The amplification products (5 µl) were electrophoresed on 1.5% (w/v) molecular grade agarose (Bio-Rad) gels in 1 × TAE (Tris-acetate, EDTA, pH8.0) at a constant 100 V for 3 h.

3.4.3. Discrimination of gram-positive LAB cocci by BOXA2R-PCR

BOXA2R-fingerprints of various LAB isolates were further inspected for common banding patterns, with a particular focus on the Gram-positive LAB species of: *Lactococcus lactis*; *Strep. thermophilus*; *Leuconostoc mesenteroides* and *Enterococcus* since these coccus- or coccoid-shaped LAB species are not always easily differentiated phenotypically.

The specific patterns for *Strep. thermophilus*, *Leucon. mesenteroides* and *Lc. lactis* and *cremoris* were generated, placing them in four separate clusters on the phylogenetic tree (see Fig. 7), while no typical profile was observed for enterococci (see Figs. 6 and 7). *Strep. thermophilus* isolates clustered together producing two characteristic intensive bands of ~300 bp and ~1600 bp, common to all tested strains. Two less intensive bands of ~390 bp and ~1 Kb were present in most strains, while the presence or absence of other fragments was variable among isolates. *Leucon. mesenteroides* strains also grouped together in a clearly identifying clade. Their fingerprints were characterized by distinctive bands of ~380 bp and ~1200 bp size. The characteristic profiles and the clear separation of the *Leuconostoc* and *Strep. thermophilus* isolates is also visually observable (Fig. 7, from the left: isolates 5–7 and 11–13, respectively). *Lc. lactis* ssp *lactis* and *Lc. lactis* ssp *cremoris* strains each grouped together into separate clusters (Fig. 7, isolates 12–13 and 14–15, respectively). To confirm that the speciation of the natural isolates deduced from the BOXA2R-PCR profiles was correct, several isolates per species were subjected to 16S rRNA sequencing (see Table 2). The BOXA2R-PCR species assignment of *Strep. thermophilus*, *Leucon. mesenteroides* and *Lc. lactis* and *cremoris* isolates matched the 16S rRNA findings.

The profiles of *Enterococcus* sp. isolates looked very different to each other, which was presumably the result of the different enterococcal species they belonged to. *Ent. faecalis* ET4 and *Ent. durans/faecium* ET5 clustered in the same group, but were evidently phylogenetically distant. These two strains, as well as BA31, seemed phylogenetically closer to the *Strep. thermophilus* clade, whereas the *Ent. faecium* ET2 was placed just out of the *lactis* clade.

1. HyperLadder I; 2–4. *Strep. thermophilus*: BA28, BA27, BA29; 5–6. *Leuconostoc* sp: FDL12, BA30; *7. Leucon. mesenteroides* ssp *cremoris* CSIRO4202^{T}; 8. *Ent. faecium* ET2; 9. *Enterococcus faecalis* BA31; 10. *Enterococcus durans/faecium* ET5; 11. *Enterococcus faecalis* ET4; 12–13. *Lc. lactis* ssp *cremoris* BA32, BA33; 14. *Lc. lactis* ssp *lactis* biovar *diacetylactis* FD6; 15. *Lc. lactis* ssp *lactis* BA34. B) The corresponding dendrogram displaying the genetic distances between isolates was obtained using the UPGMA method.

4. Discussion

Repetitive-PCR using a single primer or the combination of primers has been previously applied in studies of genetic diversity in lactic acid bacteria isolated from raw milk and traditional fermented dairy products as well as for the typing of *Lactococcus lactis* strains (Callon et al., 2004), (Terzic-Vidojevic et al., 2007), (Odamaki et al., 2011), (Mohammed et al., 2009), (Prodelalova et al., 2005). Successful differentiation was dependant on the primer used. Rep-PCR performed with the primer pair Rep-1R-Dt/REP2-D was deemed inadequate as a sole method for the accurate identification of *Lactococcus*, *Enterococcus* and *Streptococcus* cheese isolates as it was not possible to assign them to a species (Callon et al., 2004). Discrimination between L. *lactis* subspecies was unsuccessful using the primer LL-Rep1 (Prodelalova et al., 2005). Repetitive-PCR using the $(GTG)_5$ oligonucleotide primer could not distinguish isolates of the *Lc. lactis* subspecies (Terzic-Vidojevic et al., 2007), (Odamaki et al., 2011) and could not properly cluster isolates without the application of an additional method (Zamfir et al., 2006). Rep-PCR characterization of *Lactococcus* sp. strains with BOXA1R primer was ambiguous (Mohammed et al., 2009). The use of rep-PCR employing LcRep-H, which is a mixture of three designed repetitive primers proved successful for the genotyping of *Lc. lactis* at both subspecies and strain level (Odamaki et al., 2011). It was demonstrated in this current work that genotyping of *Lactococcus lactis* strains using the single primer BOXA2R in the repetitive-PCR resulted in distinctive and informative fingerprint profiles and that *Lc. lactis* and *cremoris* could be clearly segregated, which suggests that BOXA2R has potential for *Lactococcus lactis* subspecies-specific typing.

It was not possible to explicitly identify biovar *diacetylactis* among the *Lc. lactis* strains without further testing for this phenotypic trait. Difficulties in distinguishing *Lc. lactis* ssp *lactis* and *L. lactis* ssp *lactis*

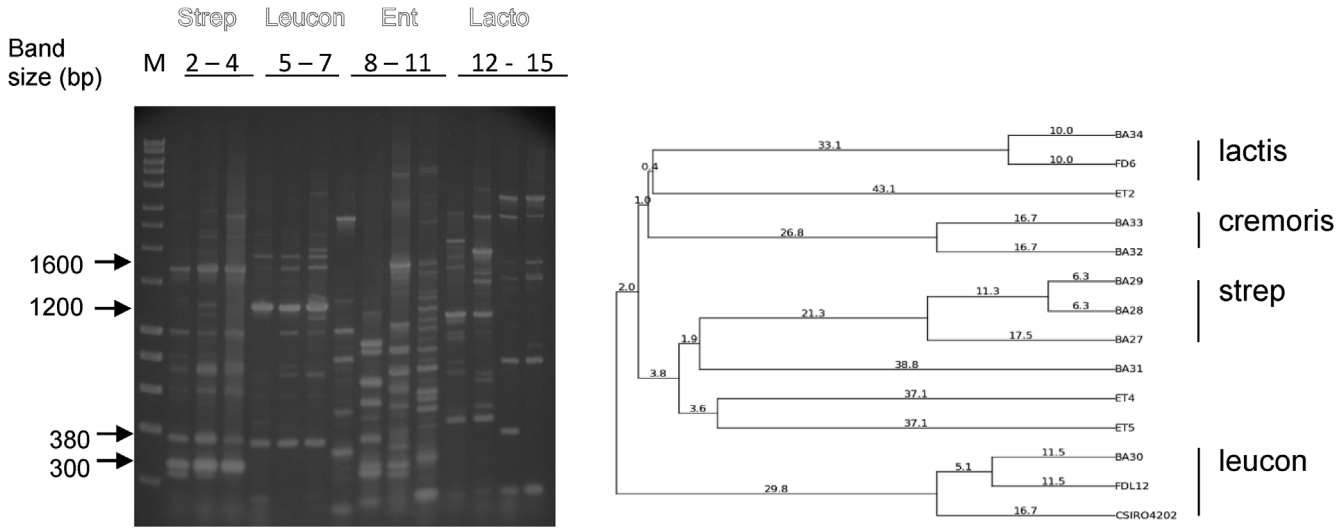

Fig. 7. A) BOXA2R-PCR fingerprints of the representatives of the Gram-positive cocci LAB genera.

biovar *diacetylactis* strains by genetic methods have been reported earlier, where they were indistinguishable by rep-PCR genomic fingerprinting using $(GTG)_5$ primer (Rademaker et al., 2007), LcREP-H primer (Odamaki et al., 2011) and by AFLP (Kutahya et al., 2011) or MLSA (Rademaker et al., 2007). However, BOXA2R-PCR fingerprinting appeared useful to distinguish if an unknown citrate fermenting bacterium belongs to a genus or species other than the common dairy bacteria that can metabolize citrate and produce diacetyl and other flavor compounds, such as Lactococci and Leuconostoc.

The value of the BOXA2R-PCR as a strain fingerprinting tool in dairy microbiology was demonstrated on isolates from various Australian dairy products. The majority belonged to *Lactococcus lactis*, *Streptococcus thermophilus*, and *Enterococcus* sp plus different *Lactobacillus* species. Some *Enterococcus* species are human pathogens (Ogier and Serror, 2008), whereas *Ec. faecalis*, *Ec. faecium* and *Ec. durans* are often encountered in raw milk and artisanal cheeses (Callon et al., 2004), (Terzic-Vidojevic et al., 2007), (Edalatian et al., 2012). While these LAB species are commonly present in dairy products, the detection in some dairy products of *Staphylococcus,* and in particular *Ochrobactrum anthropi* is uncommon. Staphylococci have been isolated from a range of foods, including meat, cheese and milk, but no case of illness related to their consumption in dairy products has been reported (Irlinger, 2008). *Ochrobactrum anthropi*, a Gram-negative rod bacteria phylogenetically closely related to the pathogen *Brucella abortus* (Scholz et al., 2008) is normally a saprophyte that inhabits soil and can colonize a variety of habitats including plants, animals and human (Chain et al., 2011). The finding of these contaminant species in the samples product should be considered undesirable from food safety and quality aspects.

The role of dairy yeasts in the products from which they were isolated could only be a matter of speculation. Some yeast strains such as *Kl. marxianus* and *Pichia cactophila* can contribute to the flavor and aroma of dairy products (Celinska et al., 2018), while others may be linked to product spoilage. *Candida zeynaloides* and *Candida parapsilosis* are among the most common non-starter yeast species isolated from milk, brines and cheeses. These yeasts could be introduced into the dairy ecosystem from various potential sources including environmental inputs, workers or cheesemaking equipment (Banjara and Hallen-Adams, 2015).

Further advantage of the BOXA2R-PCR concerned its potential to separate Gram-positive coccal LAB: *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus thermophiles* from each other and from the *Enterococcus* species. The strains of these LAB species can have overlapping phenotypic characteristics, which make their correct speciation difficult when employing physiological and biochemical methods (Callon et al., 2004), (Corroler et al., 1998). For example, *Enterococcus* sp. can grow under a wide temperature range (15–45 °C) and can be misidentified as either a *Lactococcus* or a *Strep. thermophilus*. Some lactoccocci, particularly from artisanal products can grow under conditions not specified by the traditional key for species identification, such as in 6.5% NaCl and at 10 °C and 45 °C (Callon et al., 2004). *Leuconostoc* sp. may grow on media used for isolation of *Lactococcus* sp. and can metabolize citrate like *Lc. lactis* ssp *lactis* biovar *diacetylactis* and some species of *Enterococcus* (*E. faecalis*). The Enterococcus isolates tested in this work were highly heterogenous, hence their BOXA2R-PCR profiles did not produce any characteristic recognizable profile. The observation that *Lc. garviae* ssp *garvieae* ATCC43921 clustered phylogenetically with *Lc. lactis* was not surprising considering that these two species are both phenotypically similar (Fortina et al., 2003) and have a close genetic relationship as inferred from the maximum likelihood tree based on the concatenated amino acid sequences of *Lactococcus* core genes (Yu et al., 2017). The similarity of BOXA2R-PCR patterns between these two species should be considered when exploring the diversity of any artisanal products that are commonly known to be associated with the presence *Lc. garviae* (Hoffman, 1987). For these products, to positively genetically discriminate *Lc. garviae* isolates would require a species-specific PCR identification test, such as one that can target the 16S rRNA gene (Zlotkin et al., 1998) or the 16S−23S ribosomal RNA (rRNA) internal transcribed spacer region (Dang et al., 2012).

A good correlation of BOXA2R-PCR typing with previous genotyping data on reference and other well-researched lactococcal strains obtained by other methods was observed both in terms of subspecies speciation and their relatedness. For example, strain NCDO712, which was renamed C2 when deposited in CSIRO, Australia, and its plasmid-free derivative MG1363 belong to the same group of closely related strains having a lactis phenotype, but a *cremoris* genotype (Kelly et al., 2010). This is in agreement with BOXA2R-PCR results, which classified them as *cremoris* subspecies yet resolved subtle differences between the two strains (see Fig. 4). Further, according to their PFGE patterns with *Sma*I, *Lc. lactis ssp cremoris* strains E8, HP and SK11 (the phage-resistant derivative of AM1) were representatives of related groups of strains (Kelly et al., 2010); HP and FG2 are closely related strains with some chromosomal rearrangements, which cluster together, but separately from SK11and AM2 (Kelly et al., 2010); AM1 and AM2 display identical fingerprints pointing to a clonal relationship. BOXA2R-PCR profiles of the strains E8, HP and AM1 also show related, but different fingerprint profiles thereby demonstrating good correlation between the PFGE and BOXA2R typing results. These observations are also in agreement with

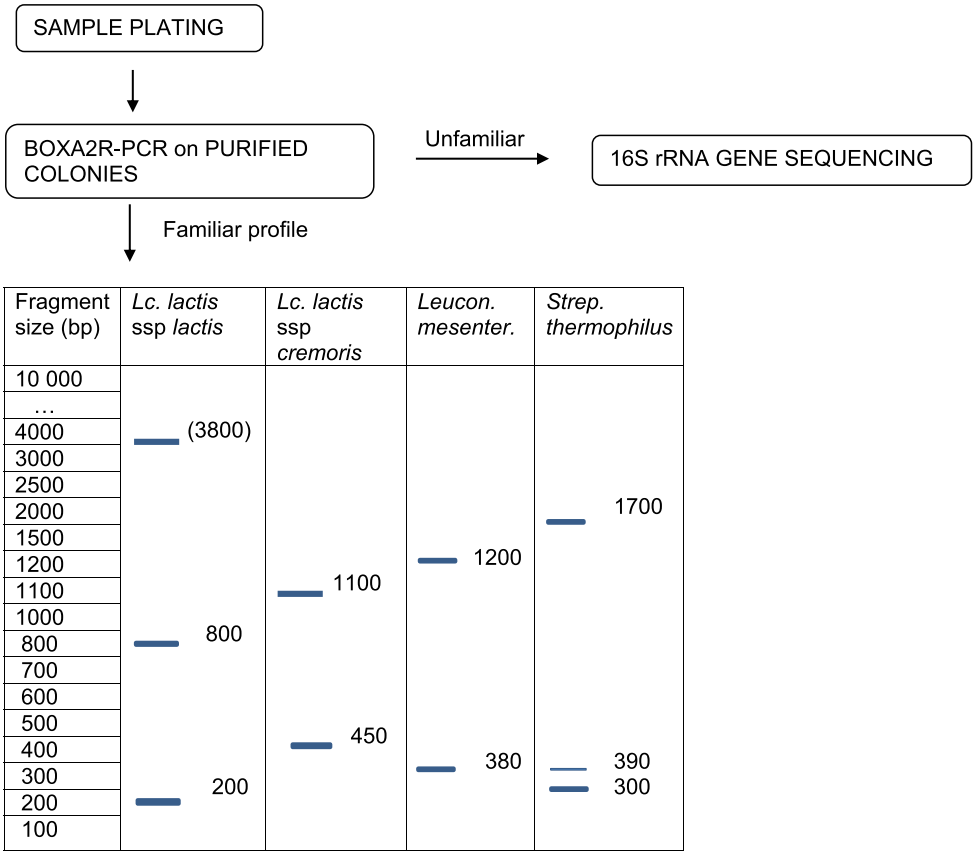

Fig. 8. Schematic representation of the common LAB genera identification by BOXA2R-PCR.

analysis performed by the high-resolution AFLP protocol that showed a clonal relationship between AM1 and AM2 and their placement with HP into a separate sub-cluster within the *cremoris* genotype (Kutahya et al., 2011).

Though it has been long established that repetitive PCR using the single primer BOXA2R can produce distinguishing fingerprint profiles for many different bacterial genera (Koeuth et al., 1995), the ERIC and REP repetitive primers have been more widely used. From our preliminary screening experiments aimed at investigating the genotyping potential of various repetitive primers for the application to the dairy microbiota, the latter two did not appear suitable, as they were not sufficiently informative. In relation to dairy bacteria, BOXA2R has been employed for genotyping of *Enterococcus faecium* (Edalatian et al., 2012), *Leuconostoc* species (Alegria et al., 2013), *Lactococcus lactis* and *Lactobacillus plantarum* (Alegria et al., 2010). BOXA2R-PCR fingerprinting of LAB isolates in this study was performed following the PCR profile by Malathum et al. (1998), which was originally applied to the typing of clinical isolates of *Enterococcus faecalis* at the subspecies level. The method involved a lower annealing temperature (40 °C vs 52 °C) and higher number of PCR cycles (35 vs 30) compared to the profile used by Koeuth et al. (1995). The annealing temperature of 40 °C was recommended as a compensation for the lower GC content of the oligonucleotide primer BOXA2R (Koeuth et al., 1995). It is presumed that the lowered specificity of the PCR reaction under these conditions enabled fingerprinting of a broad range of dairy associated microorganisms, including sporadically detected Gram-positive and Gram-negative contaminants.

It was demonstrated in this study that colony PCR can be a valid alternative to extracted DNA as no qualitative differences in BOXA2R-PCR fingerprint profiles were observed between the two sources of DNA. Rep-PCR on the minimally processed (Versalovic et al., 1994) or unprocessed colonies (Louws et al., 1994) has been reported earlier to yield genomic patterns indistinguishable from those produced with purified DNA. On occasions, though colony sources resulted in minor variations in band intensity, presumably due to loading of a non-standardized amount of DNA, the profiles' authenticity were not compromised. High reproducibility of the BOXA2R-PCR method across both templates was achieved (95–100%) with no statistically significant difference ($P = 0.118$).

In summary, this work has demonstrated that BOXA2R has potential use as a single primer for repetitive-PCR identification of dairy bacteria, in particular the most common dairy cocci. Further work will explore the general use of the method to differentiate and classify bacteria.

5. Conclusions

The choice of BOXA2R as a single primer in repetitive-PCR genomic fingerprinting offers several benefits in genotyping the microorganisms associated with dairy products; it generates unique and distinctive fingerprint profiles of individual bacterial and yeast strains; it enables classification of *Lc. lactis* subspecies into a genotype *lactis* or *cremoris* and the discrimination of *Lc. lactis* strains, including closely related ones, in a single step (*Lc. lactis sub-species and strain specificity);* it is applicable for the presumptive assignment of Gram-positive cocci commonly encountered in dairy products to *Lactococcus lactis*, *Stretococcus thermophilus* and *Leuconostoc mesenteroides*, and colonies containing intact cells are a sufficient source of template DNA.

BOXA2R-PCR can be particularly useful for the preliminary screening of large numbers of unknown dairy isolates, for example in strain selection processes or rationalization of culture collections, facilitating fast identification of commonly present LAB species, which minimizes the number of strains required to undergo further phenotypic characterization or complex genome studies.

The current methodology for the identification of catalase-negative

Gram-positive bacteria isolated from a dairy environment may require the use of several genus- and species-specific PCR reactions (Wullschleger et al., 2013). The use of BOXA2R-PCR as an alternative strategy provides a fast and simple approach for strain screening and their preliminary identification. The applied molecular protocol involves: 1) plating a sample on agar medium; 2) performing BOXA2R-PCR on morphologically different colonies or a chosen number of purified colonies depending on the sample's expected complexity; 3) genetic identification of *Lactococcus lactis*, *Strep. thermophilus* and *Leuconostoc mesenteroides*; and 4) 16S rRNA gene sequencing of purified colonies that have unfamiliar profile (see Fig. 8).

Direct application of the method on vegetative cells instead of extracted DNA (colony-PCR) significantly reduces time and cost of screening process. In addition to other advantages of PCR methods, speed and cost related, the BOXA2R-PCR fingerprint patterns are informative, the method is easy to use and interpret, has high resolving power, and is reproducible under defined conditions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.02.011.

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