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Diversity and dynamics of bacterial populations during spontaneous sorghum fermentations used to produce *ting*, a South African food

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ABSTRACT

Ting is a spontaneously fermented sorghum food that is popular for its sour taste and unique flavour. Insight of the microbial diversity and population dynamics during sorghum fermentations is an essential component of the development of starter cultures for commercial production of *ting*. In this study, bacterial populations associated with spontaneous sorghum fermentations were examined using a culture-independent strategy based on denaturing gradient gel electrophoresis and sequence analysis of V3-16S rRNA gene amplicons, and a culture-dependent strategy using conventional isolation based on culturing followed by 16S rRNA and/or *pheS* gene sequence analysis. The entire fermentation process was monitored over a 54 h period and two phases were observed with respect to pH evolution and microbial succession. The first phase of the process (0–6 h) was characterized by relatively high pH conditions and the presence of *Enterococcus mundtii*, albeit that this species was only detected with the culture-dependent approach. The second phase of the fermentation process (12–54 h) was characterized by increased acidity and the predominance of a broader range of lactic acid bacteria, including *Lactococcus lactis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Weissella cibaria*, *Enterococcus faecalis*, and a close relative of *Lactobacillus curvatus*, as well as some members of the *Enterobacteriaceae* family. The *Lb. curvatus*-like species was only detected with PCR-DGGE, while the majority of the other species was only detected using the culture-dependent approach. These findings highlighted the fact that a combination of both approaches was essential in revealing the microbial diversity and dynamics during spontaneous sorghum fermentations.

Introduction

Sorghum is widely cultivated in arid and semi-arid regions of the world, and is considered to be the fifth most important cereal after wheat, maize, rice and barley [19]. However, sorghum as a main dietary constituent is usually associated with under-nourishment due to the lack of certain essential amino acids [38,57] and the presence of anti-nutritional factors [31]. These shortcomings may be overcome by fermentation, which not only improves the nutritive value of the commodity, but also enhances its sensory properties [4,11,32]. As a result, various sorghum-based fermented foods with unique and appealing characteristics are produced wherever sorghum is cultivated. These include *injera* [23], *kisra* [51], *ogi* [2], *mahewu* [7], *uji* [48], *muramba* [52], *bushera* [53], *togwa* [46], and *ting* [6].

Ting is a sour porridge made by cooking fermented sorghum [6]. It is frequently used as a weaning food for infants in rural South Africa because it is inexpensive to prepare and does not require refrigeration or re-heating prior to consumption [43]. Due to its appetizing taste, adults also consume *ting* at major ceremonies such as weddings and funerals. In such traditional preparations, sorghum undergoes spontaneous and uncontrolled fermentation steered by microflora endogenous to the sorghum, as well as those associated with the preparation equipment and local environments. Consequently, conventional *ting* preparations vary greatly with respect to product quality, taste and acceptability [69]. Also, little is known about the microorganisms that participate in this fermentation. Although lactic acid bacteria [LAB] were previously isolated from fermented sorghum and identified as *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus rhamnosus* [47], their involvement in the *ting* fermentation process and the possible role of other microbes remains to be determined. Such information is crucial for developing starter cultures that result in reduced fermentation time and *ting* with consistent microbiological and sensory qualities.

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To study the diversity and dynamics of microbial populations associated with specific fermented foods, a combination of culture-dependent and culture-independent approaches are typically applied. Culture-dependent approaches represent the only means of recovering microorganisms from the fermented substrate [50], although it is widely recognized that they do not allow analysis of true diversity and/or population dynamics [3,49,58]. On the other hand, various studies have shown that culture-independent approaches, such as denaturing gradient gel electrophoresis of 16S rRNA gene amplicons (PCR-DGGE), provide a more reliable and reproducible means for studying microbial populations in complex food ecosystems [9,16,65,75].

In this study, the diversity and dynamics of the microbial populations associated with the process of *ting* sorghum fermentation were investigated using a combination of culture-dependent and culture-independent methods. PCR-DGGE of the V3 region of the 16S rRNA gene was used to monitor the succession of dominant microbial populations from the onset to the end of spontaneous sorghum fermentations. In parallel, conventional culturing and sequence-based approaches using the 16S rRNA and phenylalanyl-tRNA synthase (*pheS*) genes were employed to enumerate, isolate and identify the bacterial groups involved in these fermentations. This study is the first to investigate extensively the bacterial diversity and dynamics in the sorghum fermentation process, and is relevant for future development of appropriate and effective starter cultures.

Materials and methods

Sorghum fermentations

Fermentations were conducted at three different temperatures with sorghum flour obtained from two commercial sources in South Africa (i.e., King Food Corporation, Potchefstroom, and Nola Pvt Ltd., Randfontein). Individual sorghum slurries were prepared by mixing the respective sorghum flours thoroughly with sterile luke-warm (*ca.* 40 °C) distilled water (1:1 [w/v]) in sterile glass containers. The glass containers were then covered and incubated at 20, 25 and 30 °C for 54 h. These fermentations were undertaken over a period of six months (September 2004 to February 2005; one fermentation per month) and repeated during the following six months (March–August 2005). During the incubation period, 50 mL samples of the fermenting sorghum slurries were aseptically collected at the start of fermentation ($t = 0$ h) and at 6-h intervals until the end of fermentation ($t = 54$ h). Each of the collected samples was divided into one 20 mL and two 15 mL aliquots, which were respectively used for culture-dependent analyses, pH determination with a Beckman model Ø 34 pH meter (Beckman Coulter, Fullerton, CA, USA), and immediately frozen and stored at −20 °C for later DNA extractions.

Enumeration, isolation and primary phenotypic characterization of bacteria

For culture-based enumeration and isolations, 10 g of the fermented sorghum were added to 90 mL of buffered peptone water (0.1% [w/v] peptone, 0.85% [w/v] NaCl; pH 7.2) and vortexed for 3 min to obtain homogenous mixtures. From these mixtures, 10-fold dilutions were prepared in the same diluent and then surface inoculated onto the following selective media: MRS-5 [49] (Oxoid, Basingstoke, UK) for lactobacilli, M17 (Oxoid) for lactococci, ESA (*Enterococcus* selective agar; Merck, South Africa) for enterococci, violet red bile agar (VRBA, Oxoid) to obtain Gram-negative counts (GNC) and plate count agar (PCA, Oxoid) to obtain numbers of total aerobic bacteria. Plates were incubated at 30 °C for 24–48 h for MRS-5, M17 and ESA, 37 °C for 24–48 h for VRBA, and 30 °C for 72 h for PCA. For total aerobic and LAB counts, plates with between 30 and 300 colonies were selected for enumeration, while plates containing between 15 and 150 colonies, >0.5 mm in diameter, were selected for determining GNC on VRBA. The counts were recorded as averages of three determinations and expressed as colony forming units (cfu) g^{-1}.

Following incubation, colonies on the various growth media were grouped according to macroscopic and microscopic appearance. Representative colonies were then randomly picked and subcultured to obtain pure cultures, after which they were routinely grown in the appropriate broth media. LAB isolates were grown in MRS broth, while enterobacterial isolates were grown in nutrient broth using the incubation conditions described. All pure cultures were examined using light microscopy to score cell morphology, motility and Gram stain. Catalase and oxidase activity were tested using 3% hydrogen peroxide and tetramethyl-*p*-phenylenediamine (TMPD), respectively [64]. Randomly selected isolates were also subjected to carbohydrate profile analysis using the API 50 CL and API 20E systems (API Systems, bioMérieux, France). All cultures were stored at −20 °C in sterile Eppendorf tubes containing the appropriate broth media supplemented with 20% (v/v) glycerol as a cryoprotectant.

16S rRNA and pheS sequence-based identification of pure cultures

DNA was extracted from pure cultures using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) or the method described by Pitcher et al. [62]. The 16S rRNA gene was amplified for each of the isolated pure cultures with the eubacterial universal primers 27F [44] and 1507R [34]. PCR mixtures contained 0.05 U μL^{-1} of *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and PCR buffer containing NH_2SO_4 (Fermentas), 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 1–2 ng μL^{-1} of template DNA and 8% (v/v) dimethyl sulphoxide. The PCR cycling conditions consisted of denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 30 s, extension at 72 °C for 1 min, and a final extension for 10 min at 72 °C. The resulting 16S rRNA amplicons were sequenced with the original primers by Inqaba Biotech (Pretoria, South Africa). The *pheS* gene was amplified with primers PheS-21-F and PheS-23-R, as described by Naser et al. [56]. The resulting *pheS* amplicons were purified using the Nucleofast 96 PCR clean-up membrane system (Machery-Nagel, Germany) and sequenced using the original *pheS* PCR primers, the BigDye® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems).

All raw sequence files were inspected and corrected, where necessary, using Chromas Lite 2.0 (Technelysium) and BioEdit v. 5.0.9 [28]. Sequences were compared to those in the nucleotide database of the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) using *blastn* to obtain preliminary identifications for the isolated bacteria. To refine these identifications, the sequences generated in this study were subjected to phylogenetic analyses. For this purpose, 16S rRNA and *pheS* gene sequence alignments were generated using multiple sequence alignment based on Fast Fourier Transform (MAFFT v. 6) [37]. These alignments also included the sequence information for the relevant type strains of species in the genera *Lactobacillus*, *Weissella*, *Lactococcus* and *Enterococcus*, which were obtained from GenBank or from a taxonomic reference framework of *pheS* consisting of sequences for *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* strains [70]. To determine the best-fit evolutionary models for the datasets, PAUP* v. 4.0b1 [72], together with Modeltest v. 3.7 [63], were used. The calculated parameters were then used to construct neighbor-joining (NJ)

Table 1
Average microbial counts (cfu g^{-1}) at the start and end of spontaneous sorghum fermentations (25 °C) obtained on different selective media.

Isolation medium[a]	Targeted groups	Time point	Commercial sorghum flour[b]	
			K.F.C.	Nola
VRBA	Gram-negative bacteria	$t = 0$ h	8.45×10^3	8.3×10^3
		$t = 54$ h	9.0×10^6	8.8×10^6
ESA	Enterococci	$t = 0$ h	2×10^3	1.8×10^3
		$t = 54$ h	3.62×10^8	3.5×10^8
MRS-5	Lactobacilli	$t = 0$ h	9×10^3	9.1×10^3
		$t = 54$ h	3.91×10^9	3.89×10^9
M17	Lactococci	$t = 0$ h	3×10^3	2.8×10^3
		$t = 54$ h	2.8×10^9	2.92×10^9
PCA	Aerobic bacteria	$t = 0$ h	1.9×10^5	1.5×10^5
		$t = 54$ h	3.9×10^9	2.3×10^9

[a] See text for information on suppliers of media.
[b] K.F.C. and Nola refer to commercial sorghum flour that was obtained from King Food Corporation and Nola Pvt Ltd., respectively.

distance-based [67] phylogenetic trees with PAUP*, and maximum likelihood (ML) trees with PhyML v. 2.4.3 [27]. Branch support was estimated with non-parametric bootstrap analysis based on 1000 replicates and the same parameters as before.

PCR-DGGE

In order to prepare high quality DNA for PCR-DGGE analyses, individual 15 mL samples were vortexed for 10 min in the presence of glass beads (2% [v/v], 150 μm-diameter beads; Sigma–Aldrich) and then centrifuged for 5 min at $1500 \times g$ to remove the beads and remaining large sorghum particles. The bacterial cells in 1.5 mL of the individual supernatants were harvested by centrifugation at $5000 \times g$ for 15 min. The pelleted cells were frozen at −20 °C for 2 h and washed with sterile distilled water, after which DNA was extracted using the Wizard® Genomic DNA Purification Kit.

For PCR-DGGE analysis, the V3 region of the bacterial 16S rRNA gene was amplified with primer 518R and primer F357-GC, which has a 40-base GC-clamp at its 5′-end [55]. PCR was performed as described previously [75] on a MyCycler™ thermal cycler (BioRad, Hercules, USA). The 16S rRNA V3 region was also amplified from 13 different bacterial reference strains, and the resulting amplicons were combined for use as a DGGE reference standard [75]. The reference standard comprised amplicons from *Bacteroides fragilis* (DSM 1396), *Bacteroides thetaiotaomicron* (LMG 11262), *Weissella cibaria* (LMG 17699), *Enterococcus flavescens* (LMG 13518T), *E. solitarius* (LMG 12890T), *Leuconostoc fructosum* (LMG 9498T), *Bacillus subtilis* (LMG 7135T), *Clostridium butyricum* (LMG 1212), *Lb. rhamnosus* (LMG 6400T), *Bifidobacterium longum* subsp. *longum* (LMG 13197T), *Bifidobacterium bifidum* (LMG 11041T), *Bifidobacterium animalis* subsp. *lactis* (LMG 18314T), and *Bifidobacterium dentium* (LMG 11045T). DGGE was performed according to the procedure described by Muyzer et al. [55] with slight modifications, as described previously [75]. All PCR-DGGE experiments were performed in duplicate. The inclusion of the reference standard on gels allowed for digital normalization and comparison of the fragment profiles on different DGGE gels by using BioNumerics v.4.0 [75].

Sequence analysis of PCR-DGGE fragments

To determine the likely taxonomic identity of the bacteria for which individual 16S rRNA V3 PCR-DGGE fragments were generated, the most intense fragments at each of the different fermentation sampling time points ($t = 0$ h to $t = 54$ h) were excised from DGGE gels. The gel slices were then incubated overnight at 4 °C in sterile distilled water [68], after which the eluted DNA was used as template to re-amplify the specific fragments using the same PCR and cycling conditions as described above. After confirming that the re-amplified fragments co-migrated with the expected 16S rRNA V3 PCR fragments of the original samples on DGGE gels, the re-amplified fragments were subjected to another round of PCR using primers 518R and 357F to remove the GC-clamp from the amplicons. Primer 357F is the same as primer F357-GC, except that is lacks the GC-clamp at its 5′-end [55]. The resulting products were then purified with the QIAquick PCR purification kit (QIAGEN, Germany) and sequenced using primers 518R and 357F, and the same procedure as described above for the *pheS* amplicons. The resulting raw sequence data were inspected and corrected where necessary and individual 16S rRNA V3 sequences were compared using *blastn* to those in the NCBI nucleotide database. By making use of BioEdit, the 16S rRNA V3 sequences were also compared to the complete 16S rRNA sequences obtained for the pure cultures.

Results

Enumeration and primary characterization of bacteria

Enumeration of total and specific groups of bacteria was carried out on five different types of media (PCA, MRS-5, M17, VRBA and ESA). However, these enumerations were performed for the fermentations at 25 °C only, as similar PCR-DGGE profiles were obtained at all three fermentation temperatures (see below). Total bacterial counts were initially low, but increased considerably towards the end of the fermentation (Table 1). Although LAB were predominant on most media (except VRBA), none of the media were sufficiently specific to allow growth of only particular LAB species. For example, both Gram-positive cocci and rods were isolated indiscriminately from MRS-5, ESA and M17 media.

Based on macroscopic and microscopic observations, 192 isolates associated with the fermentation process using both sorghum sources were randomly selected. Of these, all Gram-positive, catalase-negative, non-motile and oxidase-negative isolates were considered presumptive LAB, and all Gram-negative, oxidase-negative and catalase-positive rods were considered presumptive *Enterobacteriaceae*. Carbohydrate fermentation profile comparisons further allowed a set of 32 isolates representative of the diversity of bacteria dominant at the beginning and end of the fermentation process to be selected. This set of 32 isolates was subsequently subjected to 16S rRNA and *pheS* sequence-based identification (Table 2).

16S rRNA and pheS sequence-based identification of pure cultures

Two separate 16S rRNA data sets were created to determine the possible identities of the isolates from this study (GenBank

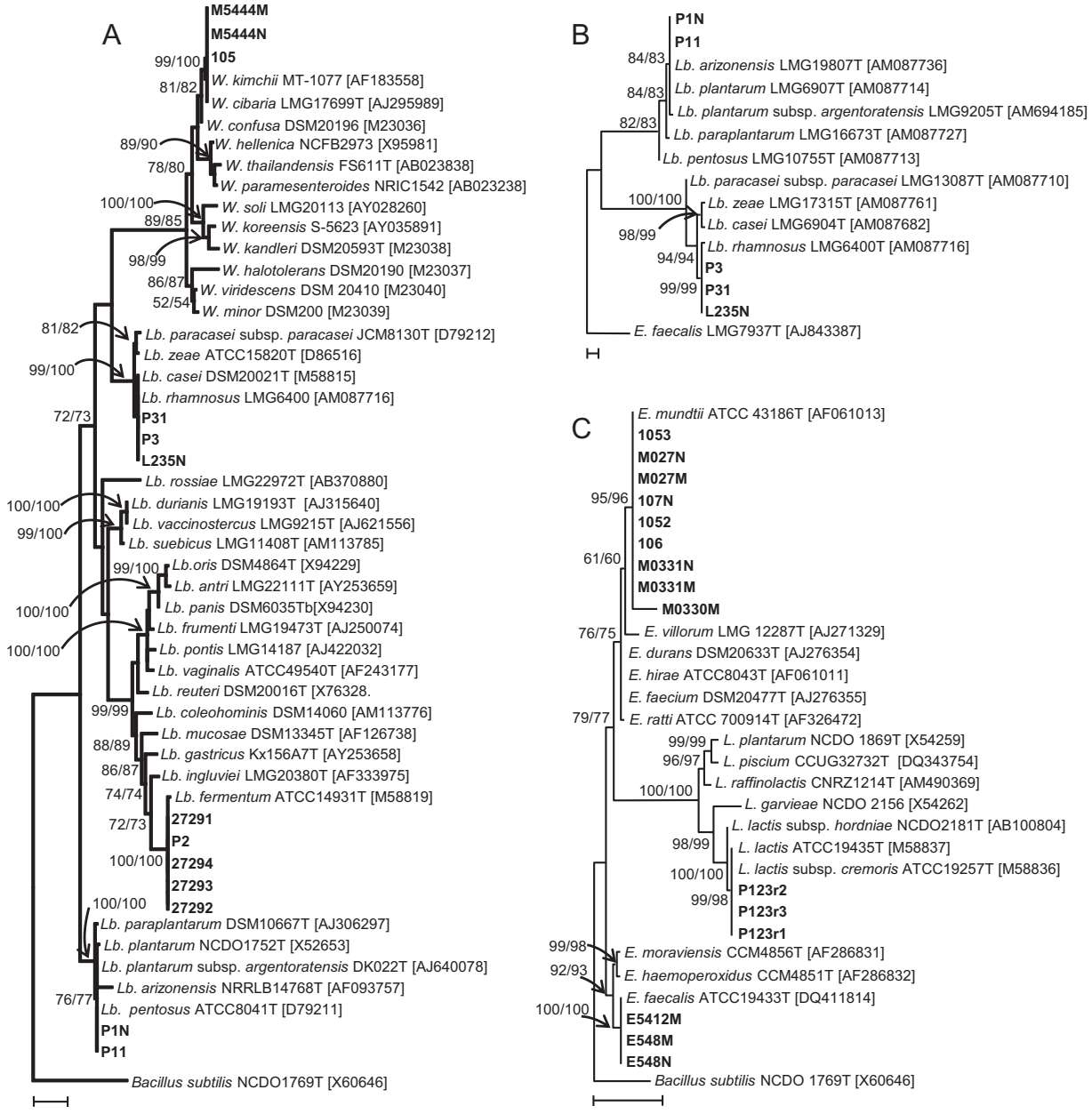

Fig. 1. Phylogenies for the bacterial isolates obtained from spontaneously fermented *ting* sorghum. Panels A and B illustrate the results of maximum likelihood (ML) phylogenetic analyses based on 16S rRNA and *pheS* gene sequences, respectively, for species in the genera *Lactobacillus* and *Weissella*. Note that *W. kimchii* and *Lb. arizonensis* are later heterotypic synonyms of *W. cibaria* [15] and *Lb. plantarum* [40], respectively. The results of the 16S rRNA ML analysis for species in the genera *Lactococcus* and *Enterococcus* are indicated in panel C. In all cases, trees with similar topologies were generated with neighbor-joining (NJ) distance analysis, and bootstrap branch support values (>60%) are indicated at the internodes (ML/NJ). Isolates obtained from fermented sorghum are indicated in bold. For the type strains, GenBank sequence accession numbers are indicated in brackets. *Bacillus subtilis* (A and C) and *Enterococcus faecalis* (B) were used as outgroups. For the respective analyses, the two 16S rRNA datasets utilized Tamura and Nei's [73] substitution model and the *pheS* dataset utilized the General Time Reversible model [66], all three with a proportion of invariable sites and gamma correction for among site variation.

accession numbers GU372694–GU372721). One included 16S rRNA gene sequences for the putative *Lactobacillus* and *Weissella* species (i.e., the Gram-positive rod-shaped bacteria), while the other included putative *Lactococcus* and *Enterococcus* species (i.e., the Gram-positive coccoid bacteria). These alignments also included the full-length or near full-length 16S rRNA sequences for the type strains of the species in these genera that were obtained from GenBank. Within the *Lactobacillus* and *Weissella* phylogeny (Fig. 1A), isolates P11 and P1N formed part of a clade containing *Lb. plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus* and *Lactobacillus arizonensis*, while P31, L235N and P3 were most closely related to *Lb. rhamnosus* and *Lactobacillus casei*. The 16S rRNA sequence similarities between the taxa within the two clades were respectively >99.9%. However, phylogenetic analysis based on aligned *pheS* sequences (Fig. 1B) allowed unambiguous assignment of isolates P11 and P1N to *Lb. plantarum* (>99.91% *pheS* sequence similarity), and isolates P31, L235N and P3 to *Lb. rhamnosus* (>99.96% *pheS* sequence similarity) (GenBank accession numbers GU372722–GU372726). The 16S rRNA phylogeny (Fig. 1A) indicated that isolates 27291, 27292, 27293, 27294 and P2 probably represented *Lb. fermentum* (99.99% sequence similarity), and isolates M5444M, M5444N and 105 were most closely related to *W. cibaria* (99.99% sequence similarity). Within the *Lactococcus* and *Enterococcus* phylogeny (Fig. 1C), isolates P123r1, P123r2, P123r3 grouped with *Lactococcus lactis*, isolates M027N, M027M, M0331M, M0331N, 107N, 106, 1052 and 1053 grouped with *Enterococcus*

Table 2
Likely species identities of bacteria isolated at the beginning and end of sorghum fermentations.

Isolate	Sorghum source[a]	Sampling time (h)	Identification[b]
M027M	K.F.C.	0	*E. mundtii*
M027N	Nola	0	*E. mundtii*
M0331M	K.F.C.	0	*E. mundtii*
M0331N	Nola	0	*E. mundtii*
M030M	K.F.C.	0	*E. mundtii*
107N	Nola	0	*E. mundtii*
106	K.F.C.	0	*E. mundtii*
1052	Nola	0	*E. mundtii*
1053	K.F.C.	0	*E. mundtii*
E548M	K.F.C.	54	*E. faecalis*
E548N	Nola	54	*E. faecalis*
E5412M	K.F.C.	54	*E. faecalis*
P11	K.F.C.	54	*Lb. plantarum*
P1N	Nola	54	*Lb. plantarum*
P3	K.F.C.	54	*Lb. rhamnosus*
P31	Nola	54	*Lb. rhamnosus*
L235N	Nola	54	*Lb. rhamnosus*
P2	K.F.C.	54	*Lb. fermentum*
27291	K.F.C.	54	*Lb. fermentum*
27292	Nola	54	*Lb. fermentum*
27293	Nola	54	*Lb. fermentum*
27294	Nola	54	*Lb. fermentum*
M5444M	K.F.C.	54	*W. cibaria*
M5444N	Nola	54	*W. cibaria*
105	K.F.C.	54	*W. cibaria*
P123r1	K.F.C.	54	*L. lactis*
P123r2	Nola	54	*L. lactis*
P123r3	Nola	54	*L. lactis*
V5422M	K.F.C.	54	*Enterobacteriaceae*
V5423N	Nola	54	*Enterobacteriaceae*
V5430	Nola	54	*Enterobacteriaceae*
V5431	K.F.C.	54	*Enterobacteriaceae*

[a] Sorghum source refers to the commercial company that produced the sorghum flour (K.F.C. for King Food Corporation and Nola for Nola Pvt Ltd.).
[b] Identification obtained with sequence analysis of 16S rRNA and *pheS* genes (Fig. 1).

mundtii, and isolates E548N, E5412M and E548M clustered with *Enterococcus faecalis* (in all cases with 16S rRNA sequence similarity values >99.9%).

PCR-DGGE analysis

The diversity and dynamics of microflora during spontaneous fermentation of sorghum flour were studied using PCR-DGGE of the *ca*. 250-bp V3 hypervariable region of the 16S rRNA gene. Based on the PCR-DGGE profiles and pH of the growth medium, the overall fermentation process appeared to be characterized by two phases (Fig. 2). The first phase (0–6 h) was associated with a relatively small change in pH (6.64–6.0) and DGGE profiles showing extremely low complexity, mainly consisting of one intense fragment that was already present at $t = 0$ h. The second phase (12–54 h) was characterized by a more complex DGGE pattern including 12 major fragments most of which remained present until the end of fermentation. During this phase of the fermentation, the pH also dropped from 5.69 to 3.79, after which it remained constant. Similar pH and PCR-DGGE profiles were obtained for the fermentations with both of the commercial sorghum flour brands included in this study. All duplicate samples generated identical PCR-DGGE profiles during the course of spontaneous fermentation, emphasizing the reproducibility of this method throughout the study. Likewise, similar pH and PCR-DGGE profiles were obtained for the fermentations conducted at 20, 25 and 30 °C.

In order to assign species identities to the major PCR-DGGE fragments visualized during the fermentation process (Fig. 2), the V3-16S rRNA sequences from extracted and purified DGGE fragments were compared to full-length 16S rRNA gene sequences obtained for the pure cultures (Fig. 1). These analyses showed that DGGE fragments 10 and 12 probably corresponded with members of the LAB (i.e. *L. lactis* and *W. cibaria*) as their sequences were identical to those determined respectively for the isolates representing these species (see Fig. 1). Fragments 2–8 probably represented members of the *Enterobacteriaceae* genera *Pantoea* or *Enterobacter*, as their sequences displayed ≥98% similarity to the sequences obtained from GenBank for *Pantoea* spp. (e.g., *Pantoea agglomerans*; GenBank accession number AB571245) and *Enterobacter* sp. (*Enterobacter* sp. strain s172; GenBank accession number HM196843), as well as those for isolates V5422M, V5423N, V5430 and V5431. The sequence for PCR-DGGE fragment 11 did not match the full-length 16S rRNA gene sequences obtained for any of the pure cultures. According to the *blastn* results, this fragment shares 98% sequence similarity with that of the 16S rRNA sequence of *Lactobacillus curvatus*. Fragment 1, the only one associated with the first fermentation stage (0–6 h), most likely represented the sorghum chloroplast 16S rRNA gene based on its high sequence similarity (98%) to that of the model monocotyledonous plant *Zea mays*.

Fragment 10, assigned to *L. lactis*, appeared at $t = 18$ h and remained present until the end of fermentation in all experiments. Once it appeared, the relative intensity of this fragment remained constant or even increased throughout fermentation. In contrast, fragments representing other bacterial groups, especially those assigned to *Enterobacteriaceae*, decreased during the overall fermentation process.

Bacterial diversity detected with culture-independent and culture-dependent approaches

Table 3 shows a comparison of the bacterial diversity associated with spontaneous sorghum fermentations assessed using culture-independent and culture-dependent approaches. Overall, many bacterial groups in the *ting* samples remained undetected using PCR-DGGE. For example, *E. mundtii* was isolated at time $t = 0$ h (Table 2) but was not represented by a fragment in the DGGE profiles obtained from the corresponding sample. Also, culture-dependent approaches allowed isolation and identification

Table 3
Comparison of the bacterial diversity in *ting* sorghum fermentations at 25 °C using culture-independent and culture-dependent approaches.

Time of detection	Culture-independent approach[a]	Culture-dependent approach[b]
$t = 0$ h	None	*E. mundtii*
$t = 54$ h	*Lactococcus lactis* *Weissella cibaria* *Lactobacillus curvatus* *Enterobacteriaceae* (4 distinct sequences)	*L. lactis* *W. cibaria* *E. faecalis* *Lb. plantarum* *Lb. fermentum* *Lb. rhamnosus* *Enterobacteriaceae* (4 isolates)

[a] Identifications are based on sequence analysis of the V3 region of the 16S rRNA gene after PCR-DGGE (Fig. 2).
[b] Identifications are based on the results of phylogenetic analyses using the 16S rRNA and *pheS* genes (Fig. 1).

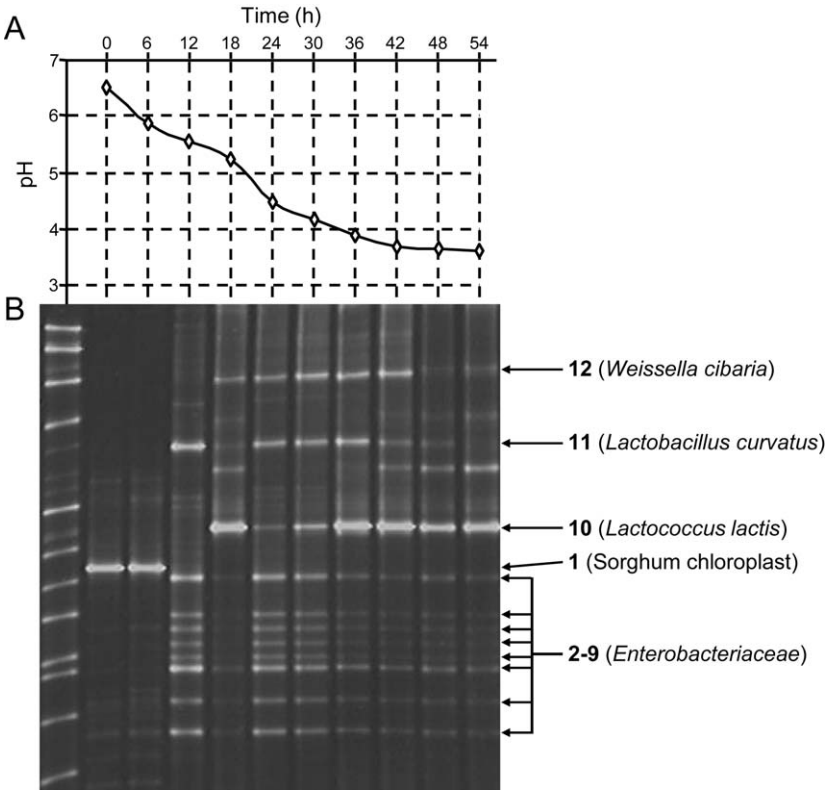

Fig. 2. Change in pH and bacterial diversity in spontaneously fermenting sorghum at 25 °C. Panel A depicts the change in pH of the fermentation, where each diamond represents the average of four independent measurements. In panel B, PCR-DGGE banding patterns representing the V3 region of the 16S rRNA gene for the bacteria involved in the fermentation are shown. The first lane of the DGGE gel contains the reference standard consisting of the V3-16S rRNA gene amplicons of 13 reference strains. The 12 amplicons that were sequenced and analyzed are indicated with arrows to the right of the gel image. The likely identities of the corresponding bacteria, as inferred from phylogenetic (Fig. 1) and *blastn* analyses, are indicated in brackets.

of *Lb. plantarum*, *Lb. rhamnosus* and *Lb. fermentum* at time $t = 54$ h, whereas their corresponding V3-16S rRNA fragments were absent from the PCR-DGGE profiles at comparable time points (Table 3). On the other hand, an isolate presumably representing *Lb. curvatus*, that was predominant in PCR-DGGE profiles at time points $t = 42$–54 h, could not be isolated from the fermented samples despite numerous attempts.

Discussion

Spontaneous sorghum fermentations used in the preparation of *ting* were characterized by two distinct phases linked to changes in bacterial diversity and pH. There was an apparent shift in microbial diversity from low-complexity PCR-DGGE profiles and relatively high pH to more complex PCR-DGGE profiles and increased acidity (Fig. 1). During the first phase, bacterial counts were very low ($<8.5 \times 10^3$ cfu g^{-1}, Table 1) and thus below the detection limit of PCR-DGGE. The single intense DGGE fragment dominating the first fermentation phase represented the V3 region of the 16S rRNA gene encoded on the sorghum chloroplast. In this respect, the finding of a chloroplast-derived amplicon is another example of the cross-reactivity of 16S rRNA gene-based PCR primers with eukaryotic DNA [36]. During the second phase, PCR-DGGE detected *L. lactis* which remained dominant until the end of the experiment. In addition, *W. cibaria* and the putatively identified *Lb. curvatus* were also among the predominant species at the end of the fermentations. These results are in agreement with the generally accepted concept that traditional fermentations are dominated by a few microbial species that are selected during the course of fermentation as a result of physiological and metabolic adaptation to the food matrix [29,35]. The relative dominance of the homofermentative *L. lactis* over other bacteria may be due to its tolerance to low pH, which may reduce growth of heterofermentative LAB [45]. Therefore, *L. lactis* can be considered as one of the most well-adapted organisms in sorghum fermentation processes. Provided that this observation is further substantiated by additional quantitative assessments, *L. lactis* strains thus may be used to control sorghum fermentations resulting in standardized *ting* products.

The LAB species associated with the production of *ting* have previously been implicated in the production of a range of fermented cereals with desirable sensory properties. *Lb. plantarum* has been isolated from fermented cereals such as *ogi* [61], *mageu* [33] and *kunun zaki* [22], while the heterofermentative *Lb. fermentum* was previously isolated from *mawe* [35] and *kenkey* [30]. Isolates identified as '*Lb. curvatus-Lb. sake*' were recovered from sorghum-based fermented weaning food [42]. Also, enterococci have been shown to form part of the microflora of certain cheeses and are responsible for sensory characteristics of the final product [20]. However, a number of bacterial taxa which are not commonly associated with food were also isolated from the spontaneously fermented sorghum. The majority of these are naturally associated with plants and are commonly found on plant-based material [51,60]. For example, the plant-associated *E. mundtii* [12] was isolated at the beginning of the process and has been reported as an environmental contaminant [9]. The same is also true for *Enterobacteriaceae* such as *Pantoea* species that generally represent plant endophytes [41], albeit that some *Pantoea* species are associated with human diseases [13].

One of the broadly recognized advantages of subjecting food materials to LAB-steered fermentation processes is the inhibitory effect this has on the growth of other microorganisms, especially food-borne pathogens [39,59]. Several previous studies have

shown that the growth of members of the *Enterobacteriaceae* and other bacteria is inhibited by the lactic acid produced during fermentation [1,60]. Some strains of *E. faecalis* and *E. faecium* may also produce bacteriocins that are active against various food-borne pathogens [21], making them suitable candidates for controlling emerging pathogens during food fermentation [8]. However, despite the prevalence of enterococci in fermented sorghum and other food products [24,26], species such as *E. faecalis* and *E. faecium* have been associated with infection that pose challenges to food safety [10]. In the current study, the presence of bacteria representing *Enterobacteriaceae* such as *Pantoea* species and *E. faecalis* during the end of the fermentations might be due to acid resistance or the presence of microenvironments in the food matrix that support the growth of these bacteria [5,76]. Overall, however, the intensity of PCR-DGGE fragments corresponding to *Enterobacteriaceae* decreased as that of *L. lactis* increased (Fig. 2). It is therefore likely that the unfavourable environment created by this and the other LAB slowed down the growth of *Enterococcus* species and the *Enterobacteriaceae* as fermentation progressed.

The comparison of culture-independent and culture-dependent approaches used in this study highlights the limitations of each approach. Some species were not detected using culture-based methods, while others were not detected using culture-independent methods. PCR-DGGE showed a significant increase in the putatively identified *Lb. curvatus* numbers from time points $t = 42–54$ h of the fermentation process, but were not isolated from the food matrix. This may be due to it entering a viable but non-cultivable state, characterized by metabolically active cells that do not produce colonies on both selective and non-selective media [25], thus illustrating one of the main benefits of culture-independent approaches over culture-dependent methods [25,54]. Overall, however, the use of culture-dependent methods allowed identification of a higher number of bacterial species from fermented sorghum than culture-independent methods (Table 3). PCR-DGGE did not allow the detection of *E. faecalis*, *E. mundtii*, *Lb. rhamnosus*, *Lb. plantarum* and *Lb. fermentum* that were isolated on culture media at the end of fermentation. This was probably because the bacteria occurred in numbers below the detection limit of PCR-DGGE [54]. In addition, biases at the level of DNA extraction and PCR specificity and efficiency [9,14,17] could also have played a role. The inability of PCR-DGGE to detect all the bacterial species associated with fermented sorghum was also observed for sourdough [49,71] and whey cultures of water buffalo mozzarella [16]. PCR-DGGE results are also strongly influenced by the intraspecific heterogeneous nature of the 16S rRNA gene region targeted [18,74], which might be the case for putative *Pantoea* and *Enterobacter* spp. detected in this study (Fig. 2).

A previous culture-dependent investigation of the microbial diversity in fermented sorghum [47] revealed the presence of some of the species also found in the current study (i.e., *Lb. rhamnosus*, *Lb. fermentum* and *Lb. plantarum*). In the earlier study, however, species such as *L. lactis* previously remained undetected. In the current study, the addition of nutritional supplements to molten MRS medium [49] probably improved the chances of isolating this species. Likewise, the use of ESA and M17 agar improved the probability of recovering *E. faecalis* that was isolated at the end of fermentation in this study, but which could not be detected on MRS in the previous study. Accurate and efficient description of bacterial populations during sorghum fermentations was therefore strongly dependent on the combined application of culture-independent and culture-dependent approaches.

The production of *ting* from spontaneously fermented sorghum flour results in immense variation in the sensory characteristics and quality of *ting*, making it a daunting task to upgrade its status to commercial level. In this study, microbial population dynamics and diversity during sorghum fermentations were analyzed using a combination of culture-independent and culture-dependent methods in order to identify the dominant bacteria. This information will be used subsequently in the development of appropriate starter cultures that may result in *ting* with standardized sensory profiles (reflecting appearance, aroma, sourness and taste) and fermentation time. However, this will require an in-depth evaluation of the contribution of such potential starter cultures to the safety and acceptability of sensory characteristics of *ting*.

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