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Bacterial diversity of aMasi, a South African fermented milk product, determined by clone library and denaturing gradient gel electrophoresis analysis

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In the present study, we investigated the bacterial diversity of aMasi, a traditional South African fermented milk product, by 16S rRNA clone library and Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Two hundred and eighty two clones from clone library were isolated and identified from aMasi, prepared from the milk of four cows from one herd in the EkuPindiseni Community, North West of Hluhluwe-iMfolozi Park in KwaZulu-Natal Province. The majority of the identified sequences corresponded to lactic acid bacteria (LAB), with the genus *Lactococcus* as major representative. The species *Lactococcus lactis* accounted for 179 of the identified clones. In addition, several species of *Lactobacillus*, *Leuconostoc* and *Enterococcus* were detected. Furthermore, several clones belonging to *Acinetobacter*, *Aeromonas* and genera within the *Enterobacteriaceae* were detected. It is important to note that human pathogens such as *Klebsiella pneumoniae* were identified in aMasi in the present study. Conversely, zoonotic bacteria such as *Brucella abortus* and *Mycobacterium bovis* were not detected in aMasi, although, they are present in the cattle population in the study area. Thirty (30) clones were identified as uncultured bacterial clones. Nine DGGE bands were successfully sequenced, of which four were classified as *L. lactis* with other bands belonging to lactobacilli, *Clostridium acidurici*, *Enterobacter* sp., *Acinetobacter baumannii* and an un-culturable bacterium. Even though there was some discrepancy between the two culture independent methods used to study the bacteriological community in aMasi, a general conclusion can be drawn, *L. lactis* may be considered as the dominant bacterium within a diverse bacterial community in this locally-produced dairy product.

Key words: South Africa, aMasi, fermented milk, microbial diversity, clone library, denaturing gradient gel electrophoresis (DGGE).

INTRODUCTION

Fermentation is a common method used to preserve milk in rural areas of South Africa and other areas with poor access to electricity and cold storage facilities (Narvhus, 2003; Mathara et al., 2008; Ukeyima et al., 2010; Franz and Holzapfel, 2011). During the fermentation process, lactic acid bacteria (LAB) produce lactic acid, which decreases the pH of the milk, causes coagulation and inhibits the growth of bacteria that leads to deterioration (Franz and Holzapfel, 2011). However, as traditional fermented milk products such as aMasi are produced at the household scale, and form an economically and nutritionally important component of local diets in rural areas, it is important to understand how the potential health benefits and public health risks vary according to local manufacture procedures, use and consumption practices. aMasi is an historically important product in many South African cultures, and is still commonly produced and consumed by cattle-owning families in rural areas. Households use traditional milk fermentation principles to generate a continuous supply of aMasi, using modifications in the duration and temperature of fermentation to attain preferred tastes and product thickness. The quality, bitterness and quantity of aMasi are also influenced by the seasonal composition, volume and availability of fresh milk in each household.

Typically, unpasteurized fresh milk (ubisi) is collected and poured into a sealable opaque container (calabash or igula) that may be pre-smoked to prevent the growth of mold. Once sealed to prevent contamination, the calabash is typically stored indoors at a warm ambient temperature (>20°C) or near a gentle source of heat. Fermentation develops over three to five days, and is accelerated by the presence of “natural” bacteria in the milk, residual bacteria on the inside of the vessel, or through contamination during the milking process. As the milk separates, the thin watery liquid (uMlaza) is removed at regular intervals, leaving a thicker white coagulant (aMasi) that is harvested when it has achieved the desired qualities, also as a result of adaptation and selection of LAB strains dominating the fermentation. Over time, more fresh unpasteurized milk is added to this stock, which together with the internal surface of the container, provides an ideal environment for bacterial growth. As few households report any pre- or post-fermentation heat treatment or filtering prior to consumption, the potential microbial diversity and public health risks of traditional dairy products requires greater investigation.

During the last two decades, numerous studies have investigated the microbial communities in traditional fermented milk products from different African countries, using traditional bacterial cultivation techniques in combination with simultaneous or subsequent biochemical and physiological differentiation of the isolates (Feresu and Muzonda, 1990; Mutukumira et al., 1996; Gadaga et al., 1999; Abedesin et al., 2001; Beukes et al., 2001; Gran et al., 2002; Mathara et al., 2004, 2008; Ukeyima et al., 2010). However, culture-dependent methods do not reflect the true bacteriological community, but rather the needs of different growth media for cultivation of certain bacteria. Based on this fact, various culture-independent methods have been developed during the last decade (Theron and Cloete, 2000; Jany and Barbier, 2008), including the use of PCR-temporal temperature gel electrophoresis (PCR-TGGE) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) to evaluate the bacterial biodiversity of raw and fermented milk (El-Baradei et al., 2007, 2008; Giannino et al., 2009; Hao et al., 2010). However, to the authors’ knowledge, less information is available on the construction of 16S rDNA clone libraries to evaluate the bacterial diversity of fermented milk (Andersen et al., 2013). We therefore address the issue of construction of a 16S rDNA clone library of aMasi and compare these results with PCR-DGGE analysis.

MATERIALS AND METHODS

Sample collection

In December 2009, fresh, untreated milk was obtained from one randomly selected rural farming household in EkuPindiseni community, located 5 km North West of Hluhluwe-iMfolozi Park in the KwaZulu-Natal Province, South Africa. This community was selected as it is: i) in close proximity to a wildlife conservation area (HiP) in which zoonotic and other diseases including bovine tuberculosis (BTB) have been documented (Jolles et al., 2006); ii) located within an area where zoonotic and potentially pathogenic milk-related diseases including BTB and brucellosis have been documented in community-owned cattle (Hesterberg et al., 2008; Geoghegan, 2012); iii) representative of local social, environmental and economic conditions, which may influence local disease risks and limit access to preventative and therapeutic veterinary and medical health services (Geoghegan, 2012). Four adult cows that are regularly milked were selected by the household livestock-owner from a single herd, totaling 30 Nguni and Brahman cross-breed cattle. Employing the typical daily household routine, lactating cattle were tethered to a tree located within the outdoor holding area (kraal), which is used overnight to protect multiple livestock species from predation and theft. Milk was manually collected from each cow into one open plastic bucket that rested directly on the muddy earth, or was held by another family member. No measures were undertaken to remove suckling calves from the kraal, which were observed to interrupt milking and required constant handling. Similarly, no hygienic measures were performed to clean or sterilize the cow udders, householders’ hands or collecting vessels prior to milking, which is consistent with a lack of access to clean piped water in EkuPindiseni.

Subsequently, the pooled milk was transferred to two sealable

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plastic bottles at ambient temperature of 26°C, before being mixed and shipped in four 50 ml Falcom tubes, under license by the Norwegian Health Department (December 2009) to the Section of Arctic Veterinary Medicine, Department of Food Hygiene, at the Norwegian School of Veterinary Science, Tromsø, Norway, for purification of DNA and construction of the clone library.

16S rRNA clone library

Bacterial DNA was extracted with QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's protocol for extraction of bacterial DNA (http://www.qiagen.com/qdm/aw/amp/dna-purification?cmpid=QVen9GAdnapurification). 200 µl of milk were used as a starting material, and the eluted DNA was stored at 4°C until further applications. The concentration and quality of DNA was determined by NanoDrop measurements. The amplification of 16S rRNA genes was done with the forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). The following was added to a 0.2 ml PCR tube: 25 pmol of each primer, 8 µl of Jump Start Red Taq Ready Mix (Sigma-Aldrich), 50 ng DNA template, and MilliQ-water to a final volume of 25 µl. The 16S rRNA genes were amplified on a Perkin Elmer GeneAmp PCR System machine (Perkin-Elmer), using the following conditions: An initial denaturation step at 94°C for 5 min, then 30 cycles with denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and elongation at 72°C for 30 s. A final elongation step at 72°C for 7 min was included to ensure complete synthesis of the amplicons. Successful amplification was confirmed by separation on a 1% agarose gel. The gel was stained with ethidium bromide, and bands were visualised by Gel Doc 2000™ Documentation System (Bio-Rad Laboratories). The PCR product was purified with ExoSAP-IT® (Affymetrix), following the manufacturer's instructions, and ligated into a pGEM®-T Easy vector (Promega). Plasmids were transformed into JM109 *Escherichia coli* cells (Promega), and cells were spread on LA-plates containing 100 µg/ml ampicillin and 20 µg/ml X-gal. White clones were selected and grown in LB-media with 100 µg/ml ampicillin. Plasmids from 350 positive colonies were purified with the QIAquick Plasmid Mini kit (Qiagen) and eluted with 50 µl EB buffer. The concentration and purity of the plasmids were determined by NanoDrop measurements.

Sequencing of the inserts was done with BigDye® terminator v3.1 (Applied Biosystems), using 27F as sequencing primer. The sequencing reactions were done according to the manufacturer's protocol (Applied Biosystems), and labelled fragments were separated and visualised on an ABI Prism 3100 Genetic Analyzer (Perkin Elmer). Sequences were individually analyzed and edited in the program ChromasPro. Edited sequences were subjected to BLAST search in the NCBI database (http://www.ncbi.nlm.nih.gov/), and subsequently all samples were aligned with selected reference sequences in the BioEdit program. Phylogenetic inferences were done by the Bayesian logarithm using the program Beast v1.6.1, and phylogenetic trees were constructed with the program Figtree v1.3.1. The sequences used for construction of the phylogenetic trees are based on a representative selection.

DNA extraction and PCR amplification

Genomic DNA was obtained using the extraction method described by He et al. (2010) with some modifications. Briefly, the aMasi was freeze-dried before transferring 200 mg sample to a 1.5 ml Eppendorf tube and centrifuged at 12.000 rpm for 10 min. The supernatant was discarded and 500 µl lysozyme lysis buffer (0.3 M sucrose, 0.025 M EDTA, 0.025 M Tris-HCl, pH 8.0) was added to each tube and mixed thoroughly. The sample was incubated for 1 h in a 37°C water bath. Each tube was gently inverted every 15 min. Then, the CTAB lysis buffer (100 mM Tris-HCl, 100 mM Na-EDTA, 1.5 M NaCl, 1% CTAB, 2% SDS, pH 8.0) was added into the tubes and mixed immediately. The tubes were incubated for 4 h in a 65°C water bath and gently inverted every 15 min. After incubation, the samples were centrifuged at 12.000 rpm for 10 min. The supernatant was poured into a clean tube and an equal volume of trichloromethane was added and gently mixed and thereafter centrifuged at 12.000 rpm for 10 min. The supernatant was transferred into a new tube and equal volume of isopropanol was added and gently mixed by inversion prior to incubation at −20°C for 30 min. After incubation, the samples were centrifuged at 12.000 rpm for 10 min and the supernatant discarded.

The 75% ethanol was aspirated off the pellet. The pellet was resuspended in 50 µl double distilled water. Genomic DNA was thereafter purified using an TIANquick Midi purification kit (TIANGEN, Beijing, China). The V3 region of the 16s rRNA gene was amplified. The primer and PCR reaction system is as described elsewhere (Liu et al., 2008; Zhou et al., 2009). Amplification consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s at 56°C for 30 s and a final extension at 72°C for 30 s. An additional final extension at 72°C for 10 min was used.

Denaturing gradient gel electrophoresis (DGGE) analysis of the V3 region

DGGE was performed as described elsewhere (Liu et al., 2008; Zhou et al., 2009). Electrophoresis was conducted with a constant voltage of 60 V at 60°C for about 16 h. Gels were stained with ethidium bromide for 20 min and photographed with UV trans-illumination.

RESULTS

16S rRNA clone library

Gel electrophoresis was run for 350 samples, and all clones had an insert of 16S rRNA gene. After NanoDrop measurements, 293 samples had a high enough concentration of DNA to be sequenced. Of these, 282 had sequences that were sufficient to construct a 16S rRNA clone library to investigate bacterial diversity of aMasi and the clones with insert of 16S rRNA were successfully sequenced and characterized through the NCBI database. Phylotypes of the 282 clones were compared using the BLAST program, and the results with their corresponding accession number are displayed in Table 1. These results are based on a similarity of ≥93% and basepair length of ≥164 showing that 194 out of 282 clones (68%) belonged to LAB. 179 of the LAB clones were different strains of *Lactococcus lactis* while 11 belonged to *Lactobacillus*, *Leuconostoc* and *Enterococcus*. In the present study, 8 clones displayed high similarity to genus *Acinetobacter*. 2 of them showed similarity to uncultured *Acinetobacter*, 5 belonged to *Acinetobacter* sp. while 1 clone was identified as *Acinetobacter johnsonii*. 4 clones were characterized as

Table 1. Identification of clones from aMasi with partial sequence of 16S rRNA genes referenced to accession no. in GenBank. Clone no. marked in bold was used to construct phylogenetic trees.

Clone number	Closest relative (obtained from BLAST search)	Accession no	Similarity (%)	No of clones showing high similarity to the closest relative
EB-82	Uncultured *Lactococcus* sp. clone Z148	EU029359.1	87	4
EB-22	*Lactococcus lactis* strain BMG 125	EU080999.1	93	1
R-6	*Lactococcus lactis* strain F6	EF204359.1	99	1
R-30	*Lactococcus lactis* strain JC10	GU936959.1	96	1
EH-59	*Lactococcus lactis* strain KLDS4.0325	GQ337877.1	98	22
R-7	*Lactococcus lactis* strain KLDS4.0424	GQ337891.1	98	1
R-27	*Lactococcus lactis* strain KLDS4.0430	GQ337892.1	97	1
R-41	*Lactococcus lactis* strain N1	HQ647114.1	98	2
R-10	*Lactococcus lactis* strain N2	HQ647115.1	98	2
EH-4	*Lactococcus lactis* strain N3	HQ647116.1	99	14
R-33	*Lactococcus lactis* strain KLDS4.0309	GQ337873.1	98	1
R-4	*Lactococcus lactis* strain KLDS4.0601	GQ337893.1	99	3
EH-73	*Lactococcus lactis* strain D23	EF204354.1	99	1
EH-26	*Lactococcus lactis* strain F124	EF204358.1	99	8
EH-6	*Lactococcus lactis* strain NM141-1	HM218559.1	99	1
EH-25	*Lactococcus lactis* subsp. *cremoris* strain SC8	AM944595.1	99	22
EB-2	*Lactococcus lactis* subsp. *lactis* strain Chr-I-str15	HM462394.1	98	1
EB-80	*Lactococcus lactis* subsp. *lactis* strain KLDS 4.0603	FJ8761117.1	97	1
EH-13	*Lactococcus lactis* subsp. *lactis* strain IMAU60156	FJ749871.1	99	44
EB-62	*Lactococcus lactis* subsp. *lactis* strain IMAU20105	FJ845005.1	99	22
EB-73	*Lactococcus lactis* subsp. *lactis* strain IMAU50170	FJ749563.1	96	1
R-26	*Lactococcus lactis* subsp. *lactis* strain NM141-1	HM218559.1	96	2
E-V	*Lactococcus lactis* subsp. *lactis* strain NM161-4	HM218648.1	98	1
R-12	*Lactococcus lactis* subsp. *lactis* I11403	AE005176.1	97	3
R-44	*Lactococcus lactis* subsp. *lactis* strain NM26-6	HM218132.1	95	1
EH-7	*Lactococcus lactis* subsp. *lactis* strain CV56	CP002365.1	99	31
EB-10	*Lactococcus lactis* subsp. *lactis* gene for 16S rRNA	AB618806.1	98	1
EB-100	*Lactococcus lactis* subsp. *lactis*	AB621973.1	98	1
R-1	*Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* strain 4001C2	GU344708.1	98	3
R-20	*Lactobacillus* sp. Rrv5	EF107621.1	95	1
EB-78	*Lactobacillus casei* strain IMAU20006	FJ844937.1	96	3
EB-90	*Lactobacillus paracasei* strain KLDS1.0653	FJ607292.1	90	2
EB-40	*Lactobacillus pentosus* strain MH53	FJ542297.1	86	1
EB-74	*Lactobacillus plantarum* strain LA445	FJ867640.1	94	4
EB-9	*Lactobacillus plantarum* strain D14	HQ853454.1	93	2
EH-17	*Leuconostoc pseudomesenteroides* strain IMAU600043	FJ749768.1	97	2
EH-7	*Leuconostoc pseudomesenteroides* strain SC8	FJ532362.1	99	2
EB-51	*Enterococcus faecalis* strain CTC328	FJ804073.1	95	1
EB-68	Uncultured *Acinetobacter* sp. clone GI5-002-G08	FJ193027.1	98	5
P-33	*Uncultured Acinetobacter* sp. clone ELC_30_27	EF464630.1	98	3
EH-37	*Acinetobacter* sp. strain SY75	FJ494707.1	99	3
EB-4	*Acinetobacter* sp. D12	EF204260.1	99	5
EH-5	*Acinetobacter* sp. strain G13	EF204259.1	98	2
EB-21	*Acinetobacter* sp. c29	AB167204.1	98	1
EH-P3	*Acinetobacter* sp. N12	AB208676.1	99	1
EB-12	*Acinetobacter johnsonii*	DQ911549.1	97	1

Table 1. Contd

R-3	*Aeromonas sobria* strain hs-1	FJ461353.1	98	1
R-5	*Citrobacter freundii* strain MH0711	EU360794.1	98	2
EB-10	*Enterobacter* sp. FMB-1	DQ855282.1	96	1
EH-58	*Enterobacter* sp. AP11	HM628704.1	96	2
EB-77	*Escherichia coli* IAI1	CU928160.1	95	1
EB-37	Uncultured *Klebsiella* sp. clone SL13	GU201565.1	98	1
R-16	*Klebsiella* sp. Cl40	EU294414.1	98	1
EH-53	*Klebsiella* sp. TJ_DMAB	JF701187.1	99	3
R-22	*Klebsiella* sp. Cl40 clone A10	GU003816.1	95	1
EH-60	*Klebsiella* sp. XW721	EU545402.1	99	4
EB-57	*Klebsiella* sp. 38	EU294412.1	93	1
EB-71	*Klebsiella* sp. DB-3	FJ711774.1	95	1
EH-28	*Klebsiella* sp. ICB369	HQ413273.1	96	2
EB-V	*Klebsiella* sp. SZ7-2	EU256398.1	98	1
EH-8	*Klebsiella ornithinolytica* strain ATCC 31898	AF129441.1	98	3
R-31	*Klebsiella pneumoniae* strain SA-C4-53	EU420942.1	98	1
EB-16	*Klebsiella pneumoniae* 342	CP000964.1	97	3
EB-13	*Kluyvera* sp. IAL9558/98 16SrRNA	AF176564.1	95	1
R-38	*Kluyvera cryocrescens* isolate TS IW 13	AM992189.1	98	1
EH-1	*Kluyvera cryocrescens* isolate WAB 1904	AM184245.1	98	1
EB-45	*Raoultella* sp. 47	FJ587229.1	95	2
EH-57	*Raoultella* sp. TJ_TMA	JF701185.1	92	1
EB-10A	*Shigella sonnei* Ss046	CP000038.1	97	1
EB-34	*Xenorhabdus nematophilia* strain PDBC SCX3	AY753196.2	91	2
	Uncultured bacterium clones			**30**
EB-2*	Uncultured bacterium partial 16S rRNA gene	FN813862.1	94	1
R-34	Uncultured bacterium clone FC04G08	FM873265.1	97	1
R-32	Uncultured bacterium clone nby263h05c1	HM808346.1	97	1
P-33	Uncultured bacterium clone BANW657	DQ264605.1	98	3
EH-70	Uncultured bacterium clone BANW663	DQ264609.1	100	2
EH-3	Uncultured bacterium clone ncd267e11c1	JF016925.1	99	1
EH-6	Uncultured bacterium clone ncd1415a07c1	JF123133.1	99	1
EH-3A	Uncultured bacterium clone ncd1651h09c1	JF146179.1	95	1
ER-15	Uncultured bacterium clone ncd2683g12c1	JF232966.1	95	2
EH-10	Uncultured bacterium clone ncd2731a08c1	JF235874.1	99	1
EH-32	Uncultured bacterium clone ncd2715b02c1	JF234928.1	98	1
ER-1	Uncultured bacterium clone ncd2763e03c1	JF237995.1	99	6
EH-10A	Uncultured bacterium clone nby238f05c1	HM811749.1	99	1
ER-45	Uncultured bacterium clone 22c06	EF515274.1	96	1
ER-19	Uncultured bacterium clone 3P-4-1-009	FJ562204.1	93	1
R-18	Uncultured gamma proteobacterium	AM421455.1	97	1
EB-21	Uncultured gamma proteobacterium	EF188656.1	98	1
P-8	Uncultured bacterium clone CE2_c06_2	EU773842.1	92	3
EB-81	Uncultured bacterium clone RPSD_1aaa04h03	EU778488.1	92	1

Klebsiella pneumoniae, while 15 clones belonged to *Klebsiella* sp. and could not be identified at species level. Other bacterial species, belonging to *Aeromonas sobria* strain hs-1, *Citrobacter freundii* MH0711, *Kluyvera cryocrescens*, *Shigella sonnei*, and the *Enterobacter*, *Kluyvera* and *Raoultella* genera were identified as less

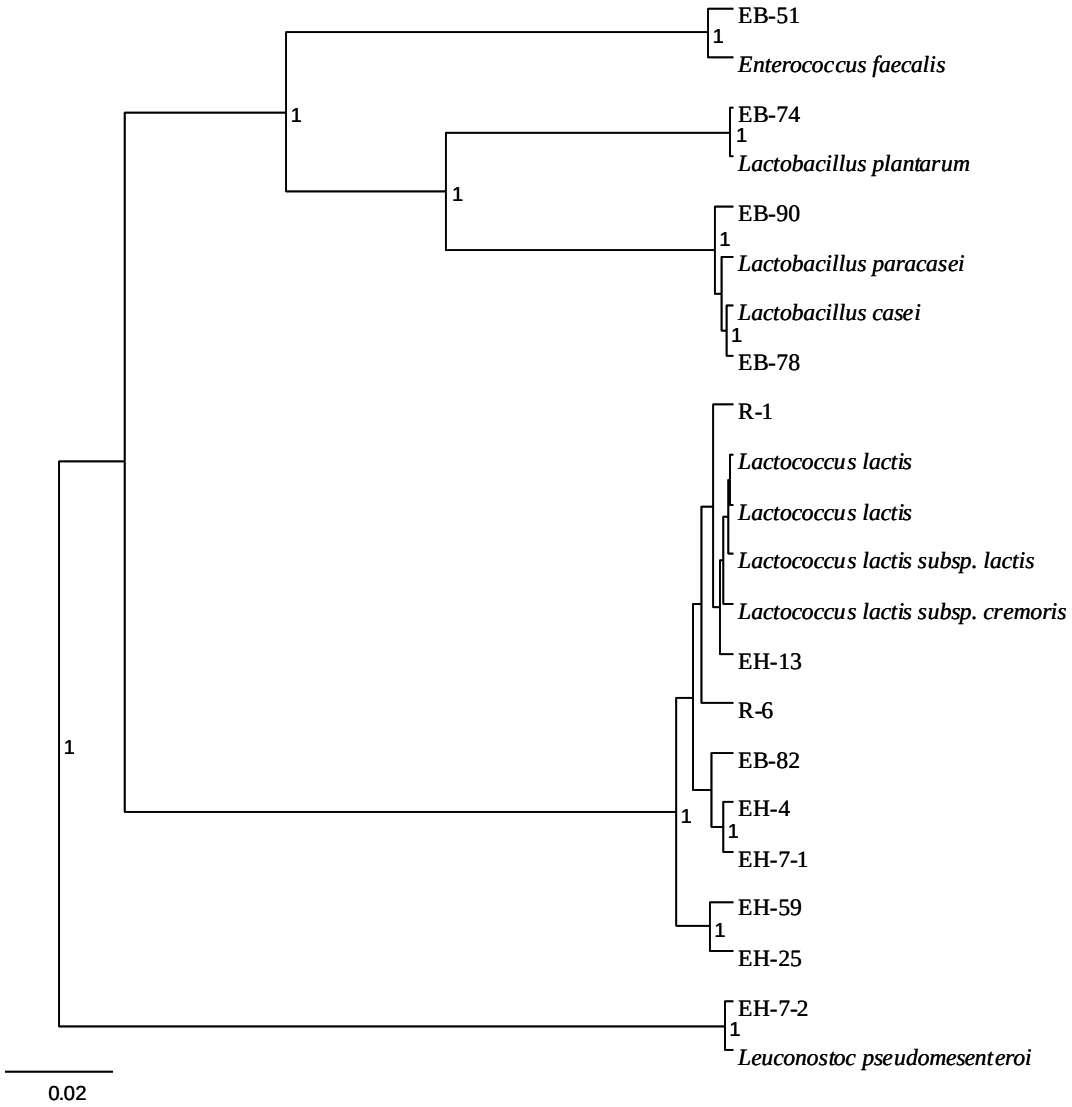

Figure 1. Phylogenetic inferences of selected clones from the aMasi 16S rDNA library. Clone sequences were aligned with sequences from the NCBI database showing highest similarities in BLAST searches. Sequences were edited and aligned with the BioEdit program, and phylogenetic analyses were done in the BEAST v6.1 program. Clones belonging to LAB are mainly grouped with *Lactococcus lactis* or *Lactobacillus* (A), while non-LAB clones affiliate either with *Acinetobacter* or *Enterobacteriaceae* (B). Clones showing low similarities with reference bacteria are shown in (C). The numbers by the nodes refer to posterior probability, and only values above 0.8 are included in the trees. The scale bars represent nucleotide substitution/site.

abundant in the aMasi milk. Of the 282 clones identified, 30 were identified as uncultured bacterium clones (Table 1). Representative selections of clones identified by 16S rRNA gene sequencing were used for phylogenetic analyses (Figure 1A, B and C).

The reference sequences used in the construction were obtained by picking out the sequences that were most similar to the clones isolated from aMasi. The phylogenetic trees show that the clones identified in the study, and which show high similarity with sequences in the NCBI database, are mainly divided into three distinct groups. The majority either belongs to LAB or *Enterobacteriaceae*, while some clones affiliate with *Acinetobacter* (Figure 1B). The phylogenetic tree based on sequences showing low similarity in BLAST searches (Figure 1C), shows that a substantial number of isolated clones represents bacteria that have not been successfully cultured.

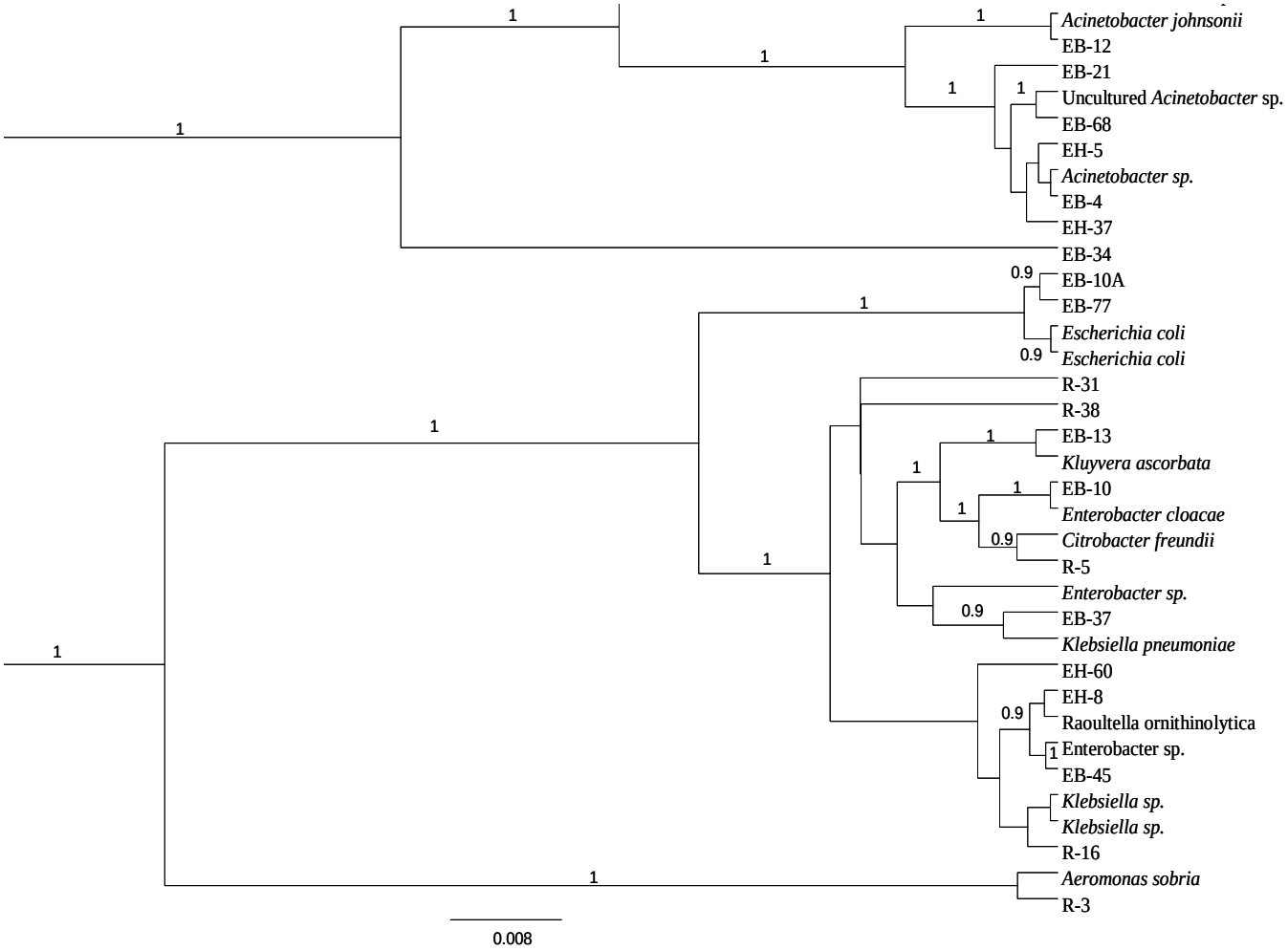

Figure 1. Contd.

DGGE analysis

DGGE fingerprint analysis of aMasi (Figure 2 and Table 2) displayed that Firmicutes were dominant in aMasi. 4 out of 9 bands belonged to *L*. *lactis*, but *Lactobacillales*, *Acinetobacter*, *Clostridium* and *Enterobacter* genera were also detected.

DISCUSSION

As conventional culture-based techniques do not present a correct picture of microbial diversity of fermented milk, we addressed the issue to investigate the bacterial diversity of the South African fermented milk product aMasi by construction of 16S rRNA clone library and denaturing gradient gel electrophoresis (DGGE) analysis. Less information is available on the construction of 16S rRNA clone libraries to evaluate the microbial diversity of milk samples (Delbes et al., 2007; Martin et al., 2007; Rasolofo et al., 2010; Raats et al., 2011; Andersen et al., 2013). PCR-DGGE has the advantage of being reliable, reproducible, rapid and somewhat inexpensive, and several studies have used the DGGE approach to analyse the bacterial community of milk (Hao et al., 2010; Miyamoto et al., 2010; Liu et al., 2012). The discrepancy determined in the bacterial diversity analysed by clone library and the PCR-DGGE method used in the present study, might be due to the fact that DGGE only can detect 1 to 2% of the microbial population representing dominant species present in microbial community pattern (Muyer et al., 1993). 16S rDNA clone library analysis was observed to be more representative of the community in qualitative and quantitative terms especially when the numbers of clones were large enough, 282 clones identified in the present study. However, by combining the

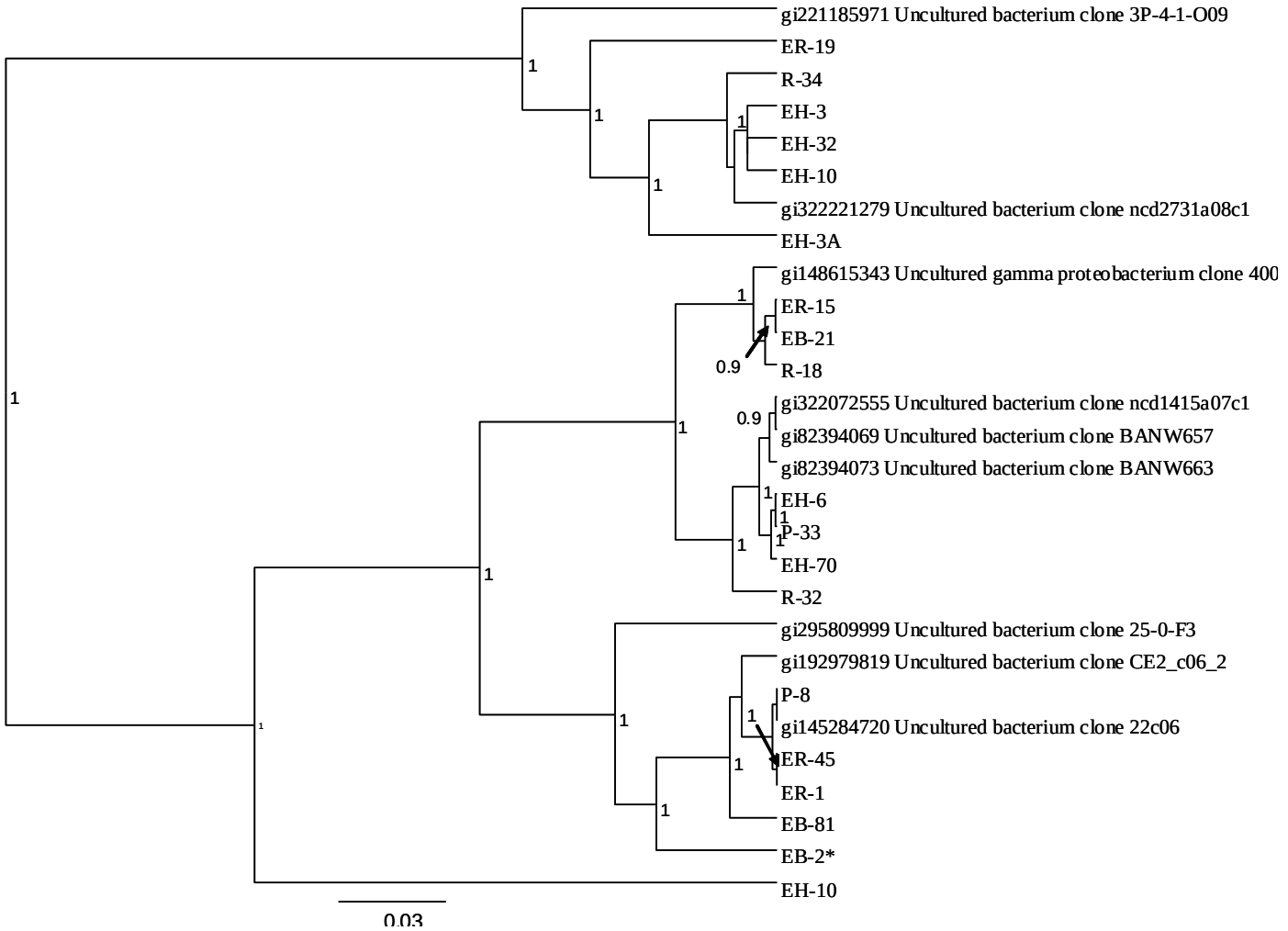

Figure 1. Contd.

two molecular methods, we could obtain a more correct picture of the bacterial diversity of aMasi.

Lactic acid bacteria (LAB)

The high numbers of LAB, comprising *Lactococcus*, *Lactobacillus* and *Leuconostoc*, were expected since the DNA was extracted from fermented cow's milk. 179 out of the 282 clones and four out of nine bands of the DGGE analysis were characterized as *L. lactis.* Strains of this species have previously been isolated from fermented milk from Zimbabwe, Kenya and South Africa (Narvhus et al., 1998; Mathara et al., 2004; Bauer et al., 2009; Mutukumira et al., 2009), but were not detected in fermented horse milk from Mongolia or fermented camel milk (Shubat) from Kazakhstan where lactobacilli were dominant (Hansen and Bjørsvik, 2009; Andersen et al., 2013). In two previous studies evaluating fermented milk produced in Zimbabwe, Gran et al. (2003a) and Mutukumira et al. (2009) isolated *L. lactis* subsp. *lactis* biovar *diacetylactis*.

In the present study, we identified 3 clones displaying high similarity to *L. lactis* subsp. *lactis* biovar *diacetylactis* strain 4001C2, which was isolated in a previous study evaluating a simple and rapid PCR-based method for specific detection and identification of 10 common LAB from dairy products (Kong and Kong, unpublished data; National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Of the identified LAB in the present study, seven clones displayed high similarity to the *Lactobacillus* spp., *L. casei*, *L. paracasei* and *L. plantarum*. To our knowledge, one or several strains of these lactobacilli have previously been isolated in studies of African fermented milk (Feresu and Muzondo, 1990; Narvhus et al., 1998; Beukes et al.,

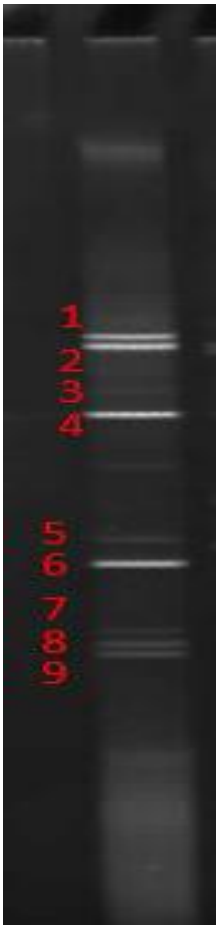

Figure 2. Denaturing gradient gel electrophoresis fingerprint of bacterial 16S rDNA amplicons of the aMasi sample.

Table 2. Representative of bacteria or clones and their relative abundance isolated from the aMasi sample.

Phylum	Band no.	Closest relative (obtained from BLAST search)	Identity (%)
Proteobacteria	8	*Enterobacter* sp. (GQ487560.1)	100
Firmicutes	1	*Lactococcus lactis* subsp. (JN194197.1)	95
Firmicutes	2	*Acinetobacter baumannii* (CP002522.2)	100
Firmicutes	4	*Clostridium acidurici* (HE582772.1)	94
Firmicutes	5	*Lactococcus lactis* (EF204360.1)	100
Firmicutes	6	*Lactococcus lactis* subsp. (JF895186.1)	91
Firmicutes	7	*Lactococcus lactis* subsp. (JN792511.1)	95
Firmicutes	9	Lactobacillales bacterium (AY581272.1)	93
Bacteria	3	Bacterium (FJ966227.1)	93

2001; Mathara et al., 2004).

Enterococci are LAB that are important in environmental, food and clinical microbiology. They are also of technical importance in the production of various fermented foods, but, due to the frequent association of strains, especially of *Enterococcus faecalis*, with nosoco-

comial infections, the use of enterococci in the food industry has become a controversial issue (Franz et al., 2005, 2011; Aguilar-Galves et al., 2012). Detection of *E. faecalis* in the present study is a concern, as the bacterium is an indicator of fecal contamination (Aguilar-Galves et al., 2012). Although, this bacterium has been reported in raw milk (Giannino et al., 2009) as well as domiati cheese (El-Zayat et al., 1995) and fermented milk products (El-Baradei et al., 2008), it represents a potential public health risk. As a cause of urinary tract infections, endocarditis and bacteremia, the severity of the risk is often related to transferable antibiotic resistance. Little scientific information is available on fermented milk products from South Africa. Beukes et al. (2001) investigated the microbial diversity of traditional fermented milks. Comparison of the bacterial diversity reported in the present study to that reported by Beukes et al. (2001) shows some similarities. *Lactococcus* was the dominant genus identified in both studies, comprising 65% in the present study versus 28% in the study of Beukes et al. (2001). However, they showed that genera belonging to *Leuconostoc* and *Lactobacillus* comprise 35 and 23% of the 336 bacteria isolated, respectively. The proportions of these genera were considerably lower in the present study. For example, only three out of 282 clones were identified as *Leuconostoc* in the present study.

Moreover, the bacterial diversity in aMasi was higher compared to that reported by Beukes et al. (2001). These findings displayed interesting differences in the microbial diversity of fermented milk products produced in the same country.

Other bacterial species of interest

As several bacterial species were retrieved in the present study that have either rarely or never previously been reported as a part of the microbial community of fermented milk, some general information is therefore presented in the following. Occurrence of pathogenic bacteria such as enterotoxigenic *E. coli*, *K. pneumoniae* and *C. freundii* in fermented milk products of Africa has previously been reported. *E. coli* has been shown to survive and grow in some traditional fermented milk (Feresu and Nayathi, 1990). In the present study, one clone showed 95% similarity to an *E. coli* described by Genoscope (unpublished data, NCBI).

In a study evaluating milk products produced at small-scale dairies in Zimbabwe, Gran et al. (2003b) reported *K. pneumoniae*. Four of the clones identified in the present study belonged to *K. pneumoniae* (Table 1). This bacterium is a well-known pathogen causing common bacterial pneumonia (Podschun and Ullmann, 1998; Aschbacher et al., 2011). The reason why *K. pneumoniae* is present in aMasi is most likely a result of contamination; handling of the milk, or via residues remaining in the containers, from which people may sometimes drink directly. Furthermore, 15 other clones detected in aMasi belonged to *Klebsiella* spp., with similarity between 95 to 98%, but these clones were not identified at species level. In the present study, 3 clones showed high similarity to *Klebsiella ornithinolytica* previously described by Drancourt et al. (2001) in a phylogenetic study of *Klebsiella* species, where the authors proposed *Raoultella* as genus name for cluster II of *Klebsiella* which includes *K. ornithinolytica.* To our knowledge, the present study is the first one reporting *K. ornithinolytica* in fermented milk.

The genus *Citrobacter* is taxonomically most closely related to *Salmonella* and *E. coli. Citrobacter* strains are normal inhabitants of human and animal intestine, but are also commonly distributed in natural environments such as soil, water, sewage and food (Sedlak et al., 1971). To our knowledge, the genus *Citrobacter* has only been reported in one previous study evaluating fermented milk (Gran et al., 2003b). One of the clones detected in the present study displayed high similarity to *C. freundii*. This bacterium is a human pathogen, but also quite normal in the human gut microbiota (Podschun and Ullmann, 1998; Aschbacher et al., 2011). Based on the present findings, we put forward the hypothesis that the bacterium might have been transferred to the milk sample by human contamination. To the authors' knowledge, the only information available on the detection of *S. sonnei* in fermented milk in the present study where clone EB-10A displayed high similarity to accession no. CP000038 described by Yang et al. (2005) in a study evaluating genome dynamics and diversity of *Shigella* species. *Aeromonas* is widely distributed in the environment, commonly occurring in surface water, sewage and both treated and untreated water (Szczuka and Kaznowski, 2004). One of the clones isolated from aMasi was identified as *A. sobria* which is reported to be a human pathogen causing gastrointestinal infection; often occuring due to direct contact with polluted water (Janda and Abbott, 2010). We suggest the presence of *A. sobria* may be derived from either human contamination, or to the poor water quality which is used to wash milking containers.

Information is available on *Acinetobacter* in fermented milk. *A. johnsonii* has been isolated from fermented milk from South Africa (Bauer et al., 2009). In the present study, clone EB-12 showed 97% similarity to *A. johnsonii* previously reported by Kim and Lee (unpublished data, NCBI). Clone library DGGE analysis revealed that 1 band showed 100% similarity to *Acinetobacter baumannii*, a bacterium previously reported by Chen et al. (2011) in a study evaluating the genome sequence of a multidrug-resistant *A. baumannii* strain. The detection of *Acinetobacter* in the present study is likely due to water used to wash the milking containers. The genus *Kluyvera*

is characterized as opportunistic pathogen. Infections are rare but have increasingly been reported (Isozaki et al., 2010). In two previous studies, *Kluyvera* was isolated as a part of the microbial ecosystem of Munster and Epoisses cheese (Ogier et al., 2004) and in Egyptian Domiati cheese (El-Baradei et al., 2007). In the present study, two clones were identified as *K. cryocrescens*. *Enterobacter* spp. are commonly reported for water, sewage and in the intestines of warm-blooded animals, and some species can cause human infections (Madigan et al., 2009). However, the genus has also been isolated from fermented milk products from Nigeria (Adebesin et al., 2001) and from aMasi, comprising three clones in the present study. DGGE analysis displayed 100% similarity of 1 band to *Clostridium acidurici*, previously reported by Yarza et al. (unpublished data, NCBI) in a study sequencing orphan species. In rural households, the production of the milk products is often performed in an environment near humans and animals, leading to contamination of the milk. The isolation of pathogenic bacteria in this study is of great concern for local public health, especially in rural areas of KwaZulu-Natal, where communities have poor access to regular human and animal health care services, and suffer high levels of HIV/AIDS, *Mycobacterium tuberculosis* and malnutrition (Geoghegan, 2012). It should therefore be a topic of further investigations.

It is worth noticing that we were unable to identify two important zoonotic agents known to be shed in milk by infected cattle, that is, *Brucella abortus* and *Mycobacterium bovis.* These pathogens are present in the cattle reservoir in our study area and are of concern in many parts of Africa (Marcotty et al., 2009). It is therefore important to regularly check aMasi in order to take into account intermittent shedding of such pathogens in milk. Special attention should be given to cows that are at high risk of shedding pathogens in milk, that is, cows in the first three months after calving.

Conclusion

Traditional fermented milk products are common in rural communities in developing countries as it is an inexpensive and easy way to conserve milk (Mensah, 1997; Gadaga et al., 1999). The temperature in South Africa is seasonally high (>20 to 40°C), and without access to cold storage facilities, milk will rapidly deteriorates. Fermentation is therefore a natural way to avoid degradation. Consumption of fermented milk products can reduce the risk of infection by opportunistic human pathogens. A diet consisting of a large part of fermented milk products is therefore important in several African countries such as Zimbabwe, Nigeria, Tanzania, Kenya, Uganda, Ethiopia and South Africa (Franz and Holzapfel, 2011). Studies of microbial diversity and the potential of functional strains in traditional fermented milk products can lead to better knowledge of the health effects related to these products (Patrignani et al., 2006).

A great part of the clones from the clone library and DGGE bands from aMasi were identified as LAB. In addition, species within the family *Enterobacteriaceae* were detected, some of which are often associated with contamination from human, environmental and animal sources. The detection of these bacterial species in fermented milk in different regions of Africa is not unexpected, since the milk is produced without any food safety control (Narvhus et al., 1998; Gadaga et al., 1999; Narvhus, 2003; Ukeyima et al., 2010). The present study provides new information about the microbial community within a traditional fermented milk product, and provides insight into potential health problems that can remain undetected using traditional culture techniques. Ideally, using molecular methods will therefore improve the focus of rural public health programs, enabling an improved targeting of behavioral and resource changes that may reduce the potential for zoonosis in vulnerable communities.

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