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Full Length Research Paper

Polyphasic taxonomic characterization of lactic acid bacteria isolated from spontaneous sorghum fermentations used to produce *ting*, a traditional South African food

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***Ting*, an indigenous cooked fermented food made from sorghum flour, is consumed extensively in South Africa. Due to the spontaneous nature of the sorghum fermentation considerable variations in the sensory and microbial quality of the end-product may occur, thus hampering large-scale production of this food. The use of starter cultures purified from the fermented sorghum may be an alternative approach to obtain *ting* of consistent quality. The aim of this study was therefore to identify the lactic acid bacteria (LAB) associated with *ting* fermentation using a polyphasic approach. Phenotypic characterization and sequence analysis of the genes encoding the 16S subunit of the ribosomal RNA (*rrs*) and phenylalanyl tRNA synthase (*pheS*) were used. The results of these analyses showed that *ting* fermentation involved at least three different species of LAB, i.e. *Lactobacillus fermentum*, *L. plantarum* and *L. rhamnosus*. To our knowledge, this is the first report of polyphasic taxonomic characterization of LAB from this food. This research forms an essential first step towards the development of relevant starter cultures to produce *ting* of consistent quality.**

Key words: lactic acid bacteria, polyphasic taxonomy, sorghum fermentation, *ting*, *pheS*.

INTRODUCTION

Ting is a fermented sour porridge made in rural South Africa (Boling and Eisener, 1982). Adults typically consume a thick form of the porridge at major ceremonies such as weddings and funerals, while a diluted form may be used as weaning food because it is relatively inexpensive to prepare and does not require refrigeration or re-heating prior to consumption (Kunene et al., 1999). *Ting* is prepared by soaking sorghum, millet or maize meal for two to three days in excess water at ambient temperature, followed by cooking to make the porridge (Boling and Eisener, 1982). During the soaking step, lactic acid bacteria (LAB) associated with the ingredients and utensils are responsible for fermentation and the resulting flavour of *ting* (Tamang, 1998; M'hir et al., 2007). As these fermentations are uncontrolled, the quality and sensory properties of different *ting* preparations may vary. However, the use of starter cultures would circumvent this problem and also facilitate the large-scale production and marketing of this food.

The development of starter cultures for a specific fermentation process usually involves isolation, selection and purification of microorganisms obtained from the fermented material (Mugula et al., 2003a). The selected microorganisms are then propagated as a mixture of pure cultures or as single-strain starters (Mugula et al., 2003a). Starter cultures that were developed from wild-type strains are generally better adapted to the specific

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food matrix (Holzapfel, 2002) and ensure that the end-product will have the expected flavour and aroma (Ayad et al., 1999). However, fermentation processes usually require constant monitoring to ensure that the micro-organisms involved remain competitive and retain their desired properties (Holzapfel, 2002). Mutations or the loss of important plasmids and contamination by other microorganisms may lead to a product of inferior quality. Accurate characterization of strains used as starter cultures is therefore of paramount importance.

Identification of LAB may be achieved using classical microbiological methods that are relatively simple to perform, but they often lack discriminatory power and reproducibility at species level (Ehrmann and Vogel, 2005). Genomic approaches using DNA fingerprint and/or sequence analyses, on the other hand, usually offer a higher level of accuracy depending on the DNA regions targeted (Gevers et al., 2001; Ehrmann and Vogel, 2005). For reliable species identification, however, a polyphasic approach is preferred in which both phenotypic and genotypic methods are used to obtain unambiguous identifications (Vandamme et al., 1996). The objective of this study was to isolate the LAB responsible for the *ting* fermentation process, and to characterize them using classical microbiological and DNA-based identification methods. For the latter, sequence analyses of the genes encoding 16S ribosomal RNA (*rrs*) and phenylalanyl tRNA synthase (*pheS*) were used.

MATERIALS AND METHODS

Sorghum fermentation and LAB isolation

Commercial packets of pure grain sorghum powder ('King Korn Mabele', King Food Corporation, Potchefstroom, South Africa) were obtained from a local supermarket. The sorghum powder (1:1, w/v) was mixed with luke-warm (*ca*. 40°C) water in glass containers to obtain a slurry. The containers were covered and incubated at 25°C for 54 h after which the pH of the mixture was measured with a Beckman model Ø 34 pH meter (Beckman Coulter, Fullerton, CA, USA). Using this approach, the experiments were carried out in triplicate.

For each fermentation, 10 g of the fermented slurry was aseptically removed at 54 h (when the pH of the slurries was around 3.8) and homogenized in 90 ml of quarter strength Ringer's solution (DAB 7, Braun, Melsungen, Germany). This 10^{-1} dilution was then serially diluted to 10^{-8} using the same diluent, after which 1 ml of each of the diluted samples was inoculated in duplicate onto MRS agar (De Man et al., 1960; Oxoid, Merck, Damstadt, Germany). For the isolation of thermophilic lactobacilli, inoculated plates were anaerobically incubated using Oxoid's Anaerobic Gas Generating kit at 42°C for 48 h. For isolation of the mesophilic lactobacilli, the plates were anaerobically incubated at 35°C for 48 h. Colonies were purified and stored at -20°C in MRS broth supplemented with 20% glycerol.

Phenotypic identification of LAB

Gram-staining, motility, oxidase and catalase tests (Ehrlich, 1956) were performed for nine representative isolates. The ability of isolates to produce gas from glucose was determined.

To determine the carbohydrate fermentation profiles of the bacteria, API 50 CHL test kits (API systems, bioMérieux, France) were used according to the manufacturer's instructions. Isolates were identified to species level using the API database and accompanying software (Johansson et al., 1995). Based on these identifications, six isolates were selected for sequence analysis.

Polymerase chain reactions (PCR)

LAB isolates were inoculated into MRS broth and incubated at 30°C overnight. DNA was extracted from these overnight cultures using the Wizard® Genomic DNA extraction kit (Promega, Madison, USA) or the phenol-chloroform method described by Gevers et al. (2001). Fragments of the *rrs* and *pheS* genes were amplified using the eubacterial universal primers 27F (5'-agagtttgatcctggctcag-3'; Lane, 1991) and 1507R (5'-taccttgttacgacttcacccca-3'; Heyndrickx et al., 1996), and the primers PheS-21-F (5'-cayccngchcgycgygayatgc-3') and PheS-23-R (5'-ggrtgraccatvccngchcc-3') described by Naser et al. (2005), respectively. The *pheS* PCR was performed as described by Naser et al. (2005). For *rrs* PCR, reaction mixtures (25 µl) contained 50 ng of the extracted DNA, 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each of primers 27F and 1507R, 8% (v/v) dimethyl sulphoxide (DMSO), 1.25 U of *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and PCR buffer containing NH_2SO_4 (Fermentas). The temperature programme consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 51°C for 30 s and primer extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Nucleotide sequencing and sequence analyses

The *rrs* amplicons were purified with a High Pure PCR Product Purification kit (Roche Applied Science, Penzberg, Germany) and sequenced in both directions with the primers used in PCR. For this purpose, the BigDye® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems) were used. The *pheS* amplicons were purified using the Nucleofast 96 PCR clean-up membrane system (Machery-Nagel, Germany) and sequenced, as described above, with the *pheS* PCR primers. The resulting sequences were analyzed using Chromas Lite 2.0 (Technelysium) and BioEdit v. 5.0.9 (Hall, 1999). The sequences were then compared to those in GenBank (National Centre for Biotechnology Information; www.ncbi.nih.gov) using the Basic Local Alignment Search Tool (Altschul et al., 1990) for nucleotide sequences (*blastn*).

Phylogenetic analyses

For phylogenetic analyses, multiple alignments were generated using Multiple Sequence Alignment, which is based on Fast Fourier Transform (MAFFT v. 6) (Katoh et al., 2005). These alignments included the *ting* LAB sequences, as well as *rrs* and *pheS* sequences for the *Lactobacillus* type strains for the *L. reuteri*, *L. plantarum* and *L. casei* groups (Naser et al. 2007) obtained from GenBank. Phylogenetic relationships based on maximum likelihood (ML) were inferred with PhyML v. 2.4.3 (Guindon and Gascuel, 2003). As indicated by Modeltest v. 3.7 (Posada and Crandall, 1998), ML analysis of the *rrs* dataset utilized Tamura and Nei's (1993) substitution model with a proportion of invariable sites (I) and gamma correction for among site variation (G), while the *pheS* dataset utilized the general time reversible (GTR) model (Tavaré, 1986) with I and G. The same best-fit parameters were also used for bootstrap analyses based on 1000 replicates.

Table 1. Differential characteristics of nine LAB isolates from fermented sorghum based on API 50 CHL analysis.

Isolate	API 50 CHL Results *								
	1.42	**3.42**	**5.42**	**2.35**	**4.35**	**5.35**	**3.30**	**4.30**	**5.30**
Substrate tested									
L-arabinose	+	+	+	+	+	+	+	+	-
Mannose	+	+[1]	+	+	+[1]	+	+[1]	+	+[1]
N-acetyl glucosamine	+	-	+	+	+	+	-	+	+
Lactose	+[2]	+	+[2]	+	+	+[2]	+	+[2]	+
Melibiose	+	+	+	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+	+	+	-
Raffinose	+	+	+	+	+	+	+	+	+
Gluconate	+	+	+	-	+	+	+	+	-
API 50 CHL identification	*Lact. plantarum*	*Lact. fermentum*	*Lact. plantarum*	*Lact. rhamnosus*	*Lact. fermentum*	*Lact. plantarum*	*Lact. fermentum*	*Lact. plantarum*	*Lact. fermentum*

* +, positive reaction; -, no reaction. [1,2] Weak reaction after 48 h.

RESULTS

Phenotypic identification of LAB

All the selected isolates were catalase-negative, non-motile and Gram-positive, and tentatively assigned to LAB. Among the nine representative LAB strains, isolates 3.42, 4.35, 3.30 and 5.30 produced gas from glucose, while isolates 1.42, 5.42, 5.35, 4.30 and 2.35 did not produce gas from glucose. All nine isolates constituted short rods. Using the API 50 CHL system, the nine isolates were shown to utilize some of the sugars tested (Table 1). *L*-arabinose, melibiose and sucrose were fermented by all isolates, except isolate 5.30. *N*-acetyl glucosamine was fermented by all isolates, except isolates 3.30 and 3.42. Gluconate was fermented by all isolates, except isolates 2.35 and 5.30. The API 50 CHL identification system assigned the isolates to *L. plantarum* (isolates 1.42, 5.42, 5.35 and 4.30), *L. fermentum* (isolates 3.42, 4.35, 3.30 and 5.30) and *L. rhamnosus* (isolate 2.35). From these, six isolates (1.42, 3.42, 2.35, 4.35, 3.30 and 5.30) were selected for further analysis.

DNA-based identification of LAB

Comparison of the *rrs* sequences for the six selected isolates (GenBank accession numbers EU825657 through EU825662) from the fermented sorghum slurries to those in GenBank indicated that they all represent *Lactobacillus* species. The BLASTN results showed that the *rrs* sequence for isolate 1.42 were most similar to those of the so-called *L. plantarum* Group (Naser et al., 2007) that includes *L. plantarum*, *L. paraplantarum* and *L. pentosus*. The *rrs* sequence for isolate 2.35 was most similar to those of the *L. casei* Group (Naser et al., 2007) that includes *L. casei*, *L. paracasei*, *L. zeae* and *L. rhamnosus*. The BLASTN results for isolates 3.42, 4.35, 3.30 and 5.30 indicated that their *rrs* sequences were most similar to that of *L. fermentum* in the *L. reuteri* Group (Naser et al., 2007). ML analysis of the *rrs* data indicated that isolates 3.42, 4.35, 3.30 and 5.30 were indeed most closely related to *L. fermentum* (Fig. 1A). The *rrs*-based ML analysis did, however, not allow us to distinguish isolates 2.35 and 1.42 from other *L. plantarum* Group and *L. casei* Group species, respectively. ML analysis of the *pheS* dataset containing sequences for the six selected LAB isolates (GenBank accession numbers EU825663 through EU825668) recovered similar groupings, but provided better resolution among the taxa included (Figure 1B).

Within the *L. plantarum* Group, isolate 1.42 grouped with known *L. plantarum* strains, and within the *L. casei* Group, isolate 2.35 grouped with *L. rhamnosus*.

DISCUSSION

To our knowledge, this is the first study investiga-

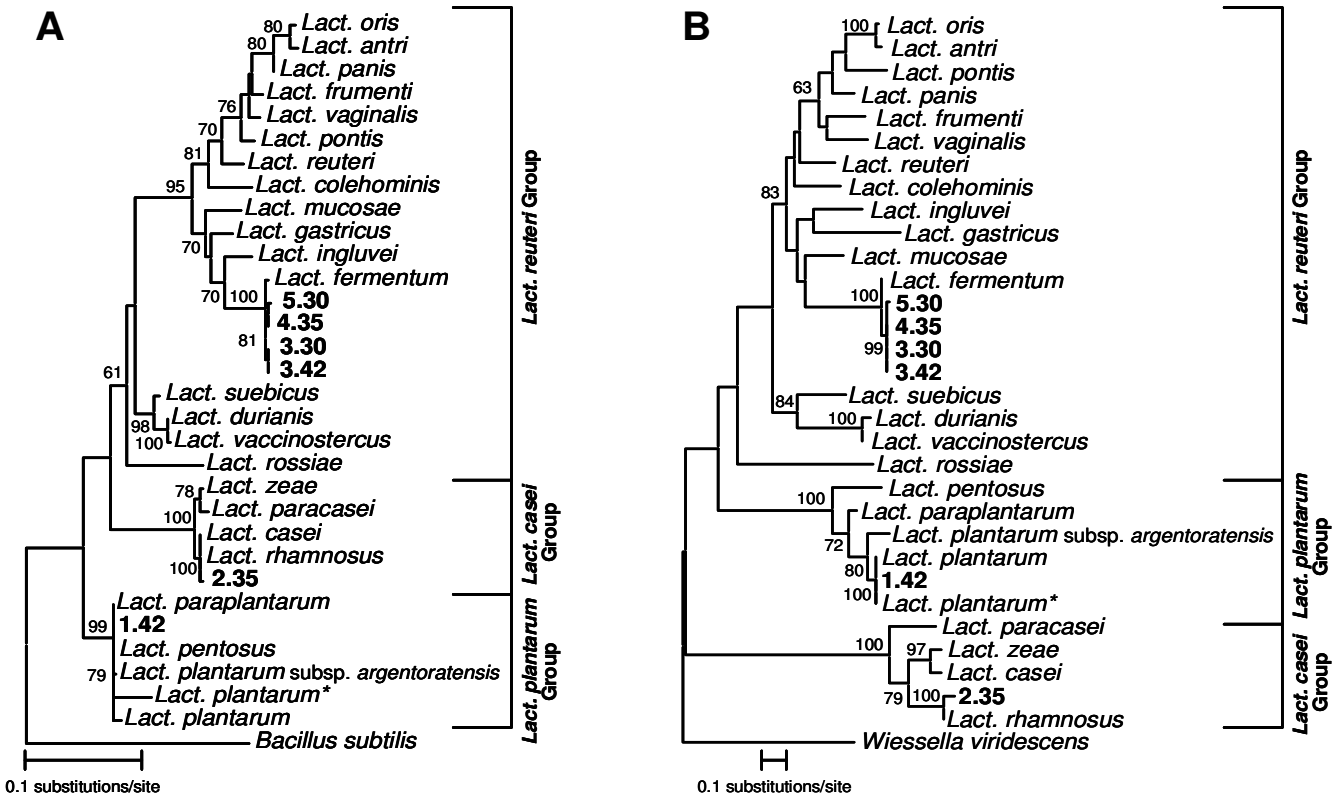

Figure 1. Maximum likelihood (ML) phylogeny for the *Lact. plantarum, Lact. casei* and *Lact. reuteri* Groups (Naser et al., 2007) based on *rrs* (A) and *pheS* gene sequences (B). Isolates from fermented sorghum slurries are indicated in bold and bootstrap values >60% based on 1000 replicates are indicated at the internodes. For the various *Lactobacillus* species, type strain numbers and GenBank accession numbers for the *rrs* and *pheS* sequences, respectively, are as follows: *Lact. antri* LMG22111T, AY253659, AM263502; *Lact. casei* LMG6904T, M58815, AM087682; *Lact. coleohominis* LMG21591T, AJ292530, AM087683; *Lact. durianis* LMG19193T, AJ315640, AM087739; *Lact. fermentum* LMG6902T, M58819, AM087693; *Lact. frumenti* LMG19473T, AJ250074, AM087741; *Lact. gastricus* LMG22113T, AY253658, AM087696; *Lact. ingluviei* LMG20380T, AF333975, AM087731; *Lact. mucosae* LMG19534T, AF126738, AM087707; *Lact. oris* LMG9848T, X94229, AM087709; *Lact. panis* LMG21658T, X94230, AM087725; *Lact. paracasei* LMG13087T, D79212, AM087710; *Lact. paraplantarum* LMG16673T, AJ306297, AM087727; *Lact. pentosus* LMG10755T, AB289240, AM087713; *Lact. plantarum* LMG6907T, X52653, AM087714; *Lact. plantarum** LMG19807, AJ965482, AM087736; *Lact. plantarum* subsp. *argentoratensis* LMG9205T, AJ640078, AM694185; *Lact. pontis* LMG14187T, AJ422032, AM087715; *Lact. rhamnosus* LMG6400T, AB008211, AM087716; *Lact. reuteri* LMG9213T, X76328, AM087728; *Lact. rossiae* LMG22972T, AB370880, AM087768; *Lact. suebicus* LMG11408T, AM113785, AM087772; *Lact. vaccinostercus* LMG9215T, AJ621556, AM087750; *Lact. vaginalis* LMG12891T, AF243177, AM087751; *Lact. zeae* LMG17315T, D86516, AM087761 (LMG = Laboratory of Microbiology, Ghent University, Belgium). *Lact. arizonensis* (type strain LMG19807) that was shown to represent a later heterotypic synonym of *Lact. plantarum* (Kostinek et al., 2005) is indicated with an asterisk. The GenBank accession numbers for *pheS* and *rrs* sequences for the outgroup taxa *Weissella viridescens* (LMG3507T) and *Bacillus subtilis* (LMG7135T) are AM711182 and X60646, respectively.

ting the identity of LAB isolated from *ting* fermentation using a polyphasic approach that combines API 50 CHL analysis and sequence-based identification. The polyphasic approach used in this study aimed to obtain an unequivocal identification of a selection of LAB isolates recovered from the fermentation of sorghum to produce *ting*. Our results suggested that three *Lactobacillus* species, i.e. *L. plantarum*, *L. fermentum* and *L. rhamnosus* are associated with this fermentation process. It has previously been reported that the API 50 CHL system does not allow accurate identification of the majority of LAB isolates. These findings have been attributed to the possible loss or acquisition of plasmids that encode many carbohydrate fermentation traits, resulting in isolates exhibiting atypical metabolic characteristics (Ahrné et al., 1989). Moreover, carbohydrate fermentation patterns across the genus *Lactobacillus* have also been shown to be incongruent with the results of DNA-based studies (Boyd et al., 2005). In addition, the subjective interpretation of similar carbohydrate fermentation patterns obtained using the API 50 CHL system may lead to atypical results (Randazzo et al., 2004). However, in this study, the use of the API 50 CHL system allowed identification of the LAB isolates to species level, the results of which correlated well with the sequence-based identification data.

Despite forming an integral part of all bacterial classifications (Vandamme et al., 1996), ribosomal gene sequences may not always allow clear-cut LAB identifications as these genes are highly conserved (Felis and Dellaglio, 2007; De Vuyst and Vancanneyt, 2007). Among the six *ting* isolates examined, only four could be identified with some level of certainty using *rrs* sequence data (Figure 1A). The other two isolates formed part of the so-called *L. plantarum* and *L. casei* Groups (Naser et al., 2007). As is the case for many other bacterial taxa, the members of these groups are known to encode *rrs* sequences that are not sufficiently polymorphic to allow species separation (Felis and Dellaglio, 2007; Gevers et al., 2005; Torriani et al., 2001). Therefore, bacterial species are increasingly defined using housekeeping loci that evolve more rapidly than *rrs* (Gevers et al., 2005). For LAB taxonomy, a number of alternative loci have been evaluated for taxonomic purposes and include protein-coding genes such as *tuf* (elongation factor Tu; Chavagnat et al., 2002), *mal* (malolactic enzyme; Groisillier and Lonvaud-Funel, 1999), *pepC* (amino peptidase C, Fortina et al., 2001), *pepN* (aminopeptidase N; Fortina et al., 2001), *htrA* (stress-inducible trypsin-like serine protease; Fortina et al., 2001), *recA* (recombinase A; Felis et al., 2001), *rpoB* (RNA polymerase beta subunit; Naser et al., 2005), *hsp60* (60-kDa heat shock protein; Blaiotta et al., 2008) and *pheS* (phenylalanyl tRNA synthase; Naser et al., 2005). Most recently, Naser et al. (2007) examined the *pheS* and *rpoB* gene sequences of 201 strains representing 98 *Lactobacillus* species and demonstrated that these regions represent highly informative taxonomic markers for the identification of *Lactobacillus* species. In the current study, we also found that *pheS* has greater discriminatory power, because it displayed sufficient interspecific variation that allowed us to unequivocally identify isolates 2.35 and 1.42 as *L. rhamnosus* and *L. plantarum*, respectively (Figure 1B).

Of the three *ting* LAB species identified, *L. fermentum* and *L. plantarum* are commonly associated with a wide range of African traditional food and beverage fermentations (Steinkraus, 1996), including *fufu* (fermented cassava), *iru* (fermented African locust bean), *kenkey* and *ogi* (fermented maize), *kukun-zaki* (fermented millet), *ugba* (fermented African oil bean) and *wara* (fermented skimmed cow's milk). In fact, *L. plantarum* is the species most commonly isolated (Olasupo et al., 1997) and has been identified as the dominant organism at the end of several natural lactic acid fermentations (Nout, 1980; Brauman et al., 1996; Olasupo et al., 1997; Mugula et al., 2003b), probably due to its acid tolerance and superior ability to utilize the substrates involved (Akinrele, 1970; Oyewole and Odunfa, 1990). It should now be possible to include the characterized isolates in starter cultures that would result in products with consistent microbiological and sensory properties. Ultimately, these starter cultures may also be used for upgrading this subsistence bioprocess technology to large-scale industrial production and marketing of *ting*. The entire process is therefore important, not only from an academic viewpoint, but also for the conservation of indigenous knowledge and technologies through the characterization and preservation of the microflora associated with this traditional fermented food.

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