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Molecular characterization and population dynamics of lactic acid bacteria during the fermentation of sorghum

by

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Submitted in partial fulfilment of the requirements for the degree of

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DECLARATION

I declare that the thesis, which I hereby submit for the degree, Philosophiae Doctor (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another University.

Signed: …… Date: ……...

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DEDICATION

This thesis is dedicated to my parents, the late Mr. Henry Mahembe and Mrs. Grace Mahembe for emphasizing the significance of a sound education.

SUMMARY

Molecular characterization and population dynamics of lactic acid bacteria during the fermentation of sorghum

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Ting is a cooked fermented sorghum food that is popular amongst southern Africans for its sour taste and unique flavour. However, major challenges are associated with large-scale production and marketing of this spontaneously fermented food due to inconsistent microbiological and sensory quality. The use of starter cultures may circumvent these limitations. Prior to engaging starter cultures, detailed knowledge of the microbial diversity and dynamics during fermentation is important. Therefore, the aim of this study was to investigate microbial diversity and dynamics during sorghum fermentations, and to clarify the role of starter cultures regarding the microbiological safety and consumer acceptance of sensory characteristics of fermented *ting*.

A culture-independent approach, based on the use of PCR-denaturing gradient gel electrophoresis (DGGE), revealed that *Lactococcus lactis*, *Lactobacillus curvatus*, *Weissella cibaria* and some *Enterobacteriaceae* were predominant at the end of spontaneous sorghum fermentations. Culture-dependent methods indicated that *Lb. fermentum*, *Lb. plantarum*, *Lb. rhamnosus*, *E. faecalis*, *E. mundtii*, *W. cibaria* and *L. lactis* were predominant at the end of fermentation. These results not only indicated the predominant bacteria during sorghum fermentation, but also indicated that a combined approach is required to reveal microbial diversity and dynamics during spontaneous sorghum fermentations.

Based on the above results, *L. lactis*, *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus* were evaluated as starter cultures for production of *ting*. All the starter cultures were able to ferment sorghum, but the lowest pH and highest lactic acid was produced in naturally fermented sorghum inoculated with *L. lactis*. This fermentation showed an increase in the number of lactic acid bacteria and yeasts, whilst pathogen counts decreased. *Ting* from this fermented gruel, in contrast to naturally fermented sorghum, had sensory properties preferred by panelists. The results indicated that the use of *L. lactis* in starter cultures may result in *ting* with consistent and acceptable attributes.

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LIST OF ABBREVIATIONS

%	percentage
°C	degrees Celsius
bp	base pair
cfu	colony forming units
ClustalX	cluster analysis version X
cm	centimetre
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ERIC	enterobacterial repetitive intergenic consensus
Fig.	figure
g	gram
h	hour
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
L.	*Lactococcus*
Le.	*Leuconostoc*
LMG	Laboratorium voor Microbiologie Gent
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
rep	repetitive extragenic palindromic
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid

TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylenediamine
U	unit
UPGMA	unweighted pair group method of arithmetic averages
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
µg	microgram
µl	microlitre

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CHAPTER ONE

LITERATURE REVIEW

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1.1 INTRODUCTION

It is generally accepted that sorghum (*Sorghum bicolor* (L.) *Moench*) originated in Ethiopia, between 5 000 and 7 000 years ago (ICRISAT, 2005). From there, sorghum was distributed along the trade and shipping routes around Africa and the Middle East to India, China and the Far East (Doggett, 1970; Kimber, 2000). Sorghum was introduced in America from West Africa through the slave trade. Currently, sorghum is mostly cultivated in the arid and the semi-arid regions of the world and is the fifth most important cereal after wheat, maize, rice and barley in terms of production (Food and Agricultural Organization, 2003; 2006).

In sub-Saharan Africa, sorghum is especially significant for food security and comes second after maize in being a staple food (Dendy, 1995). Sorghum is drought resistant by nature (Jordan and Sullivan, 1982; National Research Council, 1996; Dicko *et al*., 2006), which makes it particularly relevant to sub-Saharan Africa, as an estimated half a million families are susceptible to hunger due to drought (FAO, 2003). In addition, the ability to tolerate periods of water-logging and high temperatures (Doggett, 1988) makes sorghum an attractive crop for providing dietary energy and protein for people living in these regions.

The use of sorghum as a main constituent of the diet is usually associated with under-nourishment. This is mainly due to it lacking some essential amino acids (Neucere and Sumrell, 1979; Kazanas and Fields, 1981) and the presence of anti-nutritional factors (Hamacker *et al*., 1986; Ibrahim *et al*., 2005). However, fermentation, facilitated mainly by lactic acid bacteria (LAB), greatly improves the nutritive value and sensory properties of sorghum (Au and Fields, 1981; Chavan *et al*., 1988; Hassan and El Tinay, 1995). In this Chapter, the fermentation process and its associated advantages with specific reference to sorghum is reviewed. Aspects such as the taxonomy of LAB and DNA-based methods to study them are also emphasized.

1.2 SORGHUM-BASED FERMENTATION

The term fermentation is generally used to describe the desirable biochemical changes brought about by microorganisms and/or enzymes on primary food products (Nout and Motarjemi, 1997; Blandino *et al*., 2003). Enzymatic fermentations involve chemical reactions whereby the enzyme acts as a catalyst, as occurs when starch is converted to dextrin by α-amylase. When fermentation involves microorganisms, these are either naturally present on

the substrate or they may be deliberately added in the form of starter cultures (Nout and Motarjemi, 1997). In microbiological terms, fermentation is a type of energy-yielding microbial metabolism whereby organic substrates are incompletely oxidized and organic carbohydrates act as electron acceptors (Adams, 1990).

The four main fermentation types are alcoholic, lactic acid, acetic acid and alkali based. Yeasts usually predominate in alcoholic fermentations, resulting in the production of alcohol, whereas bacteria such as *Acetobacter* species are responsible for fermentations where alcohol is converted to acetic acid in the presence of oxygen (Blandino *et al*., 2003). The production of foods such as Japanese *natto* from cooked soybeans, *dawadawa* from African locust beans and *ogiri* from melon seeds often involve some form of alkaline fermentation, with *Bacillus subtilis* being the dominant species (Wang and Fung, 1996). LAB mainly carry out lactic acid fermentation.

LAB are essential for fermentation of most plant-based products where they usually co-exist with yeasts. In this regard, LAB play various roles that include the production of safe products through biopreservation as a result of bacterial antagonism (Oyewole, 1997; Soomro *et al*., 2002), enhancement of sensorial and nutritive value of the foods, and saving of energy through reduced cooking time (Simango, 1997). A plethora of LAB predominate in plant-based foods such as *pozol*, a Mexican maize dough (Escalante *et al*., 2001), Colombian *chicha* (Steinkraus, 1996), and *idli*, a fermented Asian food made from rice blended with black gram (Soni and Sandhu, 1991). LAB also play a central role during the production process of most fermented sorghum-based foods such as Ethiopian *injera* (Vogel *et al.*, 1993), Sudanese *kisra* (Mohammed *et al.*, 1991), Ugandan *bushera* (Muyanja *et al.*, 2003) and Tanzanian *togwa* (Mugula *et al.*, 2003).

1.2.1 Anti-nutritional compounds in sorghum

Sorghum contains significant levels of anti-nutritional compounds that include protease inhibitors, phytic acid (myoinositol hexakis) and oligosaccharides (Serna-Saldivar and Rooney, 1995). The protease inhibitors include non-specific tannins and Kunitz trypsin inhibitor. Although tannins have been shown to reduce serum lipids, increase blood clotting and reduce blood pressure (Chung *et al*., 1998), their anti-nutrititive attributes in foods far outweigh their medical value. For example, impaired utilization of minerals and vitamins and

inhibition of digestive enzymes are associated with these compounds (Chung *et al*., 1998). Together, tannins and phytic acid inhibit digestive enzymes and reduce protein availability via different modes of action. Tannins typically form complexes with proteins (Hagerman, 1989), whilst phytic acid, a highly charged molecule (Serna-Saldivar and Rooney, 1995), chelates cations such as potassium, calcium, magnesium, iron and zinc, resulting in the formation of phytate (Wodzinski and Ullah, 1996; Dvorakova, 1998; Stodolak *et al*., 2007). Oligosaccharides such as raffinose, stachyose and verbascose are responsible for causing flatulence (FAO, 1998). This is due to their indigestion by enzymes of the human gastrointestinal tract in the small intestine. Consequently, these oligosaccharides are passed to the large intestine where fermention by resident microorganisms takes place, resulting in the production of gas (FAO, 1998).

1.2.2 Nutritional and health advantages of lactic acid fermentation

Lactic acid fermentation of sorghum foods by LAB prior to cooking and/or consumption significantly alters their biochemical properties, rendering a food product with enhanced nutritional value and flavour (Towo *et al*., 2006). For instance, lactic acid fermentation improves the *in vitro* digestibility of starch (Hassan and El Tinay, 1995) and sorghum protein (Kazanas and Fields, 1981; Mertz *et al*., 1984; Taylor and Taylor, 2002; Ibrahim *et al*., 2005). The increase in *in vitro* protein digestibility may be due to a rapid decrease in pH that affects the structure of insoluble sorghum proteins such as prolamines and glutelins in such a way that they become more accessible to pepsin digestion (Taylor and Taylor, 2002).

Lactic acid fermentation has been shown to reduce the level of anti-nutritive oligosaccharides, phytate and tannins. The amount of poly- and oligosaccharides in sorghum, e.g., raffinose, stachyose and verbascose, are significantly reduced due to the action of α-galactosidases produced by some LAB that include *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Weissella paramesenteroides*, *Lactobacillus fermentum*, *Lb. brevis* and *Lb. buchneri* (Mital *et al*., 1973; Milliere *et al*., 1989). The α-galactosidases disrupt the α-D-galactosidic bonds of oligosaccharides, resulting in lowered abdominal distention and flatulence (Nout and Motarjemi, 1997). Tannin levels are also reduced by lactic acid fermentation (Lorri, 1993; Osman, 2004). The reduction may be due to metabolic processes of the microorganisms. These processes include oxidation, reduction or dissociation of the tannins as a defense mechanism against toxicity by endogenous sorghum microflora (Bvochora *et al*., 2005). Reduced levels of phytate, as a result of lactic acid fermentation, may increase B vitamins

(Kazanas and Fields, 1981; Nout and Motarjemi, 1997) and improve the availability of minerals (Marfo *et al*., 1990).

Some LAB are thought to have probiotic properties (Fuller, 1989). These include prevention of kidney stones (Campieri *et al*., 2001), treatment of heart disease (Schaafsma *et al*., 1998), control of cholesterol (Gilliland *et al*., 1985), stimulation of anti-carcinogenic action (Goldin, 1990), prevention of antibiotic-induced diarrhea (Fooks *et al*., 1999), prevention of lactose intolerance (Gilliland and Kim, 1984), prevention of food allergies (Sütas *et al*., 1996), blockage of the formation of biogenic amines (Joosten *et al*., 1996) and prevention of vaginosis in humans (Kontiokari *et al*., 2001; Reid, 2001). Lactic acid fermentation is, however, linked to certain diseases, especially in immunocompromised hosts. For instance, the D (-) lactic acid produced during fermentation may potentially be toxic to malnourished and sick children due to acidosis (Nout and Motarjeni, 1997). LAB have also been associated with clinical infections such as urethritis, endocarditis, endometritis, pneumonia, arthritis, bacteremia and meningitis (Sussman *et al*., 1986; Struve *et al*., 1988; Isenberg *et al*., 1988; Ruoff *et al*., 1988; Aguirre and Collins, 1993; Salminen *et al*., 2002; Flaherty *et al*., 2003). In addition, other members of genera such as *Leuconostoc*, *Enterococcus*, *Weissella* and *Lactobacillus* may cause dental caries (Monchois *et al*., 1999; Sbordone and Bortolaia, 2003). For these reasons, thorough characterization of LAB for use in food fermentations is of paramount importance.

1.2.3 Sensory advantages of lactic acid fermentation

During the production of many fermented foods, the development of appetizing flavour characteristics is crucial (Caplice and Fitzgerald, 1999). Particularly, during cereal fermentations, several volatile compounds are formed, which contribute to a complex blend of appetizing flavours (Chavan and Kadam, 1989). The specific flavour occurs due to secondary metabolites produced during fermentation of macromolecules such as sugars (Longo and Sanromán, 2006). The specific flavour compounds produced include diacetyl, which imparts a buttery aroma (Rankine *et al*., 1969; Davis *et al*., 1985), acetaldehyde (yoghurt flavour) (Gobbetti and Corsetti, 1997; Abd El-Salam and Alichanidis, 2004) and the amino acid alanine, a natural sweetener (Hols *et al*., 1999). Generally, these flavour compounds are products of pyruvate, which acts as an electron or hydrogen acceptor during metabolism by some LAB and yeasts, thereby perpetuating fermentation through recycling of NAD^+ (Axelsson, 2004; Fig. 1.1). Although both yeasts and bacteria may contribute to the

formation of diacetyl, the role of yeasts during lactic acid fermentation is negligible (Martineau and Henick-Kling, 1995).

Depending on the bacterial strain under investigation and the growth conditions, pyruvate may act as substrate for the following pathways: dehydrogenase pathway (aerobic metabolism), pyruvate oxidase pathway, pyruvate-formate lyase system (anaerobic) or the diacetyl/acetoin pathway (Caplice and Fitzgerald, 1999; Axelsson, 2004). When pyruvate is present in excess (compared to NAD^+ regeneration), as occurs in the presence of another carbon source such as citrate, or if an alternative electron acceptor such as oxygen is present, the diacetyl/acetoin pathway is followed (Hugenholtz, 1993; Fig. 1.1). Yeasts also convert pyruvate to diacetyl via hydroxyethyl-thiamine (acetyladehyde-thiamine pyrophosphate complex/active acetaldehyde) (Romano and Suzzi, 1996). The subsequent reactions are divided into three pathways, depending on the substrate, which interacts with the active acetaldehyde. Ultimately, pleasant sensory compounds, which include acetoin and diacetyl, are formed. Diacetyl formation usually occurs at low pH and decreased sugar concentration (Axelsson, 2004). Despite the pleasant sensory attributes imparted by these flavour compounds, high concentrations of diacetyl (above 5-7 mg/L) are considered unpleasant (Rankine *et al.*, 1969; Davis *et al*., 1985).

The pyruvate-formate lyase system is active under anaerobic conditions or when some substrate is limiting (Axelsson, 2004). This results in mixed acid fermentation wherein pyruvate and Coenzyme A (CoA) react to form formate and acetyl CoA with pyruvate-formate lyase acting as the catalyst (Fig. 1.1). The utilization of acetyl CoA results in the formation of ethanol (if it is used as an electron acceptor) or acetate (if it acts as a precursor for substrate-level phosphorylation) (Fig. 1.1), or a mixture of both. *Lb. casei* and some *Lactococcus lactis* strains mainly use this pathway (Thomas *et al*., 1979). The pyruvate dehydrogenase pathway, which is mainly active in lactococci, involves pyruvate dehydrogenase that catalyses the formation of acetyl CoA under aerobic conditions in a manner similar to the pyruvate-formate lyase system, thus resulting in the formation of acetate (Fig. 1.1). In the pyruvate oxidase pathway, pyruvate oxidase catalyses the conversion of pyruvate to acetyl-phosphate (acetyl-P) and carbon dioxide with the simultaneous formation of hydrogen peroxide. Ultimately, acetate is formed from acetyl-P (Fig. 1.1). *Lb. plantarum* was reported to produce high concentrations of acetic acid, using pyruvate oxidase under aerobic conditions (Sedewitz *et al*., 1984).

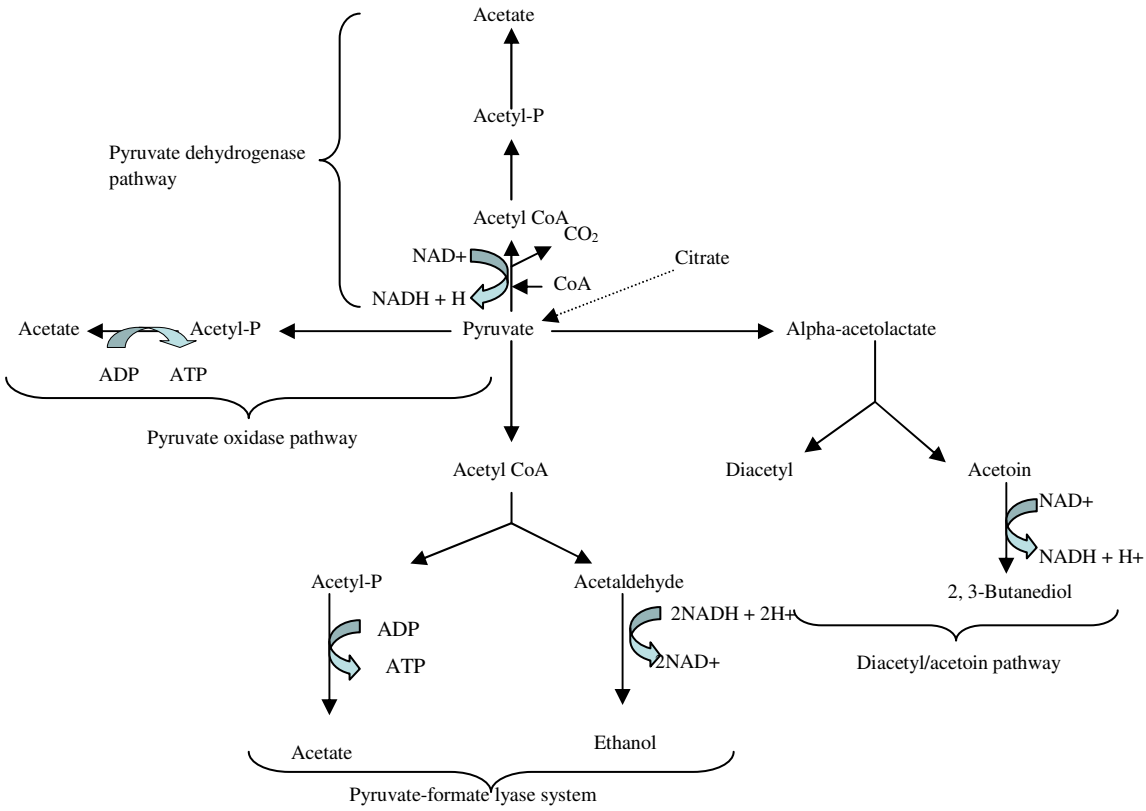

Figure 1.1 Generalized scheme for the formation of important metabolic products from pyruvate by lactic acid bacteria. Brackets indicate the four different pathways that are involved in pyruvate metabolism. Dotted arrows illustrate an external source of pyruvate. Black arrows show the direction of the reaction. Curved arrows indicate redox reactions involving NAD/NADH and energy-yielding reactions. (Adopted and modified from: Caplice and Fitzgerald, 1999; Axelsson, 2004).

Despite the sensory advantages that LAB impart to fermented foods, these bacteria may, however, also produce off-flavours in beer and wine (Caplice and Fitzgerald 1999), and in fish (Lyhs *et al*., 2002). For instance, some strains of *Lb. plantarum* may cause off-flavours through the formation of aldehydes (Kandler and Weiss, 1986; Bottazi, 1988; Henick-Kling, 1995). Furthermore, the production of diacetyl by *Pediococcus damnosus* results in cloudy beer with an acid taste (Satokari *et al*., 2000). Some species of LAB (e.g., *Lb. kunkei*) and acetic acid bacteria (e.g., *A. aceti*) may also suppress yeast growth during grape juice fermentation, resulting in sluggish or stuck fermentations (Drysdale and Fleet, 1989; Huang *et al*., 1996). This highlights the importance of accurate characterization of the LAB for use during specific fermentations.

1.2.4 Safety benefits of lactic acid fermentation

LAB render fermented foods safe for consumption by controlling the growth of pathogenic and spoilage microorganisms (Caplice and Fitzgerald, 1999). In order to create an unfavourable environment for these pathogenic and spoilage microorganisms, LAB produce a range of compounds that act as natural antimicrobials in different ways. These compounds include, but are not limited to, bacteriocins, reutericyclin, diacetyl, hydrogen peroxide, carbon dioxide and organic acids such as lactic and acetic acid (Silva *et al*., 1987; De Vuyst and Vandamme, 1994; Lavermicocca *et al*., 2000; Messens and De Vuyst, 2002; Leroy and De Vuyst, 2004). Some of these antimicrobial compounds were shown to be bactericidal or bacteristatic for a diverse range of microorganisms belonging to the following genera: *Streptococcus*, *Mycobacteria*, *Lactococcus*, *Listeria*, *Clostridium*, *Bacillus*, *Neiserria*, *Campylobacter*, and *Haemophilus* (van Kraaij *et al*., 1999; Mota-Meira *et al*., 2000; Soomro *et al*., 2002; Savadago *et al*., 2006).

Some of the antimicrobial agents, e.g., bacteriocins, organic acids and carbon dioxide, produced by LAB disrupt the cytoplasmic membranes of target organisms by virtue of different properties (Riley and Wertz, 2002). For example, lactococcin A depolarizes or makes pores in the cytoplasmic membrane, thus disrupting the integrity of this structure (Van Belkum *et al*., 1991). Reutericyclin is a highly hydrophobic and charged molecule that is produced during anaerobic growth of *Lb. reuteri* (Gänzle and Vogel, 2003). This compound is bacteriostatic or bactericidal against a broad range of food-associated spoilage and pathogenic microbes, including *Staphylococcus aureus* and *Listeria innocua*, due to its

activity as a proton ionophore, resulting in translocation of protons across the cytoplasmic membrane and consequently, dissipating the transmembrane pH gradient (Gänzle, 2004).

Organic acids such as lactic and acetic acids disrupt the cytoplasmic membranes of target microorganisms via both the dissociated and undissociated acid (Cherrington *et al*., 1991). When these organic acids are in an undissociated form, they become lipid soluble and enter the cytoplasm directly where they dissociate, releasing anions (De Vuyst and Vandamme, 1994). The growth of affected bacteria is thus inhibited, as they divert most of their energy to curbing this acidification and its effects (Adams and Nicolaides, 1997).

Carbon dioxide contributes to food safety by creating anaerobic conditions, which inhibit the growth of oxygen-dependent microorganisms such as moulds and Gram-negative bacteria (De Vuyst and Vandamme, 1994; Adams and Nicolaides, 1997). Carbon dioxide also curbs the growth of pathogenic and spoilage bacteria by disrupting enzyme-dependent reactions due to a decrease in pH (King and Nagel, 1975).

Hydrogen peroxide, whose mode of action is not clear (since LAB do not possess a true catalase system), is believed to activate the lactoperoxidase system, resulting in the formation of other antimicrobials (De Vuyst and Vandamme, 1994). Its bactericidal effect is due to oxidation of the bacterial cell, membrane lipids and sulfhydryl groups of cell proteins (Lindgren and Dobrogosz, 1990). Hydrogen peroxide may accumulate to sufficient concentrations capable of inhibiting some pathogenic and spoilage bacteria (Condon, 1987). However, peroxidases, flavoproteins and pseudocatalase are believed to decompose hydrogen peroxide, prohibiting its accumulation to effective amounts *in vivo* (Fontaine *et al*., 1996). Morever, diacetyl contributes to food safety by disrupting the arginine utilization system of Gram-negative bacteria (Jay, 1982; Caplice and Fitzgerald, 1999). However, the concentrations that are required for antimicrobial action are not acceptable in foods (De Vuyst and Vandamme, 1994).

In spite of the safety contribution of lactic acid fermentation, the inhibition of pathogenic and spoilage organisms by LAB is complex because the factors involved are difficult to quantify. These include the organism in question, temperature, level of hygiene, amount of undissociated acid and the buffering capacity of the food (Adams and Nicolaides, 1997; Nout and Motarjemi, 1997). For instance, when both the pathogen (*Escherichia coli*) and the LAB

(*Lactococcus lactis*) were added to a model weaning food simultaneously, the pathogen still grew for 5 h despite the fact that the LAB outnumbered the pathogen by more than 5 log cycles (Yusof *et al*., 1993). Consequently, the antimicrobial effect of fermentation must be an adjunct to good hygiene but not a substitute for it (Adams and Nicolaides, 1997; Nout and Motarjemi, 1997).

1.3 SORGHUM-BASED FERMENTED FOODS

Throughout the world, a plethora of fermented foods are metabolized by a variety of microorganisms to yield products with unique and appealing characteristics (Caplice and Fitzgerald, 1999; Taylor, 2003). In Africa, sorghum-based fermented foods and beverages include *injera* (Chavan and Kadam, 1989), *kisra* (Mohammed *et al*., 1991), *ogi* (Akingbala *et al*., 1981), *mahewu* (Bvochora *et al*., 1999), *uji* (Mbugua *et al*., 1984), *muramba* (Mukuru, 1992), *bushera* (Muyanja, 2003), *togwa* (Lorri and Svanberg, 1995) and *ting* (Boling and Eisener, 1982). LAB are mainly associated with most of these fermented foods (Table 1.1).

Injera is a circular, spongy-textured Ethiopian sour bread (Stewart and Getachew, 1962; Chavan and Kadam, 1989) made from sorghum, tef, corn, finger millet or barley. Over 8% of the total sorghum production in Ethiopia is used for *injera* production (Gebrekidan and Gebrettiwat, 1982). The production process for *injera* involves mixing of the mechanically dehulled grains with water, followed by addition of the starter (*ersho*) from a previous batch and fermentation for 2-3 days. A small part of the fermented mixture is gelatinized in boiling water, added back to the fermented dough and allowed to stand for 2-3 h. The resulting dough is thinned down after fermentation and poured into a thinly oiled pan, covered with a lid for 2-3 min and cooked (Parker *et al*., 1989). This food is valued for its sour flavour and high nutritional value, being rich in calcium and iron (Zegeye, 1997). Yeasts and some fungi are mainly involved in the fermentation of *injera* (Blandino *et al*., 2003; Table 1.1).

Fermented sorghum is used to make *kisra* that is baked into thin sheets to make pancakes, which are consumed throughout the Arabian Gulf, Sudan and Iraq (Eggum *et al*., 1983). *Kisra* pancakes are similar to *injera*. The fermented dough (*ajin*) is prepared by using a portion of a previous batch (back-slopping). Fermentation was found to increase the crude protein, thiamine and niacin content of *kisra* (El Tinay *et al*., 1979; Axtell *et al*., 1981; Eggum *et al*., 1983). A range of LAB, yeasts and fungi are involved in the production of *kisra* (Table 1.1).

Table 1.1 Examples of sorghum-based fermented foods, natural microflora and the methods used for characterization

Product	Country	Microorganisms isolated	Method of characterizing organisms	Reference
Togwa	Tanzania	*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, *Lb. cellobiosus*, *P. pentosaceus*, *Weissella confusa*, *Issatchenkia orientalis*, *Saccharomyces cerevisae*, *Candida pellicullosa*, *Candida tropicalis*	API 50 CHL	Mugula *et al*., 2003
Bushera	Uganda	*Lb. plantarum*, *Lb. paracasei* subsp. *paracasei*, *Lb. fermentum*, *Lb. brevis*, *Lb. delbrueckii* subsp. *delbrueckii*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranicum*, *Weissella confusa*	API 50 CHL	Muyanja *et al*., 2003
Sorghum-based fermented weaning cereal	South Africa	*Lb. plantarum*, *Leuconostoc mesenteroides*, *Lb. sake-Lb. curvatus*, *P. pentosaceus*, *P. acidilactici*, *L. lactis*	Analysis of soluble proteins, amplified fragment length polymorphism	Kunene *et al*., 2000
Injera	Ethiopia	*Candida guilliermondii*, *Pullaria* sp., *Aspergillus* sp., *Penicillium* sp., *Rhodotorulla* sp., *Homodendrum* sp., *Candida* sp., unidentified bacteria	Morphological and biochemical tests	Ashenafi, 1994; Dirar, 1993; Gashe *et al*., 1982; Steinkraus, 1983; Vogel *et al*., 1977
Kisra	Sudan	*Pediococcus pentosaceus*, *Lb. confusus*, *Lb. brevis*, *Lactobacillus* sp., *Erwinia ananas*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Candida intermedia*, *Debaryomyces hansenii*, *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp.	Morphological and biochemical tests	Mohammed *et al*., 1991
*Ogi**	Nigeria	*Lactococcus raffinolactis*, *Lactococcus garviae*, *P. pentosaceus*, *Lb. paracasei* susbsp. *tolerans*, *Lb. plantarum*, *Lb. suebicus*, *Lb. brevis*, *Pediococcus* sp., *Cephalosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* spp., *Saccharomyces cerevisae*, *Candida mycorderma*, *C. vini*	API 50 CHL	Teniola *et al*., 2005 ; Caplice and Fitzgerald, 1999

**Ogi* was prepared using maize.

In South Africa and Botswana, *ting* is used as a weaning food and is also consumed by adults (Boling and Eisener, 1982). This food represents a fermented sour porridge made traditionally by soaking sorghum flour for 2-3 days in water, followed by cooking. *Ting* is highly prized by local people for its unique taste, texture and aroma. Despite its popularity, there is little information regarding the microorganisms associated with the production of *ting*.

Togwa, a Tanzanian fermented gruel or beverage, is produced from sorghum, millet, maize, cassava or combinations of these (Mugula *et al*., 2001). It is useful as a weaning food or as a refreshment (Mugula *et al*., 2001). Like other indigenous African foods, the predominant microflora are LAB and yeasts (Table 1.1). The consumption of *togwa* is declining, probably due to the unhygienic nature of the preparation method and its poor shelf-life (Mugula *et al*., 2001).

Bushera, a non-alcoholic fermented beverage, is the most common indigenous beverage produced in the western highlands of Uganda (Muyanja *et al*., 2003). Production of *bushera* involves mixing germinated sorghum flour with boiled water, followed by cooling at ambient temperature and addition of germinated sorghum flour for starting the fermentation (Muyanja *et al*., 2003). The fermentation is dominated by LAB and normally takes place in clay pots for 1-4 days (Muyanja *et al*., 2003).

Ogi, a fermented sorghum, maize or millet gruel, is considered the most significant weaning food in West Africa and has been produced on a semi-industrial scale (Achi, 2005). *Ogi* has a sour flavour akin to that of yoghurt and a characteristic aroma, which differentiates it from other cereal-based fermented foods (Chavan and Kadam, 1989). Briefly, *ogi* is prepared by steeping the cereal grains in pots for 1-3 days, followed by wet milling and sieving of the fermented grains to yield a slurry (Steinkraus, 1998) of which the colour depends on the cereal grain. Fermentation of the grains is steered by LAB, yeasts and moulds, whilst flavour development is imparted by members of the genera *Saccharomyces* and *Candida* (Caplice and Fitzgerald, 1999; Table 1.1).

The common feature of many of these fermented foods is spontaneous fermentation, usually at household level. Despite the simple traditional method of preparation, natural fermentation involves a complex uncontrolled microbial process (Daeschel *et al*., 1987), resulting in

products with variable sensory characteristics. Such variability in product quality hampers commercial production of indigenous fermented sorghum-based foods. Nevertheless, starter cultures may be used to overcome the inconsistency in product quality associated with traditional fermented foods by improving process control.

1.4 STARTER CULTURES

Starter cultures are microbial preparations containing large numbers of cells with at least one type of microbe, which shorten and steer the fermentation process when added to the raw material (Holzapfel, 2002; Leroy and De Vuyst, 2004). These cultures are critical for the formation of the desired flavour compounds that contribute to specific sensory characteristics. In addition, starter cultures pose technological, nutritional and health advantages (De Vuyst, 2000; Hansen, 2002). A typical starter culture facilitates improved control, predictability, safety, stability against mutations and standardization of a fermentation process (Holzapfel, 1997; Holzapfel, 2002). A starter culture may consist of single or mixed strains. Although single strain cultures may improve both process control and predictability of metabolic activities, they are relatively sensitive to bacteriophage infection, spontaneous mutation and they easily lose key physiological features such as plasmid-mediated fermentation of lactose (Holzapfel, 2002). In contrast, mixed strains are relatively unaffected by fluctuating conditions of handling and storage (Holzapfel, 2002).

Starter cultures may include moulds, yeasts, lactic acid bacteria or combinations of these. Moulds such as *Penicillum nalgiovense*, *Aspergillus oryzae* or *P. chrysogenum* are important starter cultures in food products such as cheese, fermented sausages, soy sauce and *miso*. Yeasts such as *Saccharomyces*, *Candida* and *Hansenula* species are important starter cultures for plant-based material that contain relevant fermentable sugars (Holzapfel, 1997), and are especially used in spontaneous African alcoholic fermentations. However, LAB occupy a central role as starter cultures due to their long safe history of interaction with humans. Hence, they are generally regarded as safe (GRAS) (Mugula *et al*., 2003; Muyanja *et al*., 2003).

1.5 TAXONOMY OF LACTIC ACID BACTERIA (LAB)

Taxonomy, a synonym of biosystematics, involves the cataloguing of biodiversity with the aim of arranging and characterizing organisms in an organized way (Staley and Kreig, 1984).

It is divided into classification, nomenclature and identification (Vandamme *et al*., 1996). Classification is the orderly arrangement or clustering of organisms into taxonomic groups based on similarity, whilst nomenclature is the labeling of units according to international rules, and identification of organisms is the process of determining whether a query isolate belongs to one of the defined units (Staley and Kreig, 1989; Vandamme *et al*., 1996). The species is the most significant and central element of bacterial taxonomy (Vandamme *et al*., 1996) and has been described as a cluster of separate organisms, which are monophyletic, genomically coherent, and have a high degree of overall similarity with respect to independent characteristics (Rosselló-Mora and Amann, 2001). Bacterial species are usually identified using phenotypic traits, phylogenetic data or combinations of these.

Phenotypic classification usually involves carbohydrate fermentation patterns, lactic acid configuration and other growth requirements (Sharpe, 1981). Based on carbohydrate fermentation patterns, lactobacilli are classified as: (i) obligately homofermentative, (ii) facultatively heterofermentative, and (iii) obligately heterofermentative. The differences between the three fermentation types are as follow: obligate heterofermenters use the Emden-Meyerhof-Panas (EMP) pathway to convert hexose sugar to almost solely lactic acid, but not pentoses and gluconate due to the lack of phosphoketolase; facultative heterofermenters possess both aldolase and phosphoketolase, and can thus metabolize pentoses and gluconate in addition to hexoses, which are degraded using the EMP pathway; obligate heterofermenters metabolize pentoses, and hexoses are degraded via the phosphogluconate pathway with the formation of lactate, carbon dioxide, ethanol or acetic acid (Hammes and Vogel, 1995). Other phenotypic data may be obtained from the following: (i) expressed characteristics such as morphology, physiology, enzymology and serology, (ii) chemotaxonomic markers, which include cellular fatty acids and exopolysaccharides, among others, and (iii) proteins, including functionality (Vandamme *et al*., 1996).

The classification of LAB, which is based on phenotypic traits and true phylogenetic relationships inferred from rRNA gene sequences (see below), remains largely unresolved due to lack of correlation between these approaches (Vandamme *et al*., 1996; Felis and Dellaglio, 2007). For instance, the genus *Lactobacillus* is phylogenetically intermixed with some *Pediococcus* and *Leuconostoc* strains, despite their traditional separation due to differences in morphology (Stackebrandt and Teuber, 1988; Axelsson, 2004). In addition, phenotypic tests mostly fail to distinguish the genus *Carnobacterium* from that of *Lactobacillus*, and

Vagococcus from *Lactococcus* members (Axelsson, 2004). Despite this lack of correlation in some groups between phenotypic and genotypic results, phenotyping generally provides important information for separating taxa (Botina *et al*., 2006). In order to circumvent limitations inherent in either phenotyping or phylogeny, polyphasic taxonomy, involving an integration of phenotypic, genotypic and phylogenetic data, is important (Colwell, 1970; Vandamme *et al*., 1996). However, if the main thrust is speed and reliability, DNA-based techniques are more advantageous (Ehrmann and Vogel, 2005).

1.5.1 General characteristics of LAB

LAB represent a diverse and paraphyletic group of bacteria (Fig. 1.2). They constitute a heterogeneous group of Gram-positive, acid-tolerant and strictly fermentative cocci, coccobacilli or rods that produce lactic acid as the main product during carbohydrate fermentation (Axelsson, 2004; Temmermann *et al*., 2004; Ehrmann and Vogel, 2005). The heterogeneity is clearly evidenced in the genus *Weissella* that contains both coccoid and rod-shaped members (Collins *et al*., 1993). Furthermore, the largest and most heterogeneous group within the LAB group comprise lactobacilli with a G + C content of 32-55%, which is wider than that expected for members of a single genus (Goodfellow *et al*., 1997). Generally, the G + C content do not vary more than 3 and 10% for a well-defined species and genus level, respectively (Stackebrandt and Liesack, 1993).

LAB generally lack catalase, although pseudocatalase was detected in cultures grown at low sugar concentrations (Caplice and Fitzgerald, 1999). They are asporogenous and fastidious organisms and hence, occupy nutritionally rich habitats. LAB are mesophilic and their growth temperatures range from 5°C to 45°C (Caplice and Fitzgerald, 1999). This variability also applies to pH where LAB grow at pH 9.6 and pH 3.6, with the majority growing at pH 4.0-4.5 (Caplice and Fitzgerald, 1999).

1.5.2 Horizontal gene transfer (HGT) and LAB

The common ancestor of LAB was shaped by HGT (Koonin *et al*., 2001), like all lineages of bacteria (Doolittle, 1999; Jordan *et al*., 2001; Brown, 2003; Koonin, 2003; Lawrence and Hendrickson, 2003; Ochman, 2005; Choi and Kim, 2007). HGT is the transfer of genes among organisms of different species either by acquiring paralogous genes that already exist in a specific lineage, acquiring genes that do not pre-exist in the lineage or through

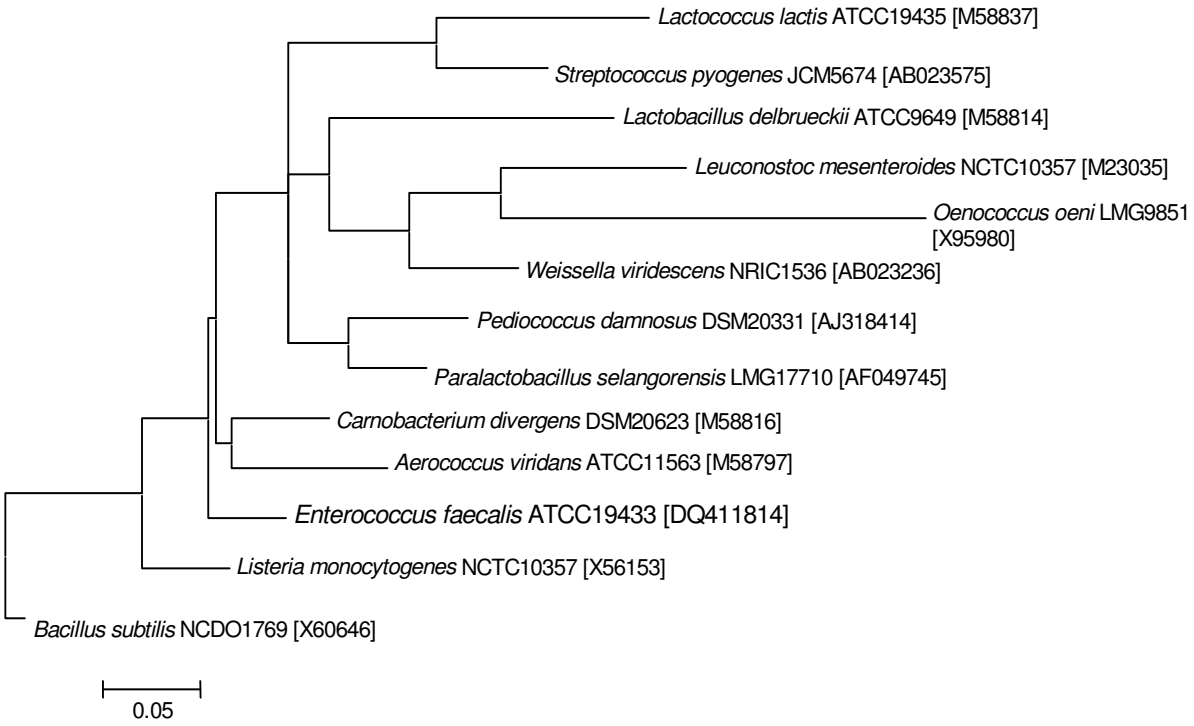

Figure 1.2 Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequence analysis, showing the relationships among type species of the representative genera from the families of *Lactobacillales*. These genera represent the families *Lactobacillaceae*, *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Leuconostoccaceae* and *Streptococcaceae* and non-*Lactobacillales*, *Listeria monocytogenes*. *Bacillus subtilis* is the outgroup. The bar indicates the number of nucleotide substitutions per site.

xenologous gene displacement (Koonin *et al*., 2001). In LAB, gene acquisition is thought to occur by conjugation or it is facilitated by bacteriophages (Wood and Warner, 2003). Consequently, duplication, acquisition and/or loss of genes occurred as these organisms underwent evolution (Makarova and Koonin, 2007). It was inferred that the ancestor of LAB might have acquired about 84 genes from various sources through HGT (Makarova and Koonin, 2007). This gene acquisition, especially of a group of peptidases, allows LAB to adapt and out-compete other microorganisms found in complex and nutritionally rich environments such as soil, silage, sewage, manure, water, plant materials, and mucous membranes such as the intestines, vagina and mouth where LAB normally reside (Axelsson, 2004; Makarova and Koonin, 2007).

HGT also influences the taxonomy of LAB. For instance, when a limited number of gene families are used for inferring phylogeny (see below), the topology of the gene trees are not concordant with species trees (Smith *et al*., 1992; McGowan *et al*., 1998; Friedrich, 2002; Ge *et al*., 2005). However, when either small subunit (SSU) rRNA genes, whole genomes or numerous housekeeping genes are used for phylogenetic analysis of organisms, HGT will have very little influence on the phylogenetic tree (Kurland, 2000; Daubin *et al*., 2003; Kurland *et al*., 2003; Snel *et al*., 2005; Choi and Kim, 2007). In other instances, HGT might result in unusual similarity of a gene originating from different genomes in comparison to orthologous genes (Koonin *et al*., 2001). The continuous genome decay through gene loss in LAB such as *Streptococcus thermophilus* explains the ongoing evolving taxonomy of these bacteria (Bolotin *et al*., 2004).

HGT plays an important part in LAB technologies, since genetic material, usually plasmids containing some characteristics, may be easily transferred among LAB, thus leading to the production of new genetic systems (Dalezios and Siebert, 2001). For instance, transferring a conjugative transposon with genes that encode for the production of nisin, an industrially important bacteriocin, to non-nisin producing strains has a huge industrial impact (Nga, 2005). Recombinant strains of *L. lactis* have been used for expression of bovine ß-lactoglobulin (Chatel *et al*., 2001) and for supplementation of lipase in pigs with pancreatic lipase defficiency (Drouault *et al*., 2002). The production of the flavour compound diacetyl by some LAB from citrate is mediated by citrate permease. The gene that codes for this enzyme is linked to a small plasmid (Kempler and McKay, 1981). Hence, recombinant strains that are deficient for this gene may not be used for production of diacetyl.

1.5.3 The order *Lactobacillales*

The order *Lactobacillales* belongs to the phylym Firmicutes, class Bacilli and comprises the LAB. Together with the *Lactobacillaceae*, this order includes five other families, as well as a number of species not currently classified in any known family (Table 1.2) (Garrity *et al*., 2001). The total number of genera within these families is 34 (Garrity *et al*. 2004; Table 1.2), with the following being important fermenters or spoilers of fermented products: *Lactobacillus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson, 2004). The valid species included in each of these genera are shown in Appendices A and B.

The family *Lactobacillaceae* consists of three genera, namely *Lactobacillus*, *Paralactobacillus* and *Pediococcus* (Garitty *et al*., 2004). The genus *Lactobacillus* currently contains 106 species, making it the largest group in the order (Felis and Delaglio, 2007; Appendix A). The genus *Pediococcus* contains 11 members. The genus *Paralactobacillus* contains only one species, *Paralactobacillus selangolensis* (Leisner *et al*., 2000), which was isolated from *chili bo*, a Malaysian food ingredient (Leisner *et al.*, 2000).

The family *Leuconostoccaceae* consists of three genera, namely *Leuconostoc*, *Oenococcus* and *Weissella*. *Leuconostoc*, the largest of these, has 23 members and some of them are associated with food (Shaw and Harding, 1989; Antunes *et al*., 2002; Kim *et al*., 2003; Chambel *et al*., 2006). *Weissella*, the second largest genus in this family, has 12 members. Although more members of this genus have been isolated from fermented food (Collins *et al*., 1993; Choi *et al*., 2002), some *Weissella* species have been detected in clinical samples (Björkroth *et al*., 2002; Shin *et al*., 2007). The genus *Oenococcus* consists of only two members, *Oenococcus oeni* and *O. kitaharae* (Appendix B).

The genera *Streptococcus* and *Lactococcus* make up the family *Streptococcaceae* (Appendix B). Of the 97 *Streptococcus* members, *Streptococcus thermophilus* is of industrial significance and it is used during the manufacture of a variety of fermented milk products. Most of the members in these genera are associated with a variety of diseases, both in humans and animals (Smith and Sherman, 1939; Bouvet *et al*., 1989; Whiley *et al*., 1999). The genus *Lactococcus* has six members with *Lactococcus lactis* playing a central role in the industrial production of various compounds such as nisin and diacetyl.

Table 1.2 Families and genera in the order *Lactobacillales

Family	Genera
Lactobacillaceae	• *Lactobacillus* • *Paralactobacillus* • *Pediococcus*
Aerococcaceae	• *Aerococcus* • *Abiotrophia* • *Dolosicoccus* • *Eremococcus* • *Facklamia* • *Globicatella* • *Ignavigranum*
Carnobacteriaceae	• *Carnobacterium* • *Agitococcus* • *Alkalibacterium* • *Allofustis* • *Alloicoccus* • *Desemzia* • *Dolosigranulum* • *Isobaculum* • *Lactosphaera* • *Marinalactibacillus* • *Trichococcus* • *Granulicatella*
Leuconostoccaceae	• *Leuconostoc* • *Oenococcus* • *Weissella*
Streptococcaceae	• *Streptococcus* • *Lactococcus*
Enterococcaceae	• *Enterococcus* • *Atopobacter* • *Mellisococcus* • *Tetragenococcus* • *Vagococcus*
Incertae sedis	• *Acetoanerobium* • *Oscillospira* • *Syntrophococcus*

*Adopted from: Garrity *et al*., 2004

Enterococcus, *Atopobacter*, *Mellisococcus*, *Tetragenococcus* and *Vagococcus* genera belong to the family *Enterococcaceae* (Garrity *et al*., 2004). The largest of these genera *Enterococcus* consists of 37 members that are associated with both foods and clinical samples (Fortina *et al*., 2004; Koort *et al*., 2004). The remaining genera consist of a total of eight members (Garrity *et al*., 2004).

The family *Aerococcaceae* consists of seven genera (Garrity *et al*., 2004; Table 1.2). The largest genus is *Facklamia*, which consists of six species (Appendix B). The genera *Abiotrophia* and *Aerococcus* have five members each. *Globicatella* genus has two members, whilst *Dolosicoccus*, *Eremococcus* and *Ignavigranum* consist of only one member per genus (Garrity *et al*., 2004; Appendix B).

The family *Carnobacteriaceae* consists of 12 genera (Garrity *et a*l., 2004; Appendix B), with a total of 24 members. The largest genus *Carnobacterium* consists of eight members, whilst the remainder contains one or two members only (Appendix B). Some members of *Carnobacteria* are associated with food spoilage (Holley *et al*., 2002).

1.6 IDENTIFICATION OF LAB ASSOCIATED WITH FERMENTED FOODS

1.6.1 Phenotypic methods

Phenotyping of bacteria involves all the identification methods that are not based on DNA or RNA (Caplice and Fitzgerald, 1999). These include morphology (e.g., shape, presence or absence of endospores or inclusion bodies), physiology and biochemical attributes such as conditions required for growth, activities of enzymes and metabolic activities. The significance of individual phenotypic characteristics may appear insignificant for determining genetic relatedness, yet, when taken together, taxa may be determined using these attributes (Vandamme *et al*., 1996).

For routine phenotyping, miniaturized identification kits such as API 50 CHL or BIOLOG, which are based on carbohydrate fermentation profiles, are often popular due to affordability (Temmerman *et al*., 2004). Although this approach is simple, affordable and provides evidence for functionality of strains, phenotyping is generally laborious, time-consuming and the efficiency and resolution may be poor, especially when complex samples with high diversity are studied (Meroth *et al*., 2003). For instance, most LAB isolates from freshwater

fish could not be identified, even at genus level, due to the poor taxonomic resolution of phenotypic methods (Gonzalez *et al*., 2000). In addition, only 38% of LAB from sourdough could be identified using the API 50 CHL system, as well as morphological and physiological characteristics (Corsetti *et al*. 2001). The fact that LAB strongly adapt to their environment and share many common attributes, makes differentiation of species using phenotypic methods challenging (e.g., Hayford *et al*., 1999; Van Reenen and Dicks, 1996). Moreover, it is challenging to compare results obtained using different phenotypic methods and similar phenotypes may not be concordant with related genotypes (Temmerman *et al*., 2004). Therefore, it is essential to use an approach that combines phenotyping and fast, reliable and reproducible DNA-based techniques with high resolution for identification of LAB associated with food fermentations (Vandamme *et al*., 1996; Nigatu, 2000).

1.6.2 DNA-based methods

LAB are characterized using a wide variety of DNA-based methods (Fig. 1.3). One DNA-based method that will allow identification of LAB to the species level is species-specific PCR (Nomura *et al*., 2002). Species-specific PCR involves the amplification of target genes, using primers that correspond to the oligonucleotide sequences present within parts of the whole genome of target organisms (Lupski and Weinstock, 1992), followed by subsequent analysis. Species-specific PCR targeting the 16S rRNA gene was used for identification of *Lb. brevis* and the method was found to be very sensitive, specific and efficient (Guarneri *et al*., 2001).

DNA-based methods that allow identification of LAB to the species and strain level include restriction fragment length polymorphism (RFLP) (Giraffa *et al*., 2002), pulsed-field gel electrophoresis (PFGE) (Ventura and Zink, 2002), randomly amplified polymorphic DNA-PCR (RAPD-PCR) (Williams *et al*., 1990), repetitive extragenic palindromic-PCR (rep-PCR) (Versalovic *et al*., 1991), amplified ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) (Vos *et al*. 1995) and phylogenetic analyses of housekeeping loci.

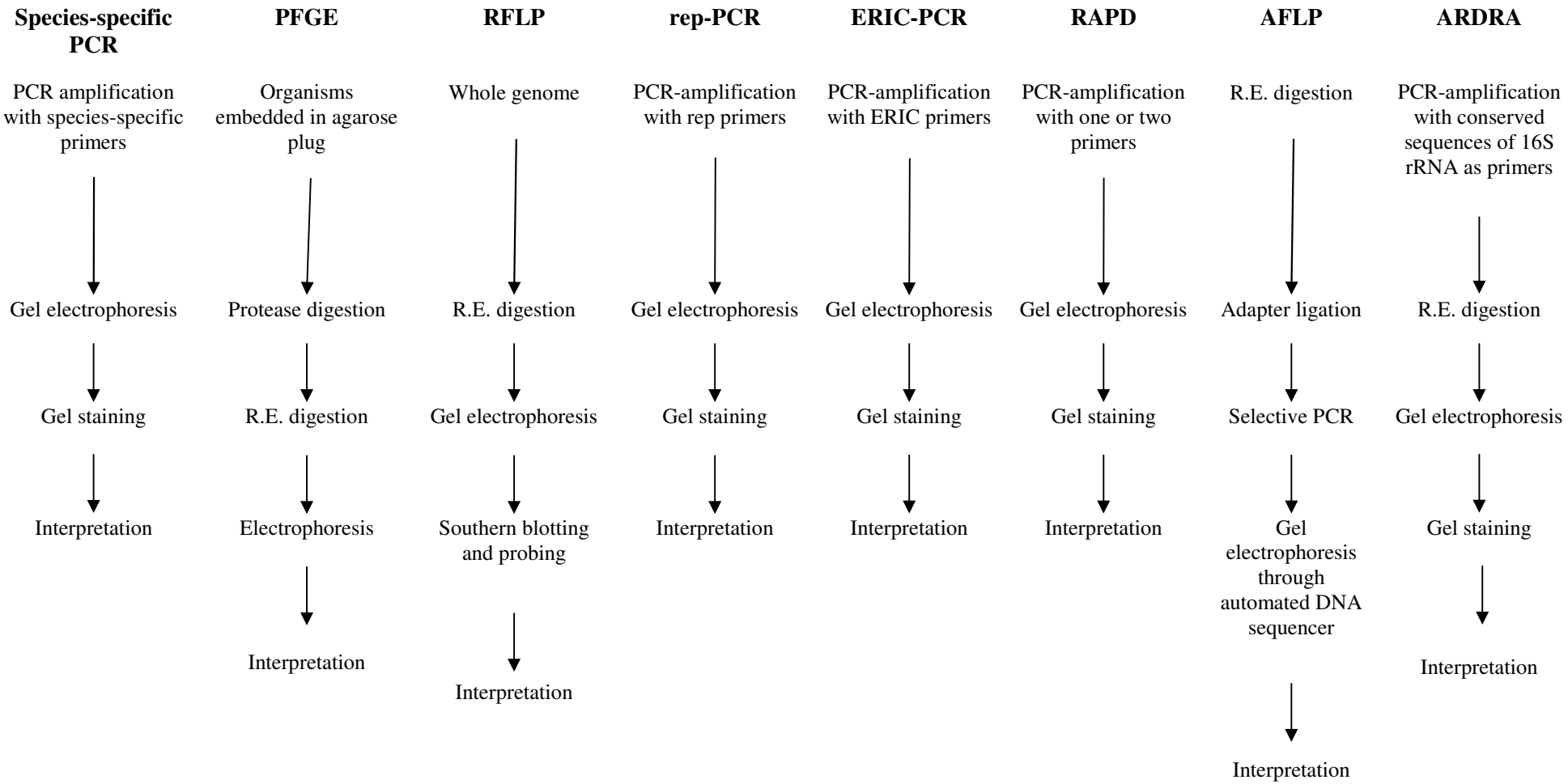

Figure 1.3 Main steps involved in DNA-based methods for characterization of LAB. R.E. represents restriction enzyme. (Adopted and modified from: Olive and Bean, 1999).

RFLP involves restriction enzyme digestion of the whole-genome, followed by size fractionation of the resultant DNA fragments by agarose gel electrophoresis. Southern blotting (Southern, 1975) may be used to transfer DNA fragments onto either a nitrocellulose or nylon membrane, followed by hybridization to a labeled homologous probe (e.g., rRNA gene probe), which allows visualization of the hybridized fragments (Vandamme *et al*., 1996). The hallmark of RFLP is high resolution up to strain level.

In ARDRA, bacterial rRNA genes are initially amplified by PCR using conserved sequences of rRNA genes as primers and the amplicon is digested with restriction endonucleases, followed by electrophoresis to obtain a fingerprint (Massol-Deya *et al*., 1995). ARDRA is fast to perform, but highly dependent on the degree of polymorphism in the region studied and the discriminatory power is often inferior (Massol-Deya *et al*., 1995) because smaller areas of the rRNA operon are targeted. Roy *et al*. (2001) used ARDRA for molecular discrimination of *Lb. helveticus*, *Lb. delbrueckii* subspecies *delbrueckii*, *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus* and *Lb. casei*, making it a suitable technique for discrimination of these closely related bacteria at subspecies level.

PFGE involves separation of large DNA representing the whole genome in an oscillating electric field (Tenover *et al*., 1995; McCartney, 2002). Excellent subspecies discrimination is the hallmark of PFGE. For instance, Ventura and Zink (2002) observed heterogeneity among isolates of *Lb. johnsonii* after using PFGE, despite the close relationship between these organisms. However, since the extraction of intact chromosomal DNA is critical to PFGE, the technique becomes labourious and expensive. Therefore, PFGE it is seldomly applied in studies where large numbers of isolates are to be characterized (Temmerman *et al*., 2004).

In RAPD-PCR fingerprinting, one or two primers (usually 10-12 bp) are arbitrarily selected and allowed to anneal to the bacterial genomic DNA template at low stringency, resulting in several amplification products of varying sizes, which are resolved electrophoretically to yield a RAPD fingerprint (Welsh and McClelland, 1990). RAPD analyses do not require prior knowledge of the target sequences. It has been used to follow unmarked starter cultures in commercial fermentations (Plengvidhya *et al*., 2004). Reguant and Bordons (2003) developed a multiplex RAPD-PCR, based on the combination of one random 10-mer and one specific 23-mer oligonucleotide in a single PCR. The method generated unique patterns and distinguished DNA profiles of strains of *Oenococcus oeni*. RAPD-PCR is simple and fast in

methodological terms, but its reproducibility is limited due to the low stringency of the PCR amplification and sensitivity to small variations in template concentration, purity, magnesium concentration and primer annealing temperature (Erlandson and Batt, 1997). Therefore, RAPD-PCR is best suited for studies where a specific bacterial strain is sought among a large number of isolates, and it is not well suited for inter-laboratory and taxonomic studies where the aim is to develop a fingerprint database.

Repetitive DNA elements allow the analysis of whole-genomes through DNA band profiles created using PCR-based techniques such as ERIC (enterobacterial repetitive intergenic consensus) sequences for typing enterobacteria (Hulton *et al*., 1991), BOX for typing *Streptococcus pneumoniae* using invertedly repeated DNA elements (Martin *et al*., 1992) and rep (repetitive extragenic palindromic) elements. In rep-PCR (Versalovic *et al*., 1991), primers anneal to repetitive parts of the chromosome and amplification occurs when the distance between the primer-binding sites are short enough to enable amplification. The advantages are high resolution (Ventura and Zink, 2002), making it suitable for analyzing closely related LAB using a $(GTG)_5$ primer, low cost, reliability (Olive and Bean, 1999) and high throughput (Gevers *et al*., 2001). In addition, standardization and inter-laboratory comparisons are feasible due to automation of rep-PCR with fluorescently labeled primers. However, very few publications are available. Bouton *et al*. (2002) used rep-PCR, RAPD-PCR and PFGE to identify selected starter strains during cheese ripening and showed that *Lb. helveticus* diminished fast, while *Lb. delbrueckii* subsp. *lactis* maintained viability during ripening.

Amplified fragment length polymorphism (AFLP) involves restriction enzyme digestion of DNA using two restriction enzymes, resulting in fragments with sticky ends. Prior to selective PCR-amplification of the fragments, adapters, which are short oligonucleotides, are ligated to the sticky ends (Zabeau and Vos, 1993). The subsequent PCR amplicons are then separated by polyacrylamide gel electrophoresis. The hallmark of AFLP is the ability to differentiate isolates at species level and below. Kunene *et al*. (2000) distinguished *Lb. plantarum* and *L. mesenteroides* isolated from different sorghum doughs using AFLP (Kunene *et al*., 2000).

Phylogenetic analyses of conserved genomic regions are increasingly being used for the identification of bacteria. Phylogenetic relationships among species are usually inferred using

rRNA genes due to the following: (i) ubiquitous nature of rRNA genes in bacteria, (ii) presence of highly conserved regions due to the essential role played by ribosomes during protein synthesis, (iii) presence of variable regions within the sequence, (iv) availability of large public domain databases (e.g., the Ribosomal Database), which enable sequence comparisons, and (v) rRNA-encoding genes are not usually influenced by horizontal gene transfer (HGT) (Woese, 1987; Axelsson, 2004; Felis and Dellaglio, 2007). However, the limitation of rRNA genes for determining phylogenetic relationships is that generally a sequence identity of less than 97% indicates a poor relationship of organisms at the genomic level (Felis and Dellaglio, 2007). Furthermore, 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). As is the case for many other bacterial taxa, LAB are known to encode 16S rRNA gene sequences that are not sufficiently polymorphic to allow species separation (Torriani *et al*., 2001; Gevers *et al.*, 2005; Felis and Dellaglio, 2007). Microorganisms are still described only as closely related even if they share identical rRNA sequences. Therefore, bacterial species are increasingly defined using multilocus sequence analysis (MLSA), involving housekeeping loci that evolve more rapidly than 16S rRNA genes (Gevers *et al.*, 2005; Kostantinidis and Tiedje, 2007).

1.7 DNA-BASED METHODS FOR STUDYING LAB COMMUNITIES

Microbial population dynamics is studied using either culture-dependent or culture-independent techniques. The limitations of culture-dependent methods for such studies are well-documented (Ampe *et al*., 1999; Walter *et al*., 2001; Randazzo *et al*., 2002). Culture-dependent methods may underestimate microbial diversity due to failure of some organisms to grow. For instance, culture-dependent methods failed to detect *Lb. delbrueckii*, despite its dominance during cheese ripening (Randazzo *et al*., 2002). Similarly, *Carnobacterium* sp. was not identified in cold-smoked salmon due to inhibition of its growth by sodium acetate in de Mann, Sharpe and Rogosa (MRS) medium (Giacomazzi *et al*., 2004). In addition, culture-dependent methods may overestimate microbial diversity due to non-selectivity of the culture media (Randazzo *et al*., 2002). Therefore, culture-independent genetic fingerprinting techniques are the methods of choice since they are easy to perform and are reproducible. These techniques include PCR-denaturing gradient gel electrophoresis (PCR-DGGE; Muyzer *et al*., 1993), single-stranded conformation polymorphism (SSCP; Orita *et al*., 1989), and terminal-restriction fragment length polymorphism (T-RFLP; Avaniss-Aghajani *et al*., 1994;

Fig. 1.4). Of these culture-independent techniques, PCR-DGGE is a frequently used method (Ercolini, 2004).

Despite their numerous advantages, culture-independent techniques with respect to food matrices are limited by DNA extraction and PCR amplification biases (Von Wintzingerode *et al*., 1997; Ercolini, 2004). DNA extraction of bacteria in complex ecosystems may be complicated due to differences in cell structure among varying taxa, leading to bias in extraction in favour of cells that are easily disrupted (Niemi *et al*., 2001; Ercolini, 2004). Niemi and co-workers (2001) concluded that PCR-DGGE profiles are a function of the method used for DNA isolation and purification. This bias in efficiency of DNA extraction may cause further bias in downstream applications such as PCR amplification (Ercolini, 2004). PCR amplification may be biased due to preferential amplification of target genes (Reysenbach *et al*., 1992). Consequently, some microorganisms in a complex ecosystem are excluded (Ercolini, 2004). Therefore, in order to obtain a true reflection of the microbial population dynamics during food fermentations, a holistic approach that combines both culture-dependent and culture-independent methods is important.

In DGGE, total bacterial DNA from the habitat of interest is extracted and a variable region of the 16S rRNA gene is PCR-amplified (Fischer and Lerman, 1983; Muyzer *et al*., 1993; Fig. 1.4). DNA amplicons of the same length but containing different DNA sequences are subjected to polyacrylamide gel electrophoresis by using a denaturing gradient established with urea and formamide. DGGE enables direct visualization of bacterial diversity and subsequent identification of community members by DNA fragment sequence analysis or hybridization with specific probes (Muyzer *et al*., 1993). Therefore, it is a suitable tool for rapid and economical investigation of microbial community dynamics during whole-food fermentation processes (ben Omar and Ampe, 2000; Meroth *et al*., 2003). Well-defined microflora produced by using starter cultures in controlled conditions are suitable for DGGE analysis, as the food substrates usually display a simple profile where each band corresponds to the expected species (Ercolini *et al*., 2004).

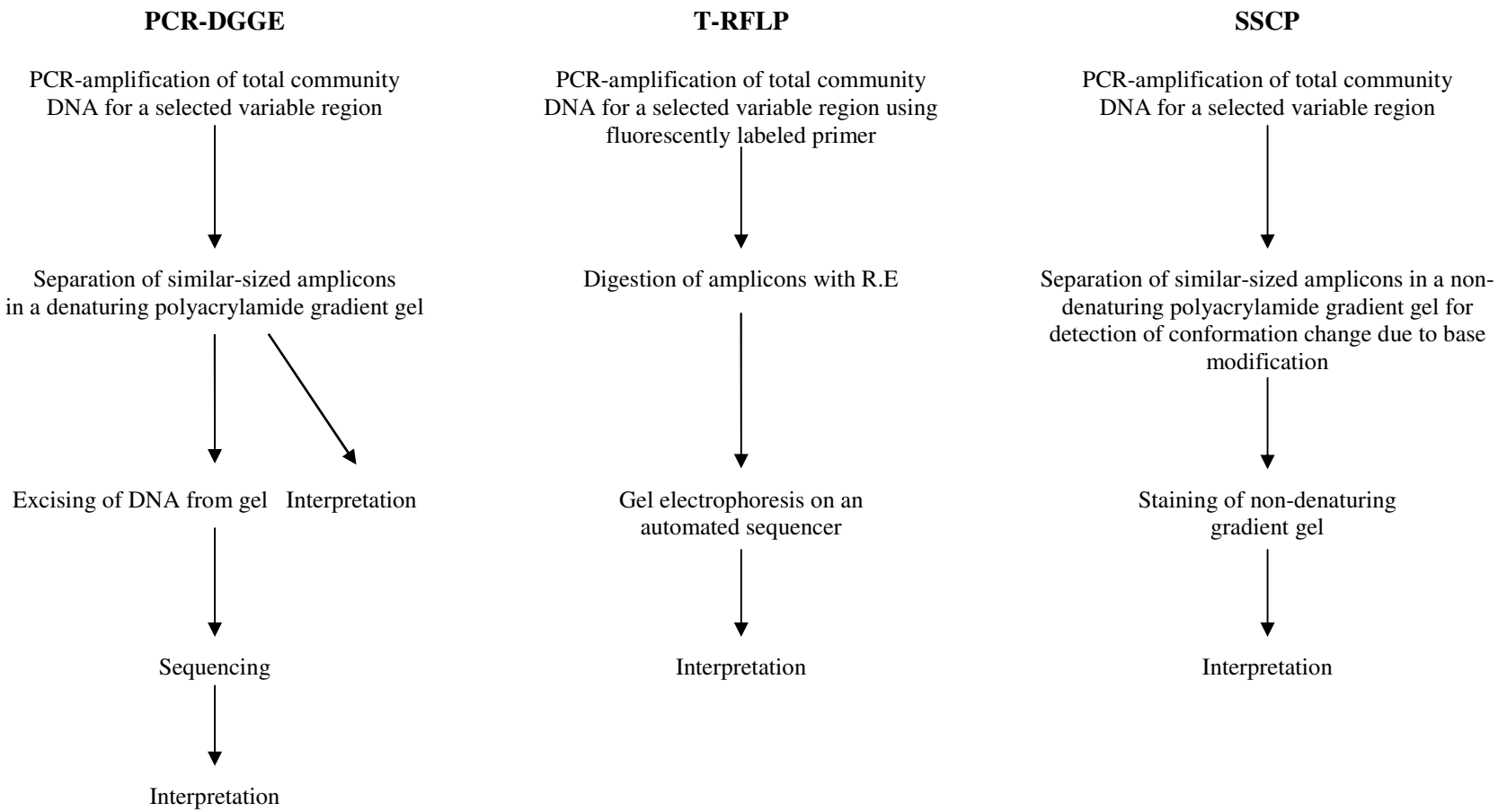

Figure. 1.4 Main steps involved in DNA-based methods for studying microbial population dynamics of LAB.

Many studies involving microbial population dynamics of LAB during food fermentations have employed PCR-DGGE. Two LAB-specific PCR-DGGE systems were used to monitor the development of starter mixtures of three commercially available sourdough starters and a baker's yeast preparation, containing various species of LAB, and *Lb. sanfranciscensis*, *Lb. pontis*, *Lb. mindensis*, *Lb. crispatus*, *Lb. johnsonii*, *Lb. frumenti* and *Lb. reuteri* were shown to be the predominant species (Meroth *et al*., 2003). Randazzo *et al*. (2002) examined microbial succession during the manufacture of artisanal Sicilian cheese using PCR-DGGE and cultivation. For total microflora, the V6-V8 variable region of the 16S rRNA gene was analyzed, whilst species-specific primers targeting the V1-V3 region were used to detect *Lactobacillus* spp. *Streptococcus thermophilus* prevailed during fermentation, whilst mesophilic bacteria were dominant in raw milk after cloning and sequencing of the 16S rRNA gene amplicons.

The major advantages of DGGE are reproducibility, reliability and speed (Muyzer, 1999). However, DGGE has particular limitations. For instance, PCR-DGGE failed to detect *Lb. acidophilus* and *Lb. casei*, declared in the probiotic yoghurt products Biospega and ABC, due to bacterial concentrations below the detection limit (Fasoli *et al*., 2003). This may also be due to the choice of PCR primers, as these are crucial for determining the resolution of DGGE (Temmerman *et al*., 2004). However, group-specific primers may improve the detection limit for less abundant groups in the microbial ecosystem. Furthermore, a single band may represent more than one strain in DGGE (Muyzer and Smalla, 1998; Sekiguchi *et al*., 2001; Van Beek and Priest, 2001; Cocolin *et al*., 2001; Meroth *et al*., 2003), a phenomenon known as co-migration. For instance, *Lb. manihotivorans*, which represented up to 13% of the total LAB of sour cassava starch, was not detected by DGGE as the PCR product migrated at the same position as that of *L. lactis* (Ampe *et al*., 2001). This may be circumvented by using a set of different primer pairs or varying the DGGE gradient (Ercolini, 2004). In addition, targeting the 16S rRNA gene to study community structure may overestimate species diversity in DGGE profiles due to the heterogeneity of this region (Farrely *et al*., 1995; Fogel *et al*., 1999). However, targeting single copy genes such as *rpoB*, encoding the RNA polymerase beta subunit, may circumvent this limitation (Dahllöf *et al*., 2000; Giacomazzi *et al*., 2004). DGGE may also be biased by the failure of PCR to distinguish between viable and non-viable cells (Uyttendaele *et al*., 1999). In some instances, PCR detected cells after autoclaving at 121°C for 15 min (Masters *et al*., 1994).

In SSCP (Orita *et al*., 1989), a single base modification can change the conformation of a single-strand DNA molecule, altering the migration distance of the molecules in a non-denaturing gel. Hence, sequence variations between DNA fragments of the same size (Hayashi, 1991; Fig. 1.4), which are usually PCR-amplified from variable regions of the 16S rRNA gene, are detected (Giraffa and Neviani, 2001). Duthoit *et al*. (2003) used SSCP analysis targeting the V2 or V3 regions of the 16S rRNA gene to study the dynamics of the microbial flora of Salers cheese without the addition of starter culture. The authors found *L. lactis*, *S. thermophilus*, *E. faecium*, *Le. mesenteroides*, *Le. pseudomesenteroides*, *Lb. plantarum* and *Lb. pentosus* to be predominant during manufacturing and ripening. In contrast to DGGE, SSCP gives at least two bands (one for each strand) and often a third one (the re-annealed dsDNA product) that represents one DNA sequence, making interpretation of the results complex, especially for studies that involve community analysis of microorganisms. The major advantage of SSCP is that it is rapid to perform and automatization of the technique by capillary electrophoresis allows microbial dynamics of many samples to be analyzed (Duthoit *et al*., 2003). The discriminatory power and reproducibility of SSCP analysis is usually most effective for fragments up to 400 bp (Vaneechoutte, 1996). PCR-SSCP detects bacterial populations that make up 1% or more of a bacterial community (Lee *et al*., 1996).

In T-RFLP, the target gene is amplified from the community DNA using standard PCR techniques with a 5' fluorescently tagged primer (Avaniss-Aghajani *et al*., 1994; Liu *et al*., 1997; Fig. 1.4), and the amplification products are digested with restriction enzymes and electrophoresed on an automated sequencer. Only the restriction fragment containing labeled primer is detected by the automated system. In general, each population of the community contributes a terminal fragment of one size (Moeseneder *et al*., 1999; Osborn *et al*., 2000; Dunbar *et al*., 2001). Hence, direct comparisons can be made rapidly and easily using the large sequence database of the Ribosomal Database Project (Marsh *et al*., 2000; Dunbar *et al*., 2001). However, there is a possibility that two different species could have the same restriction site in their 16S rRNA gene sequence, resulting in an identical peak (Moeseneder *et al*., 1999). Nevertheless, the use of different enzymes increases information for discriminating samples (Moeseneder *et al*., 1999). T-RFLP of amplified 16S rRNA genes was successfully used to study the dynamics of metabolically active mixed dairy cultures consisting of *L. lactis* subsp. *lactis* and *Le. citreum* (Sánchez *et al*., 2006).

1.8 CONCLUSIONS AND AIMS OF THIS INVESTIGATION

In summary, most studies involving characterization of LAB isolated from fermented sorghum foods have focussed on phenotypic methods. However, if traditional sorghum-based fermented foods are to be upgraded to commercial status, whilst maintaining their unique pleasant sensory attributes, characterization of wild-strain LAB as potential starter cultures is of paramount importance. The limitations of phenotyping are well documented. Various DNA-based methods offer a rapid and more reliable alternative. However, the inherent limitations in each technique necessitate the need for a polyphasic approach. To select the most suitable starter cultures, which can adapt rapidly to the sorghum matrix and dominate fermentations, it is essential to study the dynamics of the microflora involved. A combination of both culture-dependent and culture-independent approaches may give an overview of the diversity and microbial population dynamics occurring in sorghum fermentations.

Although PFGE has excellent resolution, routine use for characterization of LAB during food fermentations is limited by the labourious nature of the technique. The use of RAPD-PCR as a method for characterization of LAB in the food industry is limited by reproducibility challenges. However, rep-PCR, which is cost-effective and reliable, has a high resolution and can be used to analyze complex genomes (Ventura and Zink, 2002). This may be an important technique in future for characterization of LAB isolated from indigenous fermented sorghum-based foods since it was shown to have good correlation with PFGE results, albeit with slightly less resolution.

Predominant and dominant wild-strain LAB starter cultures used in most trial fermentations of sorghum-based foods have been selected based on methods that relied on culture-dependent approaches. However, it is imperative in future to include culture-independent methods for such studies in order to obtain a true reflection of LAB that dominate sorghum fermentations since some microorganisms may be excluded or under-represented by cultivation on particular media. PCR-DGGE is a powerful tool for microbial population studies involving food fermentations, and it has been used to monitor microbial composition and dynamics during food fermentations (Randazzo *et al.*, 2002; Meroth *et al.*, 2003). Its current limitations, however, necessitate the use of PCR-DGGE in combination with other techniques. Therefore, an approach that combines both culture-dependent and culture-

independent methods to give an overview of microbial population dynamics in indigenous sorghum-based foods is recommended. Consequently, the aims of this study were:

- To characterize bacteria isolated from spontaneous sorghum fermentations using DNA-based techniques.
- To perform PCR-DGGE on total bacterial genomic DNA obtained from two types of commercial sorghum flour spontaneously fermented at 20, 25 and 30°C, so as to determine the diversity and microbial dynamics that occur during sorghum fermentations.
- To clarify the role of selected starter cultures of LAB with regards to the microbiological safety and consumer acceptance of the sensory characteristics of *ting*.

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CHAPTER TWO

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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ABSTRACT

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 microbiological 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 *Lactobacillus*, i.e. *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus*. This represents 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*

2.1 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 could 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 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 microorganisms 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 objectives of this study were to isolate the LAB responsible for the *ting* fermentation process, and to identify 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.

2.2 MATERIALS AND METHODS

2.2.1 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 was mixed (1:1 [w/v]) 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 (Oxoid, Basingstoke, UK). 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 30°C for 48 h. Colonies were purified and stored at -20°C in MRS broth supplemented with 20% glycerol.

2.2.2 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 also determined. To determine the carbohydrate fermentation profiles of the bacteria, API 50 CHL test kits (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.

2.2.3 DNA-based identification of LAB

2.2.3.1 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 16S rRNA 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 16S rRNA genes, 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.

2.2.3.2 Nucleotide sequencing and sequence analyses

The 16S rRNA gene 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*).

2.2.3.3 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 16S rRNA and *pheS* gene sequences for the *Lactobacillus* type strains for the *Lb. reuteri*, *Lb. plantarum* and *Lb. 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 16S rRNA gene sequence 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.

2.3 RESULTS

2.3.1 Phenotypic identification of LAB

All the selected isolates were catalase-negative, non-motile and Gram-positive, and were thus tentatively assigned to LAB. Among the nine representative LAB isolates, 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 2.1). *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 *Lb. plantarum* (isolates 1.42, 5.42, 5.35 and 4.30), *Lb. fermentum* (isolates 3.42, 4.35, 3.30 and 5.30) and *Lb. 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.

2.3.2 DNA-based identification of LAB

Comparison of the 16S rRNA gene 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 16S rRNA gene sequence for isolate 1.42 was most similar to those of the

Table 2.1 Differential characteristics of nine LAB isolates from fermented sorghum, based on API 50 CHL analysis

	API 50 CHL Results*								
Isolate	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	*Lb. plantarum*	*Lb. fermentum*	*Lb. plantarum*	*Lb. rhamnosus*	*Lb. fermentum*	*Lb. plantarum*	*Lb. fermentum*	*Lb. plantarum*	*Lb. fermentum*

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

so-called *Lb. plantarum* Group (Naser *et al*., 2007) that includes *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus*. The 16S rRNA gene sequence for isolate 2.35 was most similar to those of the *Lb. casei* Group (Naser *et al*., 2007) that includes *Lb. casei*, *Lb. paracasei*, *Lb. zeae* and *Lb. rhamnosus*. The *blastn* results for isolates 3.42, 4.35, 3.30 and 5.30 indicated that their 16S rRNA gene sequences were most similar to that of *Lb. fermentum* in the *Lb. reuteri* Group (Naser *et al*., 2007). ML analysis of the 16S rRNA gene sequence data indicated that isolates 3.42, 4.35, 3.30 and 5.30 were indeed most closely related to *Lb. fermentum* (Fig. 2.1A). The 16S rRNA gene sequence-based ML analysis did, however, not allow discrimination of isolates 2.35 and 1.42 from other *Lb. plantarum* Group and *Lb. 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 (Fig. 2.1B). Within the *Lb. plantarum* Group, isolate 1.42 grouped with known *Lb. plantarum* strains, and within the *Lb. casei* Group, isolate 2.35 grouped with *Lb. rhamnosus*.

2.4 DISCUSSION

This represents the first study investigating 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*. The results suggested that three *Lactobacillus* species, i.e. *Lb. plantarum*, *Lb. fermentum* and *Lb. 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, 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.

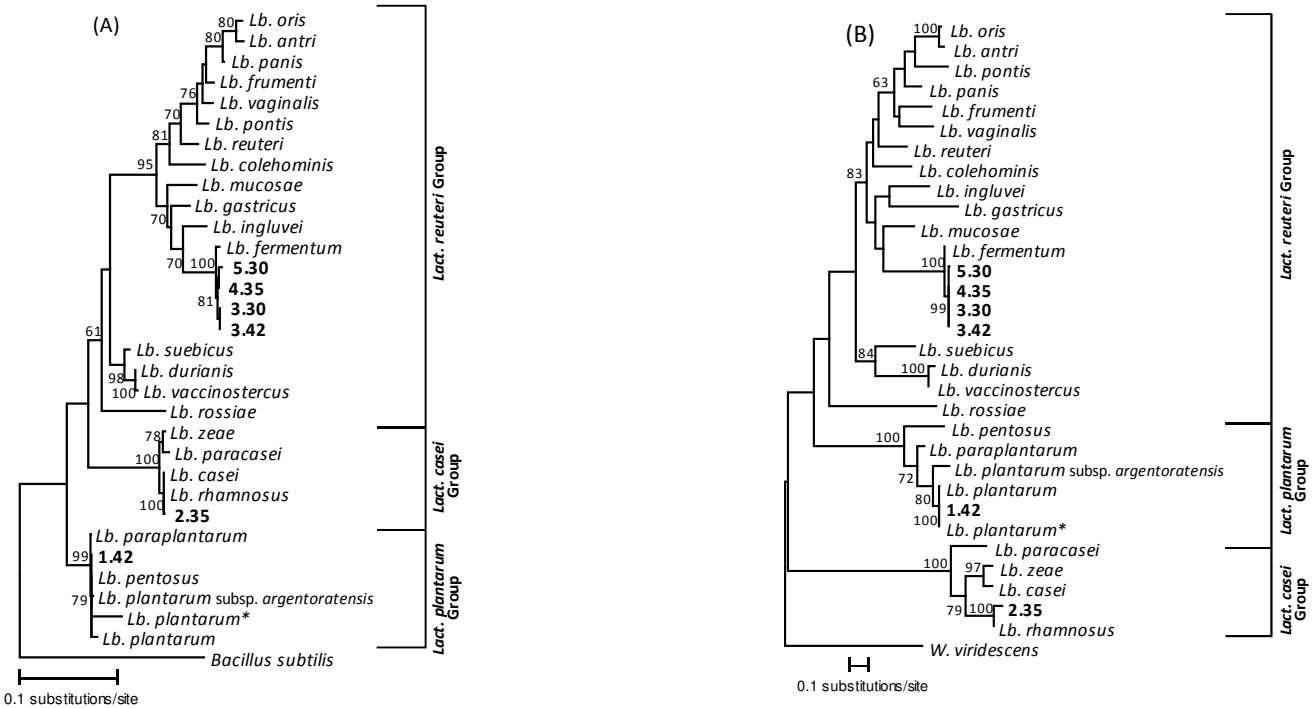

Figure 2.1 Maximum likelihood (ML) phylogeny for the *Lb. plantarum*, *Lb. casei* and *Lb. reuteri* Groups (Naser *et al*., 2007), based on 16S rRNA (A) and *pheS* (B) gene sequences. Isolates from fermented sorghum slurries are indicated in bold and bootstrap values >60%, based on 1000 replicates, are indicated at the internodes. For the various *Lactobacillus* species, type strain numbers and GenBank accession numbers for the 16S rRNA and *pheS* sequences, respectively, are as follows: *Lb. antri* LMG22111T, AY253659, AM263502; *Lb. casei* LMG6904T, M58815, AM087682; *Lb. coleohominis* LMG21591T, AJ292530, AM087683; *Lb. durianis* LMG19193T, AJ315640, AM087739; *Lb. fermentum* LMG6902T, M58819, AM087693; *Lb. frumenti* LMG19473T, AJ250074, AM087741; *Lb. gastricus* LMG22113T, AY253658, AM087696; *Lb. ingluviei* LMG20380T, AF333975, AM087731; *Lb. mucosae* LMG19534T, AF126738, AM087707; *Lb. oris* LMG9848T, X94229, AM087709; *Lb. panis* LMG21658T, X94230, AM087725; *Lb. paracasei* LMG13087T, D79212, AM087710; *Lb. paraplantarum* LMG16673T, AJ306297, AM087727; *Lb. pentosus* LMG10755T, AB289240, AM087713; *Lb. plantarum* LMG6907T, X52653, AM087714; *Lb. plantarum** LMG19807T, AJ965482, AM087736; *Lb. plantarum* subsp. *argentoratensis* LMG9205T, AJ640078, AM694185; *Lb. pontis* LMG14187T, AJ422032, AM087715; *Lb. rhamnosus* LMG6400T, AB008211, AM087716; *Lb. reuteri* LMG9213T, X76328, AM087728; *Lb. rossiae* LMG22972T, AB370880, AM087768; *Lb. suebicus* LMG11408T, AM113785, AM087772; *Lb. vaccinostercus* LMG9215T, AJ621556, AM087750; *Lb. vaginalis* LMG12891T, AF243177, AM087751; *Lb. zeae* LMG17315T, D86516, AM087761 (LMG = Laboratory of Microbiology, Ghent University, Belgium). *Lb. arizonensis* (type strain LMG19807) that was shown to represent a later heterotypic synonym of *Lb. plantarum* (Kostinek *et al*., 2005) is indicated with an asterisk. The GenBank accession numbers for *pheS* and 16S rRNA gene sequences for the outgroup taxa *Weissella viridescens* (LMG3507T) and *Bacillus subtilis* (LMG7135T) are AM711182 and X60646, respectively.

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* LAB isolates examined, only four could be identified with some level of certainty using 16S rRNA gene sequence data (Fig. 2.1A). The other two isolates formed part of the so-called *Lb. plantarum* and *Lb. casei* Groups (Naser *et al*., 2007). As is the case for many other bacterial taxa, the members of these groups are known to encode 16S rRNA gene sequences that are not sufficiently polymorphic to allow species separation (Torriani *et al*., 2001; Gevers *et al.*, 2005; Felis and Dellaglio, 2007). Therefore, bacterial species are increasingly being defined using housekeeping loci that evolve more rapidly than 16S rRNA genes (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* (aminopeptidase 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, it was also found that *pheS* has greater discriminatory power, because it displayed sufficient interspecific variation that allowed the unequivocal identification of isolates 2.35 and 1.42 as *Lb. rhamnosus* and *Lb. plantarum*, respectively (Fig. 2.1B).

Of the three *ting* LAB species identified, *Lb. fermentum* and *Lb. 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, *Lb. 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). This is probably due to its acid tolerance and superior ability to utilize the substrates involved (Akinrele, 1970; Oyewole and Odunfa,

1990). It should 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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CHAPTER THREE

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 cooked South African food that is popular for its sour taste and unique flavour. Insight of microbial diversity and population dynamics during sorghum fermentations is an initial but imperative step in the development of starter cultures for commercial production of *ting*. In this study, microorganisms associated with spontaneous fermentations of two sorghum types were examined using culture-independent and culture-dependent methods. Culture-independent polymerase chain reaction (PCR) of the V3 region of the 16S rRNA gene and analysis of the resulting amplicons using denaturing gradient gel electrophoresis (DGGE), followed by sequence analyses of the most intense bands, revealed that lactic acid bacteria (LAB), including close relatives of *Lactococcus lactis*, *Lactobacillus curvatus*, *Weissella cibaria* and some members of the *Enterobacteriaceae* family, were predominant at the end of fermentation in both sorghum types. *L. lactis*, however, showed the most intense band. Culture-dependent methods, involving isolation of the various bacteria, followed by 16S rRNA gene sequence analyses indicated that *Enterococcus mundtii* was present at the start of fermentation, whilst *Lb. fermentum*, *Lb. plantarum*, *Lb. rhamnosus*, *E. faecalis*, *W. cibaria* and *L. lactis* were predominant at the end of fermentation. *Lb. curvatus* was not detected in culture media, but it was predominant in DGGE gels. Likewise, *Lb. fermentum*, *Lb. plantarum*, *Lb. rhamnosus*, *E. faecalis* and *E. mundtii* were isolated in culture media, but they were absent in DGGE gels. Therefore, the combined approach was effective in revealing the microbial diversity and dynamics during spontaneous sorghum fermentations.

Key words: culture-independent; culture-dependent; microbial diversity; population dynamics; PCR-DGGE; fermentation

3.1 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 (Food and Agricultural Organisation, 2006). However, sorghum as a main dietary constituent is usually associated with under-nourishment due to the lack of certain essential amino acids (Neucere and Sumrell, 1979; Kazanas and Fields, 1981) and the presence of anti-nutritional factors (Hamacker *et al*., 1986; Ibrahim *et al*., 2005). These shortcomings may be overcome by fermentation that is primarily facilitated by lactic acid bacteria (LAB) (Au and Fields, 1981; Chavan *et al*., 1988; Hassan and El Tinay, 1995). In addition to improving the nutritive value of this commodity, fermentation also enhances its sensory properties (Au and Fields, 1981; Chavan *et al*., 1988; Hassan and El Tinay, 1995). As a result, various sorghum-based fermented foods with unique and appealing characteristics are produced whereever sorghum is cultivated (Caplice and Fitzgerald, 1999; Taylor, 2003). These include *injera* (Gebrekidan and Gebrettiwat, 1982), *kisra* (Mohammed *et al*., 1991), *ogi* (Akingbala *et al*., 1981), *mahewu* (Bvochora *et al*., 1999), *uji* (Mbugua, 1984), *muramba* (Mukuru, 1992), *bushera* (Muyanja, 2003), *togwa* (Lorri and Svanberg, 1995), and *ting* (Boling and Eisener, 1982).

Ting is a sour porridge made by cooking fermented sorghum (Boling and Eisener, 1982). It is frequently used as a weaning food for infants in rural South Africa because it is relatively inexpensive to prepare and does not require refrigeration or re-heating prior to consumption (Kunene *et al*., 1999). 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 (Sanni, 1993). Also, little is known about the microorganisms that participate in this fermentation. Although bacteria were previously isolated from fermented sorghum and identified as *Lactobacillus plantarum*, *Lb. fermentum* and *Lb. rhamnosus* (Chapter 2), their involvement in the 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 quality.

To study the diversity and dynamics of microbial populations associated with specific fermented foods, a combination of culture-dependent and -independent approaches are typically applied. Culture-dependent approaches represents the only means of recovering microorganisms from the fermented substrate (Miambi *et al*., 2003), although it is widely recognized that they do not allow analysis of true diversity and/or polulation dynamics (Ampe *et al*., 1999; Meroth *et al*., 2003; Nielsen *et al*., 2007). On the other hand, various studies have shown that culture-independent approaches provide more reliable and reproducible (Ercolini, 2004) means for studying microbial populations in complex ecosystems such as food matrices (Ercolini, 2004; Rantsiou and Cocolin, 2006; Renouf *et al*., 2006; Camu *et al*., 2007). These approaches are based on methods such as PCR-DGGE (denaturing gradient gel electrophoresis; Muyzer *et al*., 1993), SSCP (single-stranded conformation polymorphism; Orita *et al*., 1989), T-RFLP (terminal-restriction fragment length polymorphism; Liu *et al*., 1997) and LH-PCR (length heterogeneity-PCR; Ritchie *et al*., 2000) analyses of a specific gene fragment (usually the 16S ribosomal RNA gene, *rrs*).

The aim of this study was to systematically and effectively describe the microbial populations associated with fermented sorghum, using a combination of culture-dependent and culture-independent methods. For the culture-independent analysis, PCR-DGGE was used to monitor the succession of dominant microbial populations from the onset to the end of spontaneous fermentations of sorghum. In parallel with the PCR-DGGE experiments, culture-dependent methods were used to enumerate, isolate and identify the bacteria involved in the fermentations by making use of phylogenetic analyses of the genes encoding 16S rRNA and phenylalanyl-tRNA synthase (*pheS*).

3.2 MATERIALS AND METHODS

3.2.1 Sorghum fermentations

In this study, fermentations were conducted at three different temperatures using sorghum flour obtained from two commercial sources in South Africa (King Food Corporation, Potchefstroom and Nola Pvt Ltd, Randfontein). For this purpose, individual sorghum slurries were prepared by mixing the respective sorghum flours thoroughly with sterile luke-warm (ca. 40°C) 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. All fermentations were done in duplicate. During the incubation period, 50-ml samples of the fermenting sorghum slurries were aseptically

collected at the start of fermentation (t = 0 h) and after 6, 12, 18, 24, 30, 36, 42, 48 and 54 h of fermentation. The pH of a 15-ml aliquot of each sample was determined using a Beckman model Ø 34 pH meter (Beckman Coulter, Fullerton, CA, USA) by recording the averages of measurements taken in triplicate. For each of the collected samples, 15-ml aliquots were immediately frozen and stored at -20°C for later DNA extractions, while the remainder was used for culture-dependent analyses.

3.2.2 PCR-DGGE analyses

To prepare good quality DNA for the PCR-DGGE analyses, most of the sorghum was first removed from each of the various collected slurry samples, as follows. The 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 1 500 × *g* to remove the beads and large sorghum particles. The bacterial cells in 1.5 ml of the individual supernatants were harvested by centrifugation at 5 000 × *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 (Promega, Madison, USA).

For PCR-DGGE analyses, the V3 region of the bacterial 16S rRNA gene was targeted using the primers 518R (5'-attaccgcggctgg-3'; Muyzer *et al*., 1993) and F357-GC. Primer F357-GC is similar to primer F357 (5'-tacgggaggcagcag-3', Rainey *et al*., 1996), except that it includes a 40-base GC-clamp (5'-cgcccgccgcgcgcggcgggcggggcgggggcacggggg-3'; Muyzer *et al*., 1993) at its 5'-end to avoid complete melting of PCR products during DGGE (see below). PCR was performed according to Van der Meulen *et al*. (2007) on a MyCycler™ thermal cycler (BioRad, Hercules, USA) with the following cycling conditions: denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 45 s, extension at 72°C for 1 min and final extension for 7 min at 72°C.

The 16S rRNA V3 region was also amplified from 13 different bacteria and combined to use as a DGGE reference ladder (Van der Meulen *et al*., 2007). These amplicons were generated using the same primers and cycling conditions as above. The bacteria comprised *Bacteriodes fragilis* (DSM 1396), *Bacteroides thetaiomicron* (LMG 11262), *Weissella cibaria* (LMG 17699), *Enterococcus flavescens* (LMG 13518T), *Enterococcus solitarius* (LMG 12890T), *Leuconostoc fructosum* (LMG 9498T), *Bacillus subtilis* (LMG 7135T), *Clostridium*

butyricum (LMG 1212), *Lactobacillus rhamnosus* (LMG 6400T), *Bifidobacterium longum* (LMG 13197T), *Bifidobacterium bifidum* (LMG 11041T), *Bifidobacterium lactis* (LMG 18314T), and *Bifidobacterium dentium* (LMG 11045T).

DGGE was essentially performed according to the procedure described by Muyzer *et al.* (1993) with slight modifications, as described previously (Van der Meulen *et al.*, 2007). The incorporation of a reference ladder on each DGGE gel allowed digital normalization of the band profiles by using the BioNumerics v. 4.0 software package and comparing with a standard reference (Van der Meulen *et al.*, 2007). Consequently, migration distances between different gels could be compared. All PCR-DGGE experiments were performed in duplicate.

To determine the identity of the bacteria for which individual 16S rRNA V3 PCR-DGGE fragments were generated, the most intense bands 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, after which the eluted DNA was used as template to re-amplify the specific fragments using identical PCR conditions and primers as 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. The resulting products were then purified with the QIAquick PCR purification kit (QIAGEN, Germany) and sequenced in both directions using primers 518R and 357F, the BigDye® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems, USA) and an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems).

3.2.3 Enumeration, isolation and characterization of bacteria

For the fermented sorghum flour slurries, triplicate 10-fold serial dilutions were carried out by transferring 10-g samples to 90 ml of buffered peptone water (0.1% [w/v] peptone, 0.85% [w/v] NaCl; pH 7.2), followed by vortexing for 3 min to obtain homogenous mixtures. One (1) ml of this 10^{-1} diluted homogenate was then transferred into 9 ml of the same diluent. Aliquots (100 µl) of the dilutions were surface inoculated onto the following selective media: MRS-5 (Oxoid, Basingstoke, UK) for lactobacilli (Meroth *et al.*, 2003), M17 for lactococci (Terzaghi and Sandine, 1975), *Enterococcus* selective agar (ESA) for enterococci (Slanetz and Bartley, 1957), Violet red bile agar (VRBA, Oxoid) to obtain Gram-negative counts

(GNC) (Mossel *et al*., 1986) and plate count agar (PCA, Oxoid) to obtain numbers of total aerobic bacteria (Reasoner and Geldreich, 1985). The 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 counts and LAB counts, plates with colonies between 30 and 300 were selected for enumeration, whilst colonies between 15 and 150, and greater than 0.5 mm in diameter, were selected for GNC on VRBA. The counts were recorded as averages of three determinations and they were expressed as colony forming units (cfu) per gram.

After incubation, colonies on the various growth media were grouped according to macroscopic and microscopic appearance. Representative colonies were then randomly picked and sub-cultured to obtain pure cultures, after which they were routinely grown in appropriate broth media. *Lactobacillus*, *Weissella* and *Lactococcus* species were grown in MRS broth, whilst *Enterobacteriaceae* were grown in nutrient broth using the incubation conditions described. All pure cultures were microscopically examined using light microscopy to score cell morphology, motility and Gram stain. Catalase and oxidase activity were evaluated using 3% hydrogen peroxide (Morgulis, 1921) and tetramethyl-*p*-phenylenediamine (TMPD), respectively (Ehrlich, 1956). All cultures were stored at -20°C in sterile Eppendorf tubes, containing the appropriate broth media supplemented with 20% (v/v) glycerol as cryoprotectant.

3.2.4 PCR and sequencing of the 16S rRNA and *pheS* genes from 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*. (1989). The *pheS* gene was amplified for each of the isolated pure cultures using the primers PheS-21-F (5'-cayccngchcgycgygayatgc-3') and PheS-23-R (5'-ggrtgraccatvccngchcc-3'), as described by Naser *et al*. (2005). Amplicons were purified using the Nucleofast 96 PCR clean-up membrane system (Machery-Nagel, Germany) and sequenced, as described above, using the original *pheS* PCR primers.

The gene encoding 16S rRNA was amplified for each of the isolated pure cultures using the eubacterial universal primers 27F (5'-agagtttgatcctggctcag-3'; Lane, 1991) and 1507R (5'-taccttgttacgacttcacccca-3'; Heyndrickx *et al*., 1996). PCR mixtures contained 1.25 U 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, 50 ng of template DNA and 8% (v/v) dimethyl sulphoxide (DMSO). The PCR cycling conditions consisted of an initial denaturation step at 95°C for 10 min, and 35 cycles with denaturation at 94°C for 1 min, primer annealing at 51°C for 30 s and primer extension at 72°C for 1 min, followed by final extension at 72°C for 10 min and cooling to 4°C. The resulting 16S rRNA PCR products were sequenced with the original primers by Inqaba Biotech (Pretoria, South Africa).

3.2.5 Sequence and phylogenetic analyses

All raw sequence files were inspected and corrected, where necessary, using Chromas Lite 2.0 (Technelysium) and BioEdit v. 5.0.9 (Hall, 1999). To match the sequences obtained for individual 16S rRNA V3 fragments excised from DGGE gels with those obtained for the complete 16S rRNA gene of the pure cultures, these sequences were compared in BioEdit. All sequences were also compared to those in the nucleotide database of the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) using *blastn* (Altschul *et al*., 1990) to obtain preliminary identifications for the isolated bacteria.

Alignments for the complete 16S rRNA gene and *pheS* data determined in this study were generated using Multiple sequence Alignment based on Fast Fourier Transform (MAFFT v. 6, Katoh *et al*., 2002; 2005). 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. To determine the best-fit evolutionary models for the datasets, PAUP* v. 4.0b1 (Swofford, 2002), together with Modeltest v. 3.7 (Posada and Crandall, 1998), were used. The calculated parameters were then used to construct Neighbor-Joining (NJ) distance-based (Saitou and Nei, 1987) phylogenies with PAUP*, and maximum likelihood (ML) phylogenies with PhyML v. 2.4.3 (Guindon and Gascuel, 2003). Branch support for the various NJ and ML trees were determined using non-parametric bootstrap analysis based on 1000 replicates and the same parameters as before.

3.3 RESULTS

3.3.1 Enumeration and presumptive identification of bacteria

In order to select representative isolates that were dominant at the beginning and end of the fermentation of both sorghum samples at 25°C, enumeration of total bacteria and specific

groups of microorganisms were carried out by cultivation on five different types of media (PCA, MRS-5, M17, VRBA and ESA). Enumerations of total bacteria at 20°C and 30°C were not performed, as PCR-DGGE profiles were similar for fermentations performed at 20, 25 and 30°C. Total counts were initially low, but increased considerably toward the end of the fermentation (Table 3.1). Although LAB were predominant in 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.

A total of 192 isolates from both commercial sorghum sources and the various media were randomly selected. Of these, all Gram-positive, catalase-negative, non-motile and oxidase-negative isolates were considered presumptive LAB, whilst all Gram-negative, catalase-positive and oxidase-negative rods were presumptive *Enterobacteriaceae*. A total of 32 representative isolates were selected for further characterization (Table 3.2).

3.3.2 PCR-DGGE analyses

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, the overall fermentation process appeared to be divided into two stages (Fig. 3.1). The first stage (0-6 h) was associated with a relatively small change in pH (6.64-6.0) and simple DGGE profiles, mainly consisting of one intense band that was already present at t = 0 h. The second stage (12-54 h) was characterized by a more complex pattern, including 12 different fragments that remained almost the same until the end of fermentation. From 12-48 h of the second stage, the pH dropped from 5.69 to 3.79, after which it remained constant. Similar results were obtained for both of the commercial sorghum flour brands included in this study. All duplicate samples also generated identical PCR-DGGE profiles during the course of spontaneous fermentation, emphasizing the reproducibility of PCR-DGGE. The PCR-DGGE profiles for fermentations at 20, 25 and 30°C were also similar.

Table 3.1 Microbial counts (cfu/g) at the start and end of spontaneous sorghum fermentations (25°C) obtained on different selective media

Media	t = 0 h (K.F.C*)	t =54 h (K.F.C)	t = 0 h (Nola*)	t = 54 h (Nola)
VRBA	8.45×10^3	9.0×10^6	8.3×10^3	8.8×10^6
ESA	2×10^3	3.62×10^8	1.8×10^3	3.5×10^8
MRS-5	9×10^3	3.91×10^9	9.1×10^3	3.89×10^9
M17	3×10^3	2.8×10^9	2.8×10^3	2.92×10^9
PCA	1.9×10^5	3.9×10^9	1.5×10^5	2.3×10^9

* K.F.C and Nola refer to commercial sorghum flour from King Food Corporation and Nola Pvt Ltd.

Table 3.2 Likely species identities of bacteria isolated at the beginning and end of sorghum fermentations

Sample identification[1]	Sorghum source[2]	Sampling time (h)	DNA-based identification[3]
M027M	K.F.C	0	*E. mundtii*
M033N	Nola	0	*E. mundtii*
M0331M	K.F.C	0	*E. mundtii*
107N	Nola	0	*E. mundtii*
M027N	Nola	0	*E. mundtii*
M030M	K.F.C	0	Novel, related to *E. mundtii*
106	K.F.C	0	*E. mundtii*
1052	Nola	0	*E. mundtii*
1053	K.F.C	0	*E. mundtii*
P11	K.F.C	54	*Lb. plantarum*
P3	K.F.C	54	*Lb. rhamnosus*
P31	Nola	54	*Lb. rhamnosus*
L235N	Nola	54	*Lb. rhamnosus*
P2M	K.F.C	54	*Lb. fermentum*
M5444M	K.F.C	54	*W. cibaria*
M5444N	Nola	54	*W. cibaria*
E548M	K.F.C	54	*E. faecalis*
E548N	Nola	54	*E. faecalis*
E5412M	K.F.C	54	*E. faecalis*
27291	K.F.C	54	*Lb. fermentum*
27292	Nola	54	*Lb. fermentum*
27293	Nola	54	*Lb. fermentum*
27294	Nola	54	*Lb. fermentum*
P1N	Nola	54	*Lb. plantarum*
P123r1	K.F.C	54	*L. lactis*
P123r2	Nola	54	*L. lactis*
P123r3	Nola	54	*L. lactis*
105	K.F.C	54	*W. cibaria*
V5422M	K.F.C	54	*Pantoea* species
V5423N	Nola	54	*Enterobacteriaceae*
V5430	Nola	54	*Enterobacteriaceae*
V5431	K.F.C	54	*Enterobacteriaceae*

[1] Sample identification refers to the arbitrary identification of bacterial isolates.
[2] Sorghum source refers to the commercial company that produces the sorghum flour (K.F.C for King Food Corporation and Nola for Nola Pvt Ltd).
[3] DNA-based identification refers to the final identification of the isolate obtained from the results of sequence analysis of 16S rRNA and *pheS* genes.

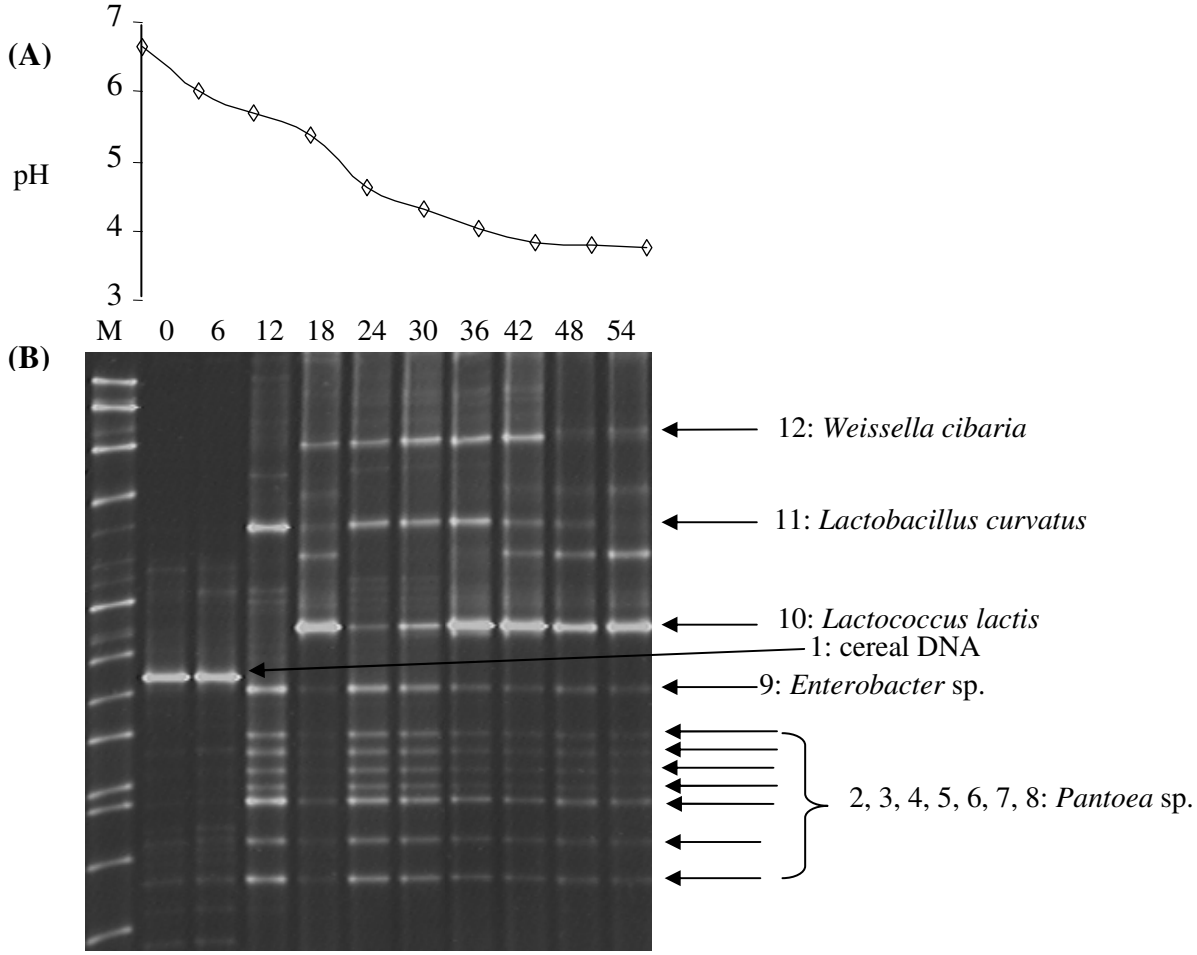


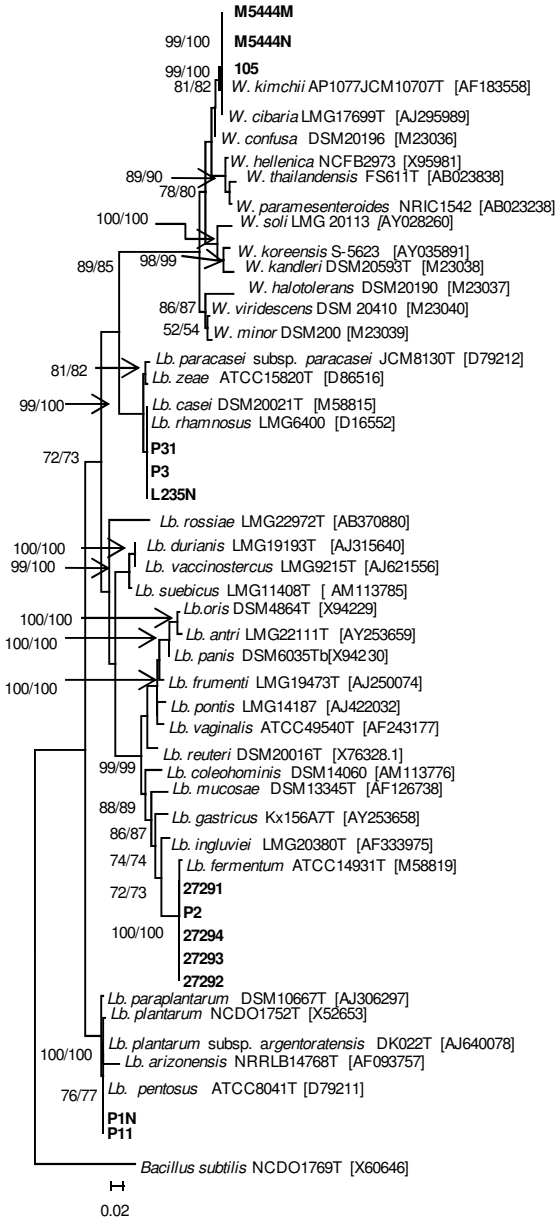
Figure 3.1 Change in pH (A), as well as PCR-DGGE banding patterns (B) representing the 16S rRNA gene fragments of the bacteria involved in the spontaneous fermentation of sorghum at 25°C. Lane M: 16S rRNA V3 marker generated for this study with bands corresponding to *Bacteriodes fragilis* (DSM 1396), *Bacteroides thetaiomicron* (LMG 11262), *Weissella cibaria* (LMG 17699), *Enterococcus flavescens* (LMG 13518T), *Enterococcus solitarius* (LMG 12890T), *Leuconostoc fructosum* (LMG 9498T), *Bacillus subtilis* (LMG 7135T), *Clostridium butyricum* (LMG 1212), *Lactobacillus rhamnosus* (LMG 6400T), *Bifidobacterium longum* (LMG 13197T), *Bifidobacterium bifidum* (LMG 11041T), *Bifidobacterium lactis* (LMG 18314T), and *Bifidobacterium dentium* (LMG 11045T). Sampling time points for PCR-DGGE analysis and pH measurements are indicated at times 0, 6, 12, 18, 24, 30, 36, 42, 48 and 54 h. The identity of the different bacterial species, as suggested by sequence and phylogenetic analyses (Figs. 3.2 and 3.3), are indicated by arrows to the right-hand side of the gel image. Similar PCR-DGGE profiles were generated for fermentations at 25 and 30°C and for both sorghum sources.

To determine the identity of the bacteria represented by the various PCR-DGGE fragments, comparisons of V3 sequences to the full-length 16S rRNA gene sequences obtained from pure cultures and phylogenetic analysis (see below) were used. These analyses also showed that two PCR-DGGE fragments associated with the second fermentation stage represented members of the LAB (i.e. *L. lactis* and *W. cibaria*) and eight fragments represented *Enterobacteriaceae* (i.e. *Pantoea* sp. and *Enterobacter* sp.). The sequence of only one of the PCR-DGGE fragments evident at the end of the fermentation did not match the full-length 16S rRNA gene sequences obtained from any of the pure cultures, and based on the *blastn* results it most likely represents *Lb. curvatus*. The single band associated with the first fermentation stage (0-6 h) most likely represents the sorghum chloroplast 16S rRNA gene, because its sequence is most similar to that of the model monocotyledonous plant *Zea mays*. The *L. lactis* amplicon appeared at t = 18 h and remained until the end of fermentation in all experiments. Once it appeared the intensity of the *L. lactis* band remained constant or increased throughout the fermentation, while those representing the other bacteria, specifically the *Enterobacteriaceae*, decreased during the overall fermentation process.

3.3.3 Phylogenetic analyses using 16S rRNA and *pheS* gene sequences

Two separate data sets were created for the bacteria examined in this study. One included 16S rRNA gene sequences for putative *Lactobacillus* and *Weissella* species (i.e. the Gram-positive rod-shaped bacteria), while the other included *Lactococcus* and *Enterococcus* species (i.e. the Gram-positive coccoid bacteria). These alignments also included the full-length or near full-length sequences for the type strains of the species in these genera that were obtained from GenBank. For each of the datasets, similar trees were infered using NJ and ML methods (Figs. 3.2 and 3.3). Within the *Lactobacillus* and *Weissella* phylogeny (Fig. 3.2), isolates P11 and P1N formed part of a clade containing *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus* and *Lb. arizonensis*, while P31, L235N and P3 were most closely related to *Lb. rhamnosus* and *Lb. casei*. However, phylogenetic analysis of *pheS* sequences clearly showed that isolates P11 and P1N represented *Lb. plantarum*, while P31, L235N and P3 represented *Lb. rhamnosus*. Isolates 27291, 27292, 27293, 27294 and P2 grouped together and represented *Lb. fermentum*, and isolates M5444N, M5444N and 105 were most closely related to *W. cibaria* (Fig. 3.2A). Within the *Lactococcus* and *Enterococcus* phylogeny (Fig. 3.3), isolates P123r1, P123r2, P123r3 represented *L. lactis*, and isolates M027N, M027M, M0331M, M0331N, 107N, 106 and 1052 represented *E. mundtii*. The isolates E548N, E5412M and E548M clustered with *E. faecalis* (Fig. 3.3).

(A)


M5444M
99/100 M5444N
99/100 105
81/82 W. kimchii AP1077JCM10707T [AF183558]
W. cibaria LMG17699T [AJ295989]
W. confusa DSM20196 [M23036]
W. hellenica NCFB2973 [X95981]
89/90 W. thailandensis FS611T [AB023838]
78/80
W. paramesenteroides NRIC1542 [AB023238]
100/100 W. soli LMG 20113 [AY028260]
W. koreensis S-5623 [AY035891]
89/85 98/99 W. kandleri DSM20593T [M23038]
W. halotolerans DSM20190 [M23037]
86/87 W. viridescens DSM 20410 [M23040]
52/54 W. minor DSM200 [M23039]
Lb. paracasei subsp. paracasei JCM8130T [D79212]
81/82 Lb. zeae ATCC15820T [D86516]
99/100 Lb. casei DSM20021T [M58815]
Lb. rhamnosus LMG6400 [D16552]
72/73 P31
P3
L235N
Lb. rossiae LMG22972T [AB370880]
100/100 Lb. durianis LMG19193T [AJ315640]
99/100 Lb. vaccinostercus LMG9215T [AJ621556]
Lb. suebicus LMG11408T [AM113785]
100/100 Lb.oris DSM4864T [X94229]
100/100 Lb. antri LMG22111T [AY253659]
Lb. panis DSM6035Tb[X94230]
100/100 Lb. frumenti LMG19473T [AJ250074]
Lb. pontis LMG14187 [AJ422032]
Lb. vaginalis ATCC49540T [AF243177]
Lb. reuteri DSM20016T [X76328.1]
99/99 Lb. coleohominis DSM14060 [AM113776]
Lb. mucosae DSM13345T [AF126738]
88/89 Lb. gastricus Kx156A7T [AY253658]
86/87 Lb. ingluviei LMG20380T [AF333975]
74/74 Lb. fermentum ATCC14931T [M58819]
72/73 27291
P2
100/100 27294
27293
27292
Lb. paraplantarum DSM10667T [AJ306297]
Lb. plantarum NCDO1752T [X52653]
Lb. plantarum subsp. argentoratensis DK022T [AJ640078]
100/100 Lb. arizonensis NRRLB14768T [AF093757]
76/77 Lb. pentosus ATCC8041T [D79211]
P1N
P11
Bacillus subtilis NCDO1769T [X60646]
0.02

(B)

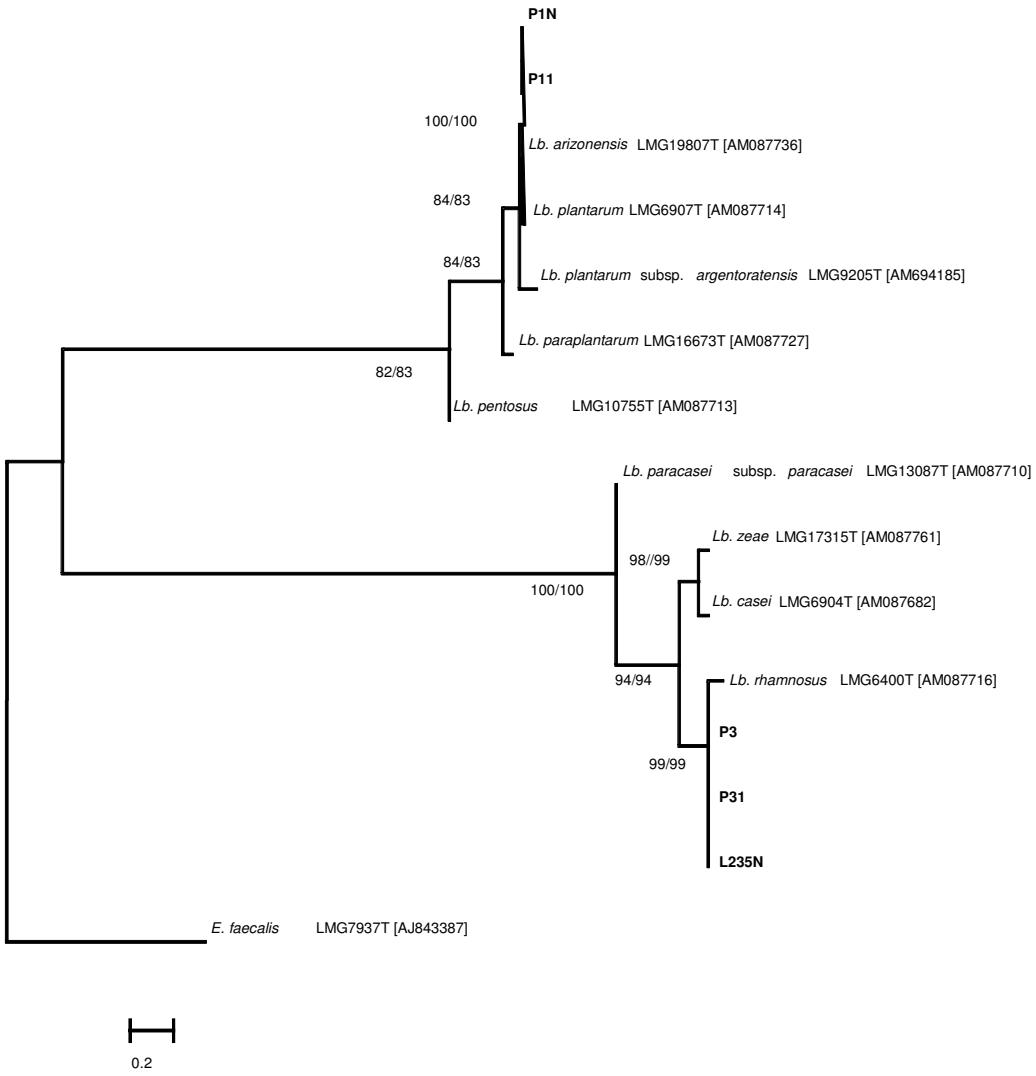


Figure 3.2 Maximum likelihood (ML) phylogeny of species in the genera *Lactobacillus* and *Weissella*, based on 16S rRNA (A) and *pheS* (B) gene sequences. Trees with similar topologies were inferred using Neighbor-Joining (NJ) distance analysis. Isolates obtained from fermented sorghum are indicated in bold. All species names are proceeded by strain numbers and GenBank accession numbers in brackets. *Bacillus subtilis* (A) and *Enterococcus faecalis* (B) were used as the outgroups and bootstrap values (> 60%), based on 1000 replications, are shown at internodes, as follows: ML/NJ. *Weissella kimchii* is a later heterotypic synonym of *Weissella cibaria* (Ennahar and Cai, 2004). *Lb. arizonensis* is a later heterotypic synonym of *Lb. plantarum* (Kostinek *et al*., 2005).

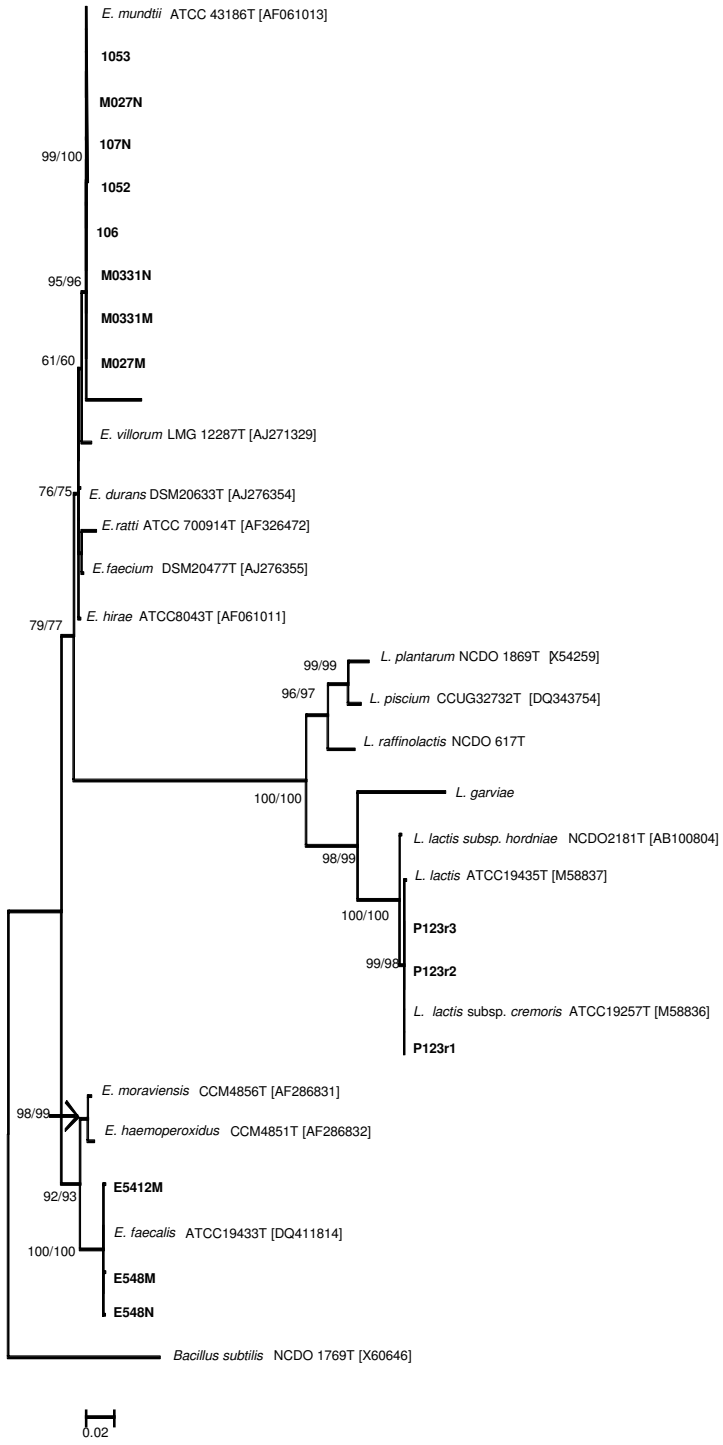


Figure 3.3 Maximum likelihood (ML) phylogeny of species in the genera *Lactococcus* and *Enterococcus*, based on 16S rRNA gene sequences. Trees with similar topologies were inferred using Neighbor-Joining (NJ) distance analysis. Isolates obtained from fermented sorghum are indicated in bold. All species names are preceded by strain numbers and GenBank accession numbers in brackets. *Bacillus subtilis* was used as the outgroup and bootstrap values (> 60%), based on 1000 replications, are shown at internodes, as follows: ML/NJ.

3.3.4 Detection of bacterial diversity using culture-independent and culture-dependent approaches

Table 3.3 shows a comparison of the diversity of bacteria identified during spontaneous sorghum fermentations using culture-independent and culture-dependent approaches. Overall, PCR-DGGE appeared to be unable to detect most of the bacteria in the *ting* samples. For example, at time t = 0 h, *E. mundtii* was found to be present using culture-dependent methods (Table 3.2), although PCR-DGGE did not allow its detection, as all profiles at time t = 0 h for both sorghum brands at 20, 25 and 30°C included only the sorghum chloroplast 16S rRNA gene fragment (Fig. 3.1). Also, culture-dependent approaches allowed isolation and identification of *Lb. plantarum*, *Lb. rhamnosus* and *Lb. fermentum* at time t = 54 h, although their 16S rRNA gene fragments were absent from the PCR-DGGE profiles at comparable time points in both sorghum types at 20, 25 and 30°C (Table 3.3). However, an isolate presumably representing *Lb. curvatus* and that was predominant in PCR-DGGE profiles at time points t = 42-54 h was not isolated from the fermented samples, despite numerous attempts.

3.4 DISCUSSION

The use of culture-independent PCR-DGGE, in combination with culture-dependent microbiological methods, allowed for the effective description of the diversity and dynamics of the bacterial populations involved in the spontaneous fermentation process to produce *ting*. The results illustrated that various LAB and members of the *Enterobacteriaceae* were predominant at the end of fermentation, with *L. lactis* appearing to play a more important role compared to other bacteria. Consequently, *L. lactis* may be used to control sorghum fermentations, resulting in standardized products. The research presented here represents the first extensive study investigating bacterial diversity and dynamics in this ecosystem, and is relevant for development of appropriate and effective starter cultures.

In this study, species-level identification was primarily based on 16S rRNA gene sequences. This approach allowed positive identification of most of the bacteria involved in sorghum fermentations as *E. mundtii*, *E. faecalis*, *L. lactis*, and *Lb. fermentum* or *W. cibaria*. It did, however, not allow unambiguous identification of isolates that are related to *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus* and *Lb. arizonensis*, nor those related to *Lb. rhamnosus* and *Lb. casei*. The bacteria in these two groups are each also characterized by very similar

Table 3.3 Comparison of the bacterial diversity in *ting* using culture-independent and culture-dependent approaches

Time of detection	Culture-independent approach[1]	Culture-dependent approach[2]
t = 0 h	None	*E. mundtii*
t = 54 h	*L. lactis* *W. cibaria* *Enterobacteriaceae* members (4) *Lb. curvatus*	*L. lactis* *W. cibaria* *Enterobacteriaceae* members (4) *E. faecalis* *Lb. plantarum* *Lb. fermentum* *Lb. rhamnosus*

[1] Identification was based on sequencing of the V3 region of the16S rRNA gene.
[2] Identification was based on results of sequence analysis of the16S rRNA and *pheS* genes.

phenotypes, further complicating their differentiation (Torriani *et al*., 2001; Ennahar *et al*., 2003). The fact that 16S rRNA gene sequence analysis did not allow their definitive identification is, however, not suprising as it has been shown on numerous occasions that this gene region is unable to separate closely related species (Fox *et al*., 1992; Ward, 2002; Felis and Dellaglio, 2007). Recently, Naser *et al*. (2007) introduced the use of additional genes such as *pheS* and *rpoB* for separating species of *Lactobacillus*. In this study, it was similarly found that *pheS* has greater resolution, because it displayed sufficient interspecific variation that allowed for the unequivocal identification of isolates P3, P31 and L235N as *Lb. rhamnosus*, and isolates P1N and P11 as *Lb. plantarum* (Fig. 3.2B).

Spontaneous sorghum fermentations were characterized by two well-delineated stages that are linked to bacterial diversity and pH. There was an apparent shift in microbial diversity from simple PCR-DGGE profiles and relatively high pH to more complex PCR-DGGE profiles and increased acidity (Fig. 3.1). During the first phase of fermentation the bacterial counts were very low ($< 8.5 \times 10^3$ cfu/g, Table 3.1) and below the detection limit of PCR-DGGE. The single intense PCR-DGGE band dominating the first fermentation phase represented the V3 region of the 16S rRNA gene encoded on the sorghum chloroplast, which is also a eubacterial descendent (Krajčovič and Ebringer, 1990; Martin, 1999). During the second fermentation phase, PCR-DGGE detected *L. lactis*, which remained dominant until the end of the fermentation. *W. cibaria* and the putatively identified *Lb. curvatus* were also among the predominant species at the end of the fermentations. The dominance of *L. lactis*, a homofermenter, over other bacteria may be due to its tolerance of the low pH, which reduced the growth of heterofermentative LAB (Lin *et al*., 1992). Therefore, *L. lactis* together with the other dominant species represent important bacteria that are well adapted to sorghum fermentation processes and may be suitable candidates to use as starter cultures for standardization of *ting* preparations. These results are also 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 because of good adaptation to the food matrix (Hounhouigan *et al*., 1993; Halm *et al*., 1996).

All of the LAB species associated with the production of *ting* have previously been implicated in the production of fermented cereals with desirable sensory properties. *Lb. plantarum* was isolated from fermented cereals such as *ogi* (Odunfa and Adyele, 1985), *mageu* (Hesseltine, 1979) and *kunun zaki* (Gaffa and Gaffa, 2004), whilst the heterofermentative *Lb. fermentum*

was previously isolated from *mawe* (Hounhouigan *et al*., 1993) and *kenkey* (Halm *et al*., 1993). *Lb. curvatus-Lb. sake* was isolated from sorghum-based fermented weaning food (Kunene *et al*., 2000). The production of the pleasant sensory characteristics of *ting* is likely due to some LAB such as *Lb. plantarum*, which has previously been shown to produce flavour-active compounds such as ethyl acetate and aldehydes (Rehman *et al*., 2006).

In this study, a number of bacteria not normally associated with food were isolated from spontaneously fermented sorghum. The majority of these are naturally associated with plants and commonly found on plant-based material (Nout, 1991; Mohammed *et al*., 1991). The yellow-pigmented species resembling *E. mundtii* that was isolated at the beginning of the process is typically associated with plants (Martin and Mundt, 1972) where it exhibits epiphytic relationships with them (Mundt *et al*., 1962), and has also been reported as an environmental contaminant (Camu *et al*., 2007). The same is also true for the *Pantoea* sp., which is a plant endophyte and commonly associated with rice and soybean (Kuklinsky-Sobral *et al*., 2004). However, some *Pantoea* species are associated with diseases in humans such as septic monoarthritis (De Champs *et al*., 2000). The presence of a bacterial species representing *E. faecalis* at the end of sorghum fermentations was expected, as this species is often prevalent in foods (Giraffa, 2002; Gomes *et al*., 2008). Enterococci have been shown to form part of the microflora that is present throughout the cheese production process, and are responsible for sensory characteristics of the final product (Franz *et al*., 2003). In addition, some enterococci strains, especially *E. faecalis* and *E. faecium* may produce bacteriocins that are active against a plethora of food borne pathogens (Franz *et al*., 2007), making them suitable candidates for controlling emerging pathogens during food fermentation (Callewaert *et al*., 2000). Despite the safety and pleasant sensory attributes imparted by *E. faecalis* in foods, some strains of *E. faecalis* and *E. faecium* are associated with infection that pose challenges to food safety (Carlos *et al*., 2009).

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 (Nigatu and Gashe, 1994; Kingamkono *et al*., 1995). Many previous studies have shown that the growth of members of the *Enterobacteriaceae* and other bacteria is inhibited by the lactic acid produced during fermentation (Nout, 1991; Adams and Nicolaides, 1997; Abegaz, 2007). The presence of bacteria representing *Pantoea* sp. and *E. faecalis* during and at the end of the sorghum fermentations might, however, be due to acid

resistance or the presence of microenvironments in the food matrix that support the growth of these bacteria (Wacher *et al*., 1993; ben Omar and Ampe, 2000). Similar findings were also reported for other studies where significant numbers of coliforms and *Escherichia coli* were detected during fermentations involving LAB, especially when no starter cultures were added (Feresu and Nyathi, 1990; ben Omar and Ampe, 2000; Gran *et al*., 2002; 2003). Overall, however, the intensity of PCR-DGGE bands corresponding to *Enterobacteriaceae* decreased as that of *L. lactis* increased. It is therefore likely that the unfavourable environment created by this and the other LAB slowed down the growth of *Pantoea* sp. and *E. faecalis* as fermentation progressed. This was also probably true for *E. mundtii* that was only isolated at the start of the fermentations and not thereafter.

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 to 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 (Giraffa and Neviani, 2001), which illustrates one of the main advantages of culture-independent approaches over culture-dependent methods (Muyzer and Smalla, 1998; Giraffa and Neviani, 2001). Overall, however, the use of culture-dependent methods allowed identification of more bacteria than culture-independent methods (Table 3.3). 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 (Muyzer and Smalla, 1998). Biases that exist in terms of DNA extraction and PCR (Ercolini, 2004; De Vero *et al*., 2006; Camu *et al*., 2007) 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 (Meroth *et al*., 2003) and whey cultures of water buffalo mozzarella (Ercolini *et al*., 2004). PCR-DGGE results are also strongly influenced by the intraspecific heterogeneous nature of the 16S rRNA gene region (Ueda *et al*., 1999), which in the case of *Pantoea* species, yielded multiple bands associated with apparently a single species (Fogel *et al*., 1999). 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 use of a culture-dependent approach used in a previous study (Chapter 2) also highlights the advantages of using a combination of both culture-dependent and culture-independent approaches applied in this study. Some of the strains isolated from fermented sorghum in the previous study were similar to those obtained in this study (*Lb. rhamnosus*, *Lb. fermentum* and *Lb. plantarum*). However, *L. lactis* that was isolated in this study could not be detected in the previous study, probably because the addition of five nutritional supplements to molten MRS medium (Meroth *et al*., 2003) improved the chances of isolating this LAB. Likewise, the use of ESA and M17 agar improved the probability of isolating *E. faecalis* that was isolated at the end of fermentation in this study, but could not be detected on MRS in the previous study.

In South Africa, *ting* is still produced from spontaneously fermented sorghum flour. Consequently, there is immense variation in the sensory characteristics and quality of *ting*, making it a daunting task to upgrade its status to commercial level. Detailed insight of the microbial processes occurring during spontaneous sorghum fermentations is a prerequisite for development of starter cultures that may result in the production of standardized *ting*. In this study, microbial population dynamics and diversity during sorghum fermentations were analyzed in detail, using a combination of culture-independent and culture-dependent methods, in order to get an overview of the important bacteria. Taken together, the use of these methods offered an excellent approach for increasing knowledge of microbial diversity and dynamics during spontaneous sorghum fermentations. Such information is useful, not only from an academic viewpoint, but for designing relevant starter cultures that may result in *ting* with standardized sensory profiles (appearance, aroma, sourness and taste) and fermentation time. Evaluation of the contribution of such starter cultures to the safety and acceptability of sensory characteristics of *ting* is important in future.

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CHAPTER FOUR

USE OF STARTER CULTURES OF LACTIC ACID BACTERIA IN THE PRODUCTION OF *TING*, A SOUTH AFRICAN FERMENTED FOOD

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ABSTRACT

Ting, a porridge popular amongst southern Africans for its sour taste, is produced by cooking fermented sorghum into a thick porridge. With a view to selecting the most appropriate starter for use in the production of *ting*, trial fermentations were performed using different defined starter cultures. The lactic acid bacterial strains (*Lactococcus lactis*, *Lactobacillus fermentum*, *Lb. plantarum* and *Lb. rhamnosus*) used in the starters had been isolated previously from spontaneous sorghum fermentations. All the starter cultures showed an ability to ferment the sorghum, as evidenced from lowering the pH from 6.4 to 3.79 - 4.0 and increasing the titratable acidity from 0.18% to 0.72 - 0.95% (w/w, lactic acid) in 24 h of fermentation. However, the lowest pH (3.3) and highest lactic acid (1.44%) was produced the fastest in non-sterilized sorghum fermented by *L. lactis*. This fermentation was characterized by an increase in the number of lactic acid bacteria and yeasts, while the mean aerobic plate count, Gram-negative and bacterial spore counts, as well as fungal counts decreased. Moreover, bacterial food-borne pathogens detected in naturally fermented porridge, i.e. *Bacillus cereus* and *Escherichia coli* biotype I, were absent in the *L. lactis*-inoculated fermentation batch. *Ting* produced from this fermented gruel also had sensory properties preferred by panelists. The strong sour taste and strong aroma were especially appealing to the panelists. Conversely, *ting* prepared from naturally fermented sorghum in 54 h was the least preferred by panelists.

Key words: sorghum fermentation; *ting*; lactic acid bacteria; starter culture; pathogens; antimicrobial; sensory attributes

4.1 INTRODUCTION

Fermentation of local staple food, usually cereals such as maize, sorghum or millet, is a traditional technology in Africa (Mensah, 1997; Oyewole, 1997; Holzapfel, 2002). Lactic acid bacteria (LAB) and yeasts have been reported to be predominant microorganisms in most of the African indigenous fermented foods (Nout, 1991; Olasupo *et al.*, 1997; Kunene *et al.*, 1999). Lactic acid fermentation not only produces desirable changes in the aroma, flavour and texture of fermented foods (Kandler, 1983; Hugenholtz *et al.*, 2000; Axelsson, 2004), but studies have shown that the survival and growth of pathogenic and spoilage microorganisms are also adversely affected (Nout *et al.*, 1989; Holzapfel *et al.*, 1995; Kingamkono *et al.*, 1995; Adams and Nicolaides, 1997; Magula *et al.*, 2003). Antimicrobial activity caused by growth of LAB is due to a decrease in pH to below 4 in the food products, depletion of nutrients and production of antimicrobial compounds, including bacteriocins (De Vuyst and Vandamme, 1994; Parente and Ricciardi, 1999) and various organic acids such as lactic and acetic acids (De Vuyst, 2000; Messens and De Vuyst, 2002; Mante *et al.*, 2003).

Ting, which is made from fermented sorghum, is consumed extensively in South Africa (Boling and Eisener, 1982). This food is usually prepared by soaking sorghum powder for two to three days in water. Microorganisms associated with the raw materials, equipment and local environments ferment the sorghum powder, resulting in a gruel that is mixed with boiling water, cooked and then consumed as a sour porridge. In rural and informal settlements of South Africa where electricity is not readily available, *ting* is frequently used as a weaning food for infants mainly because it is inexpensive to prepare, can be stored at ambient temperatures and does not require re-heating before consumption (Kunene *et al.*, 1999). Moreover, due to its appetizing taste, *ting* is consumed by adults at major ceremonies such as weddings and funerals. However, since the sorghum is allowed to undergo natural fermentation, variations in the sensory quality and acceptability of *ting* is frequently encountered.

The use of starter cultures may represent an appropriate approach for the control and optimization of the fermentation process in order to alleviate problems regarding the quality and acceptability of African indigenous fermented foods (Holzapfel *et al.*, 1995; Kimaryo *et al.*, 2000; Leroy and De Vuyst, 2004). The primary consideration before introducing starter cultures for traditional fermentations should be whether these would contribute significantly

to an improvement of processing conditions and product quality with respect to rapid and accelerated acidification, improved microbiological safety, an improved and more predictable fermentation process, and desirable sensory characteristics (Holzapfel, 2002; Leroy and De Vuyst, 2004).

Towards selecting an appropriate starter culture for the manufacture of *ting*, a study aimed at identifying the lactic acid flora present in the natural fermentation of sorghum had been undertaken previously. Using DNA-based molecular and bacteriological culturing approaches, the bacterial species *L. lactis*, *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus* were confirmed to be present during fermentation, but *L. lactis* appeared to be dominant during the final stages of fermentation (Chapter 3). Consequently, four starter cultures, each inclusive of *L. lactis*, were prepared by making use of the above-mentioned LAB species isolated from natural fermentations of sorghum. Thus, the aims of this study were to assess bacterial, yeast and mould populations found in sorghum before, during and after its preparation as a fermented cereal, to investigate possible antimicrobial interactions that may occur between the LAB species comprising the starter cultures, and to clarify the contribution of the LAB species with respect to the sensory characteristics of *ting*.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial isolates

Starter cultures were prepared using LAB isolates that had been isolated previously from naturally fermented sorghum and of which their identity had been confirmed using 16S rRNA gene sequence analysis. The cultures of LAB (*L. lactis*, *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus*) had been stored at -20°C in sterile Eppendorf tubes, containing DeMan, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) with 20% (v/v) glycerol.

4.2.2 Preparation of starter cultures

The LAB starter cultures were prepared using a previously reported method (Mugula *et al*., 2003a). Briefly, the LAB were individually streaked onto MRS agar (Merck) and incubated anaerobically in an anaerobic jar with anaerocult A (Merck) at 30°C for 24 h. A single colony was picked from each pure culture plate, grown overnight in MRS broth, followed by centrifugation at 655 × *g* for 15 min in a benchtop centrifuge (Eppendorf, Hamburg, Germany). The cell pellets were washed twice in buffered peptone water (0.85% [w/v] NaCl,

0.1% [w/v] peptone, pH 7.2) and then resuspended in 1 ml of the solution. This procedure achieved a culture preparation containing 10^9 colony forming units (cfu)/ml, as determined by viable counts on MRS agar. Starter cultures E1, E2, E3 and E4 were considered. E1 was prepared from *L. lactis*; E2 was prepared from *L. lactis* and *Lb. fermentum*; E3 was prepared from *L. lactis*, *Lb. fermentum* and *Lb. plantarum*; and E4 was prepared from a combination of all LAB cultures (*L. lactis*, *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus*). Suspensions of each selected strain (1 ml) were combined, made up to 20 ml with buffered peptone water, mixed thoroughly and then inoculated into individual sorghum powder slurries used in the preparation of *ting*.

4.2.3 Preparation of *ting*

Six trial fermentations were prepared (Table 4.1). In addition to untreated (non-sterilized) sorghum powder inoculated with and without *L. lactis*, sterilized sorghum powders were inoculated separately with the respective starter cultures. Commercial packets of pure grain sorghum ('King Korn Mabele', King Food Corporation, Potchefstroom, South Africa) were purchased from a local supermarket. For natural fermentations, the sorghum powder was mixed with sterile luke-warm (ca. 40°C) water (1:1 [w/v]) in glass containers to make a slurry. The containers were covered and incubated at 25°C for 54 h. For inoculated fermentations, the sorghum powder was autoclaved at 121°C for 15 min prior to preparation of the slurry, as described above. The slurry was subsequently inoculated with the appropriate LAB starter culture, thoroughly mixed and incubated at 25°C for 24 h. The cooking process involved mixing 200 ml of the resultant fermented slurry with 600 ml of boiling (96°C) water, followed by cooking for 15 min. A further 100 ml of the fermented slurry was then added slowly until a thick porridge formed. The porridge was left to simmer for 15 min and stirred intermittently.

4.2.4 pH and acidity determinations

Samples of fermenting sorghum were withdrawn at 6-h intervals starting at t = 0 h until the end of fermentation (t = 24 h for inoculated fermentations and t = 54 h for natural fermentation). pH was measured using a Beckman model Ø 34 pH meter (Beckman Coulter, Fullerton, CA, USA) after calibration with standard buffers (Merck) at pH 4.0 and 7.0. For determination of titratable acidity of the fermented sorghum slurries, 10 g of sample was thoroughly drained and then titrated against 0.1 N NaOH to a pH of 8.5, using

Table 4.1 Experimental protocol for the six sorghum fermentations

Fermentation batch	Sorghum powder[1]	Inoculum	Fermentation time (h)
NF	Untreated	Natural microflora	54
E1ns	Untreated	*L. lactis*	24
E1s	Sterilized	*L. lactis*	24
E2	Sterilized	*L. lactis* and *Lb. fermentum*	24
E3	Sterilized	*L. lactis*, *Lb. fermentum* and *Lb. plantarum*	24
E4	Sterilized	*L. lactis*, *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus*	24

[1] The respective starter cultures were inoculated directly into either slurries of untreated sorghum powder or slurries made from sorghum powder that was autoclaved at 121°C for 15 min.

phenolphthalein as indicator, as described previously (Nout *et al*., 1989). One ml of 0.1 M NaOH was taken as equivalent to 0.0090 g lactic acid (Nout *et al*., 1989). Measurements were repeated three times in each type of fermentation and the mean values recorded.

4.2.5 Microbiological analysis

4.2.5.1 Bacterial and spore counts

Sorghum powder, together with the fermented sorghum slurries and the corresponding cooked fermented *ting* porridges (Table 4.1), were aseptically processed and tested within 20 min of collection. For each sample, 10 g was homogenized in 90 ml of buffered peptone water, resulting in a 10^{-1} dilution. Ten-fold serial dilutions in the same diluent were plated onto different media and incubated for 48-72 h under either aerobic or anaerobic conditions (Table 4.2). Plates showing between 30 and 300 colony forming units (cfu), or the highest number if below 30, were counted following incubation. Bacterial spore counts were obtained by heating 10 ml of the 10^{-1} dilution at 80ºC for 10 min in a water bath, followed by cooling on ice and spread plating onto Plate Count Agar (PCA) (Merck) (Becker *et al.*, 1994). All samples were processed in duplicate.

4.2.5.2 Identification of colonies isolated from spore count plates

A total of 65 colonies, isolated from plates of the highest dilution showing growth from sorghum powder (50 isolates) and the naturally fermented sorghum slurry (15 isolates), were purified on PCA agar (Table 4.2). These isolates were subjected to standard Gram stains and catalase tests. Gram-positive, catalase-positive isolates that showed phase bright spores were identified as *Bacillus* spp. (Ehrich, 1956), while *B. cereus* was identified with a culture-based approach, as described below.

4.2.5.3 Analysis of yeasts and moulds

For isolation of yeasts, the samples (1 g of sorghum powder and aliquots of 100 µl of 10^{-1} dilutions of fermented sorghum slurries and *ting*) were plated onto Malt Extract Agar (MEA) (Merck), containing 125 mg/l of chloramphenicol (Sigma, St. Louis, MO, USA), followed by incubation at 25°C for 3-5 days. The yeast isolates were identified based on morphological characteristics (Jespersen *et al*., 2005) and by using ID32C diagnostic test kits (bioMérieux, France), assisted by computer software (bioMérieux). This identification system was based

Table 4.2 Culture conditions used for microbiological analysis of sorghum samples

Enumeration type	Incubation temperature (°C)	Plating technique	Incubation period (h)	Atmospheric conditions	Growth medium
Aerobic plate count (APC)	30	Pour-plate	72	Aerobic	Plate Count Agar
Gram-negative count (GNC)	37	Pour-plate	24-48	Aerobic	Violet Red Bile Glucose Agar
Spore count (SC)	37	Spread-plate	24-48	Aerobic	Plate Count Agar
Lactic acid bacteria count (LABC)	30	Spread-plate	48-72	Anaerobic	MRS agar with 0.1% L-cysteine (Dykes *et al*., 1991) and 0.01% cycloheximide (Nout *et al*., 1989)
Yeasts	25	Spread-plate	120	Aerobic	Malt Extract Agar (MEA) with 125 mg/l chloramphenicol
Moulds	25	Direct plating and spread plating	360	Aerobic	Potato Dextrose Agar (PDA), Malt Salt Agar (MSA), Pentochloronitrobenzene (PCNB) agar. PDA medium was supplemented with 125 mg/l chloramphenicol

on 29 carbohydrate assimilation profiles (carbohydrates, organic acids, amino acids), one susceptibility test (cycloheximide) and an esculin hydrolysis colorimetric test.

Moulds were isolated on Potato Dextrose Agar (PDA), Malt Salt Agar (MSA) and Pentachloronitrobenzene (PCNB) agar (Merck), as described previously (Rabie and Lübben, 1984; Rabie *et al*., 1997). Sorghum powder (1 g) was inoculated at almost equidistant positions on five points in Petri dishes containing the respective agar media (10 for each type of medium) and incubated at 25°C for 15 days. Aliquots of 1 ml of 10^{-1} dilutions of fermented sorghum slurries and *ting* were also streaked onto the above agar media and incubated at 25°C for 15 days. Moulds were microscopically identified based on morphological characteristics to either genus or species level, depending on the ability of fungi to form fruiting structures and spores (Pitt, 1988). The identifications were confirmed by qualified mycologists.

4.2.5.4 Analysis of food-borne bacterial pathogens

Bacillus cereus

Robertson's heated cooked meat (100 ml) (Merck) was mixed with 50 ml of the 10^{-1} dilution and incubated at 37ºC for 48 h (Kramer and Gilbert, 1989). The broth (100 µl) was spread onto Columbia blood agar (Oxoid, Basingstoke, UK) and Polymyxin-Pyruvate-Egg Yolk Mannitol-Bromothymol Blue agar (PEMBA) (Oxoid), and incubated at 37ºC for 24 h (ICMSF, 1996). Thereafter, PEMBA plates were examined for discoloration and blood agar plates for haemolysis, which is suggestive of *B. cereus*.

***Campylobacter* spp.**

Preston broth (225 ml) (Oxoid) was mixed with 25 g of sample and incubated at 42ºC for 24 h. The broth (100 µl) was plated onto blood-free *Campylobacter* agar (Oxoid) and Columbia blood agar (Oxoid), and incubated at 42ºC for 48 h under microaerophilic conditions (Beuchat, 1987).

Clostridium perfringens

Aliquots of 100 µl of the 10^{-1} dilution were spread onto Clostridial agar (Oxoid) and Salicin Tryptic Soy agar (STSA) (Difco, Detroit, MI, USA). The plates were incubated at 37ºC for

48 h aerobically and anaerobically (anaerocult A) to test for scant or no growth on aerobic plates and for growth on the anaerobic plates (SABS, 1975a, method 761).

***Escherichia coli* biotype I**

An aliquot of 1 ml of the 10^{-1} dilution was added into test tubes, containing 10 ml of MacConkey broth purple (Merck) and sterile Durham tubes, and incubated at 37ºC for 24 h. Aliquots of 100 µl of the broth from test tubes showing gas and acid production (yellow colour) was inoculated into Brilliant green bile broth (Oxoid) and Tryptone water (Oxoid), and incubated at 44ºC for 18 h. Production of gas in Brilliant green bile broth and indole in tryptone water (red colour after addition of Kovac's reagent [Merck]) indicated the presence of *E. coli* biotype I (SABS, 1975b, method 758). Gram-negative lactose fermenting colonies were subjected to further biochemical tests, as described by Schlegel (1988), and all tests were performed in duplicate.

***Escherichia coli* 0157:H7**

An aliquot of 100 µl of the 10^{-1} dilution was spread onto MacConkey sorbitol agar (Oxoid) and incubated at 37ºC for 24 h to select for sorbitol-negative *E. coli* 0157:H7 (Vernozy-Rozand, 1997).

Listeria monocytogenes

Listeria enrichment broth (225 ml) (Difco) was inoculated with 25 g of sample and incubated at 37ºC for five weeks. At the end of each week, the broth (100 µl) was spread onto *Listeria* selective agar (Oxoid), incubated at 37ºC for 24 h and examined for the presence of black colonies (Van Netten *et al*., 1988).

***Salmonella* spp.**

Pre-enrichment was carried out by homogenizing 25-g samples of sorghum powder, fermented sorghum slurries or *ting* in 225 ml of buffered peptone water, followed by incubation at 37ºC for 24 h (Arvanitidou *et al*., 1998). Selective enrichment for *Salmonella* spp. was done by inoculation of 10 ml of the buffered peptone water into 100 ml of Tetrathionate medium (Oxoid) and incubation at 43ºC for 48 h. In addition, buffered peptone water (10 ml) was inoculated into 100 ml of Selenite broth (Difco) and Gram-negative medium (Merck), and then incubated at 37ºC for 48 h (Arvanitidou *et al*., 1998). The respective broths were streaked onto Xylose, Lysine, Desoxycholate (XLD) (Difco) and

Brilliant Green Agar (BGA) (Oxoid), and incubated at 37ºC for 24 h. Lactose-negative colonies are indicative of the presence of *Salmonella* spp.

***Shigella* spp.**

Gram-negative medium (100 ml) (Merck) was mixed with 25 ml of the 10^{-1} dilution and incubated at 37ºC for 24 h. The broth (100 µl) was streaked onto XLD agar plates and incubated at 37ºC for 24 h**.** Translucent colonies are suggestive of the presence of *Shigella* spp. (Taylor, 1965).

Staphylococcus aureus

Three bottles, each containing 20 ml of *Staphylococcus* enrichment broth (Difco), were mixed with 1 ml of the 10^{-1} dilution and incubated at 37ºC for 48 h after which they were examined for gas production (SABS, 1975c, method 760). The broth (100 µl) was streaked onto Baird-Parker agar (Merck) and incubated at 37ºC for 24 h, after which plates were examined for black colonies surrounded by a clear zone that are indicative of *S. aureus*.

***Vibrio* spp.**

A 25-g sample was homogenized in 225 ml of buffered peptone water and an aliquot of 50 ml was then inoculated into two bottles containing 50 ml of *Vibrio* enrichment broth (Oxoid), followed by incubation for 24 h, one at 37ºC and one at 42ºC. Loopfuls from the surface of the broths were streaked onto Thiosulphate Citrate Bile Sucrose (TCBS) agar (Difco) and incubated at 37ºC for 24 h. Thereafter plates were examined for flat yellow colonies and/or smooth green colonies, which are indicative of *Vibrio* spp. (Madden and McCardell, 1989).

***Yersinia* spp.**

A 10-g sample was homogenized in 100 ml of phosphate-buffered saline (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) and incubated at 4ºC for four weeks (Sutherland and Varnam, 1977). Loopfuls from the broth were streaked onto *Yersinia* selective agar (Difco) and incubated aerobically at 30ºC for 24-48 h. *Yersinia* spp. ferment mannitol that result in acid production and thus are indicated by red discolouration of the colonies surrounded by a transparent zone (Schiemann, 1979).

4.2.6 Antimicrobial studies

4.2.6.1 Test for microbial inhibition by LAB isolates

The inhibitory potential of LAB cultures used in starter culture formulations were investigated *in vitro* using the Agar Well Assay method (Schillinger and Lücke, 1989; Olsen *et al*., 1995). For this purpose, MRS agar and M17 agar (Merck) were poured into Petri dishes and left to solidify for two days. A sterile cork borer was used to make circular wells into the agar. *L. lactis*, *Lb. fermentum*, *Lb. plantarum* and *Lb. rhamnosus* were each cultured separately in MRS broth (Merck) at 30°C for 24 h, and 100 µl of the cultures (1×10^8 cfu/ml) were transferred into the wells and left to diffuse into the agar for 5 h. The wells were overlaid with ca. 10 ml of soft MRS agar (0.7% agar) containing the above LAB isolates as indicator strains. The indicator lawns were prepared by adding 250 µl of a 10^{-1} dilution of an overnight culture of the indicator bacterium to 10 ml of MRS agar (Merck). The plates were incubated at 30°C for 24-48 h and the diameter of clear zones around the wells were measured and regarded as inhibitory reactions (Mante *et al*., 2003). Individual tests were carried out in duplicate.

4.2.6.2 Test for possible bacteriocin production by LAB isolates

Volumes of 100 µl of overnight LAB cultures were transferred separately into MRS agar wells and overlaid with 10 ml of MRS soft agar containing *Listeria monocytogenes* ATCC 19115, as bacteriocin indicator organism. The agar plates were incubated aerobically at 37°C for 24 h and examined for inhibitory reactions, as evidenced by zones of inhibition. The experiments were carried out in duplicate.

4.2.6.3 Test for possible bacteriocin production by LAB culture supernatants

For each of the LAB cultures tested, 10 ml of an overnight culture was centrifuged at 3 000 × *g* for 15 min at 4°C, and the cell-free supernatants filtered using a 0.20-µm sterile filter (Sartorius Minisart, Cottingen, Germany). Cell-free extracts (100 µl) were digested separately with Proteinase K (Merck) and Pronase E (Sigma), at a concentration of 1 mg/ml at 30°C for 2 h, and then tested against the *L. monocytogenes* indicator strain using the Agar Well Assay method. The agar plates were incubated aerobically at 37°C for 24 h.

4.2.6.4 Test for acid production as antimicrobial property of LAB isolates

Cell-free extracts (100 µl) of the LAB cultures were neutralized by adding 0.1 M NaOH to the supernatants to raise their pH to 6.8. Both neutralized and non-neutralized supernatants were filter-sterilized through a 0.20-µm filter (Sartorius Minisart). The neutralized and non-neutralized supernatants were tested separately against *Listeria monocytogenes* using the Agar Well Assay method.

4.2.7 Sensory evaluation of *ting*

Sensory evaluation of the different *ting* porridges was done at the sensory laboratory of the Department of Food Science, University of Pretoria, South Africa. Randomly selected three-digit numbers were used for coding the *ting* samples that were presented in random order to the consumer panel for sensory evaluation. The acceptability of aroma, appearance, mouth feel, taste, aftertaste and overall acceptance of *ting* was evaluated. The panelists scored the seven characteristics using a 9-point hedonic scale (1 = dislike extremely; 2 = dislike very much; 3 = dislike moderately; 4 = dislike slightly; 5 = neither like nor dislike; 6 = like slightly; 7 = like moderately; 8 = like very much; and 9 = like extremely) (Stone and Siddel, 1992). The panelists took between 15 and 20 min to rate the samples. A total of 50 panelists (23 men and 27 women), who were all familiar with the taste of *ting*, participated in the study. The panelists were given water and a piece of carrot before and in between tasting the *ting* samples in order to cleanse their palates. Data was recorded using Compusense® version 5.4.6 (Compusense Inc., Guelph, ON, Canada).

4.2.8 Statistical analyses

The results of the sensory analysis were subjected to analysis of variance (ANOVA), using a significance level of $P < 0.05$. Fischer's least significant difference (LSD) test was also applied to ascertain the significance of differences in the average values for each of the attributes for the different *ting* samples. Pearson's product-moment correlation coefficient (r) was used to test the correlation between pH, titratable acidity and acceptance of other sensory attributes at $P < 0.05$. All statistical analyses were performed using Statistica version 7.1 (StatSoft Inc., Tulsa, OK, USA).

4.3 RESULTS

4.3.1 Bacterial counts

Bacterial counts were determined for the sorghum powder, the fermented sorghum slurries (t = 24 h for inoculated fermentations and t = 54 h for natural fermentation) and for the corresponding cooked fermented porridges. For the sorghum powder, aerobic plate counts (APC) were the highest, followed by counts of lactic acid bacteria (LABC) and Gram-negative counts (GNC), while spore counts (SC) were the lowest. Compared to sorghum powder, the mean APC and GNC of the naturally fermented sorghum slurry increased by 2.1 and 2.07 log cfu/g, respectively, while the SC decreased by 1 log cfu/g. In contrast, the mean APC of inoculated fermented sorghum slurries decreased by more that 2 log cfu/g, while the mean SC and GNC decreased to below the detection limit (<2.00 log cfu/g). The LABC exhibited increases in excess of 5 log cfu/g in both natural and inoculated fermented sorghum slurries. In cooked fermented porridge samples, the bacterial counts (APC, SC, GNC and LABC) were below the detection limit (Table 4.3).

4.3.2 Yeast and mould counts, and identification of isolates

Yeast and mould counts were determined concurrently with bacterial counts. Compared to sorghum powder, the mean yeast and mould counts of the naturally fermented sorghum slurry increased by 1.40 and 3.19 log cfu/g, respectively. The mould count of inoculated fermented sorghum slurries decreased to below the detection limit (<2.00 log cfu/g) as did the yeast count, except for *L. lactis*-inoculated untreated sorghum which exhibited an increase of 2.58 log cfu/g in the mean yeast count. In cooked fermented porridge samples, the yeast and mould counts were below the detection limit (Table 4.3).

Yeasts isolated from sorghum powder and fermented sorghum slurries were identified by the use of ID32C diagnostic kits. The 16 isolates were tentatively identified as *Cryptococcus laurentii* (75%) and *Cryptococcus humicolus* (25%). In contrast, various different moulds were isolated from sorghum powder used in the respective fermentations. The predominant species were *Alternaria* spp., *Cladosporium* spp., *Fusarium andiyazi*, *Penicillium* spp., *Mucor* spp. and *Phoma sorghina*. Fungi that occurred at low prevalence included *Trichoderma* spp., *Rhizopus oryzae*, *F. equiseti*, *Drechslera sativus*, *Drechslera halodes*, *Aspergillus terreus*, *Curvularia eragrostidis*, *Euroteum repens*, *Nigrospora* spp. and *F. chlamydosporum*. Of these fungi, however, *Mucor* spp. and *Penicillium* spp. were detected in the naturally fermented sorghum slurry at the end of fermentation only.

Table 4.3 Microbial counts (log cfu/g) of sorghum powder, fermented sorghum slurries and corresponding cooked fermented porridge (*ting*)

Analysis	Sorghum powder	Fermentation batch[1]						Cooked *ting*
		NF	E1ns	E1s	E2	E3	E4	
APC	5.84 (± 0.02)	7.90 (± 0.09)	2.91 (± 0.02)	2.18 (± 0.02)	2.18 (± 0.03)	2. 32 (± 0.03)	2.50 (± 0.02)	<2.00
SC	2.04 (± 0.04)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
GNC	2.94 (± 0.01)	5.01 (± 0.02)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
LABC	3.85 (± 0.01)	9.21 (± 0.02)	9.83 (± 0.03)	9.37 (± 0.04)	9.59 (± 0.02)	9.70 (± 0.02)	9.82 (± 0.02)	<2.00
Yeasts	2.83 (± 0.02)	4.23 (± 0.62)	5.41 (± 0.07)	<2.00	<2.00	<2.00	<2.00	<2.00
Moulds	2.31 (± 0.02)	5.50 (± 0.13)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00

[1] Refer to Table 4.1 for details of fermentation batches.

4.3.3 Occurrence of food-borne bacterial pathogens

Of the different food-borne pathogens tested for, only *B. cereus* and *E. coli* biotype I were detected in the sorghum powder and in the naturally fermented sorghum slurry. None of the pathogens tested for were detected in any of the inoculated sorghum slurries or in any of the cooked, fermented porridge samples (Table 4.4). Based on biochemical tests, isolates belonging to *E. coli* biotype I was identified as *Klebsiella* spp., *Escherichia* spp. and *Enterobacter* spp. The 65 isolates from spore count plates of sorghum powder and the naturally fermented sorghum slurry were all catalase-positive, rod-shaped and spore-forming, and, of these, only five (7.7%) were identified as *B. cereus*.

4.3.4 Antimicrobial interactions between LAB isolates used as starter cultures

The four LAB isolates used in the formulation of starter cultures were tested for inhibitory reactions against each other, the results of which are shown in Table 4.5. Of the different LAB isolates tested, inhibitory reactions were only observed for *L. lactis* and *Lb. plantarum*. Although *L. lactis* showed strong inhibitory reactions against both *Lb. fermentum* and *Lb. rhamnosus*, no inhibitory reaction was observed against *Lb. plantarum*. Similarly, *Lb. plantarum* showed strong inhibitory reaction against *Lb. fermentum*, but exhibited less interaction with *Lb. rhamnosus* and no inhibitory activity against *L. lactis*.

To determine the mechanism of *L. lactis* and *Lb. plantarum* inhibitory activity, the culture supernatants of the LAB isolates were tested against *L. monocytogenes* as an indicator of bacteriocin production. Both of the LAB isolates showed inhibitory reactions against *L. monocytogenes*. However, after neutralization of the culture supernatants, no inhibitory reactions were observed for the respective cultures. The addition of the proteolytic enzymes Proteinase K and Pronase to the supernatants did not have an effect on their inhibitory activity against the *L. monocytogenes* indicator bacterium (results not shown). Thus, it was concluded that the inhibitory action of *L. lactis* and *Lb. plantarum* was due to acids produced rather than the production of bacteriocins or other antimicrobial compounds.

Table 4.4 Occurrence of bacterial food-borne pathogens in sorghum powder, fermented sorghum slurries and cooked *ting*

Pathogen	Sorghum powder	Fermented slurries		*Ting*
		Natural	Inoculated sorghum slurries	
B. cereus	+	+	-	-
Campylobacter spp.	-	-	-	-
C. perfringens	-	-	-	-
L. monocytogenes	-	-	-	-
Salmonella spp.	-	-	-	-
Shigella spp.	-	-	-	-
E. coli biotype I	+	+	-	-
S. aureus	-	-	-	-
Vibrio spp.	-	-	-	-
Yersinia spp.	-	-	-	-
E. coli 0157:H7	-	-	-	-

-: not detected; +: detected.

Table 4.5 Antimicrobial interactions among lactic acid bacteria used as starter cultures for sorghum fermentation

LAB test strains	LAB indicator strains			
	L. lactis	*Lb. fermentum*	*Lb. plantarum*	*Lb. rhamnosus*
L. lactis	-	++	-	++
Lb. fermentum	-	-	-	-
Lb. plantarum	-	++	-	+
Lb. rhamnosus	-	-	-	-

-: no inhibition; +: 5-8 mm inhibition zone; ++ : >8 mm inhibition zone.

4.3.5 Changes in pH and titratable acidity

The changes in pH and titratable acidity of sorghum slurries fermented naturally for 54 h and of inoculated slurries fermented for 24 h are shown in Fig. 4.1. For all six fermentations, a decrease in pH was accompanied by a simultaneous increase in titratable acidity. In the naturally fermented sorghum slurry, the pH decreased from 6.64 to 4.64 in 24 h and to pH 3.75 in 54 h, with a simultaneous increase in titratable acidity (% w/w, lactic acid) from 0.13% to 0.65% and to 0.84% in 24 and 54 h of fermentation, respectively (Fig. 4.1). After 24 h, the pH of sorghum powder slurries prepared from sterilized sorghum powder and inoculated separately with the starter cultures E1, E2, E3 and E4 decreased from 6.4 to 3.79 - 4.0, with a simultaneous increase in titratable acidity from 0.18% to 0.72% for fermentations by E2 and E3, and to 0.90% and 0.95% for fermentations by E4 and E1, respectively. Inoculation of slurry prepared from untreated (non-sterilized) sorghum powder with *L. lactis*, however, resulted in the most significant and rapid decrease in pH (3.3) and increase in acidity (1.44%) in 24 h of fermentation.

4.3.6 Sensory evaluation of *ting*

Attributes such as aroma and sourness, especially, makes *ting* very popular amongst consumers. The results of the sensory analysis (Table 4.6) indicated that for aroma, *ting* prepared from untreated sorghum powder inoculated with *L. lactis* as starter culture (batch E1ns) was more acceptable than *ting* prepared from both naturally fermented sorghum powder (batch NF) and from sterilized sorghum powder inoculated with the same starter culture (batch E1s). In addition, these samples differed significantly ($P < 0.05$) with respect to acceptability of aroma. The acceptability of *ting* prepared from batches E2, E3 and E4 did not differ significantly with respect to aroma. *Ting* prepared from batch E1ns was clearly most acceptable compared to the rest of the samples as far as sourness was concerned. On the contrary, *ting* prepared from batches E1s, E2, E3, E4 and NF did not differ significantly ($P > 0.05$) with respect to acceptability of sourness. Although there was a strong negative correlation between pH and titratable acidity (r = -0.8494; Table 4.7), there were no significant correlations ($P > 0.05$) between pH and acceptance of any of the sensory attributes. However, titratable acidity had a strong positive correlation with sourness (r = 0.8389; Table 4.7). Indeed, sourness (r = 0.8776) was among other attributes (aroma, appearance and taste) that exhibited a strong positive correlation with overall acceptability of *ting*. Although there were no significant differences ($P > 0.05$) regarding acceptability of the taste and aftertaste of

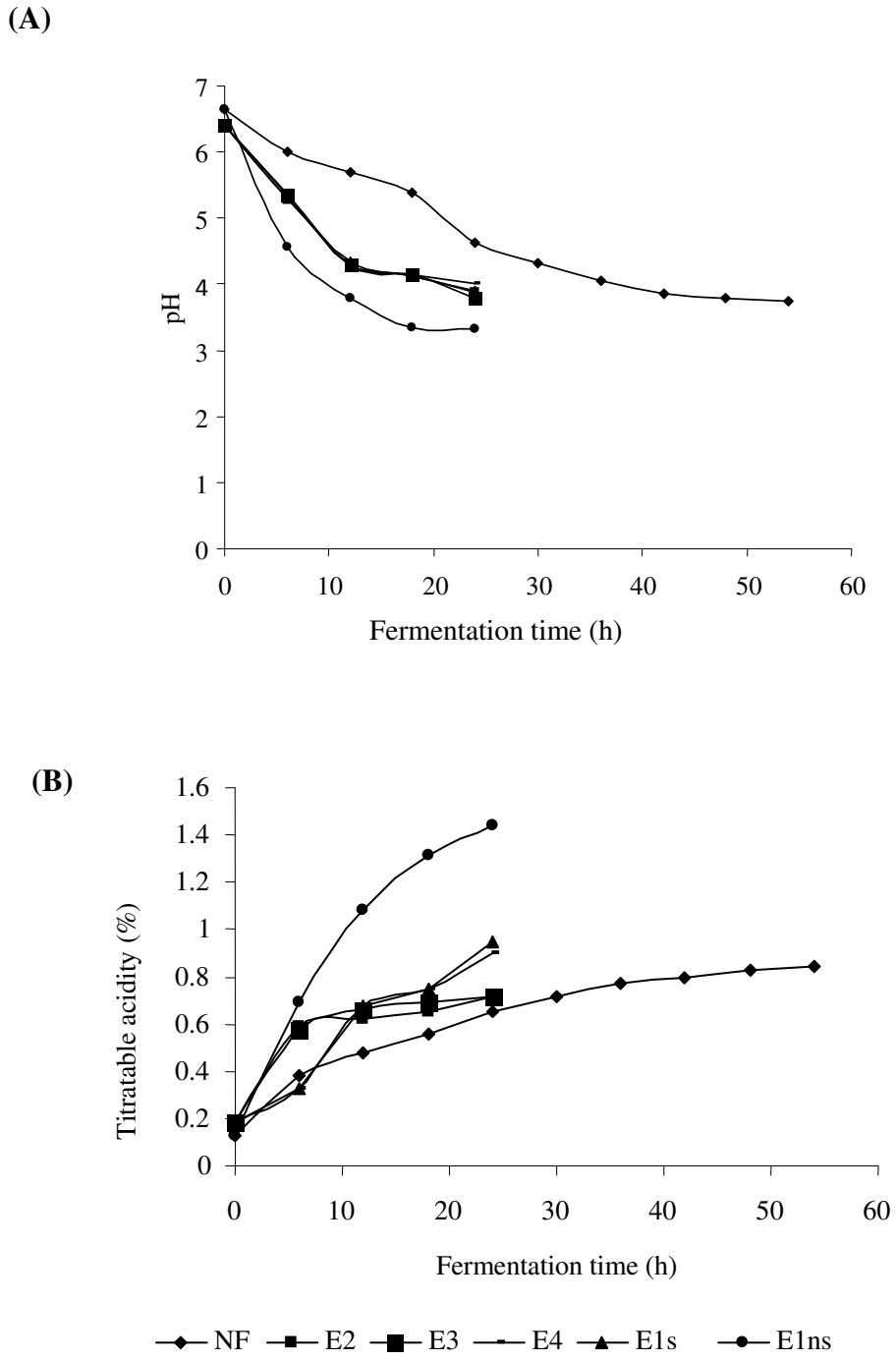

Figure 4.1 Changes in pH (A) and titratable acidity (B) in sorghum powder slurries during natural fermentation (NF) for 54 h and inoculated fermentations with different LAB starter cultures (E1, E2, E3 and E4) for 24 h. LAB starter cultures were as follow: E1, *L. lactis*; E2, *L. lactis* + *Lb. fermentum*; E3, *L. lactis* + *Lb. fermentum* + *Lb. plantarum*; E4, *L. lactis* + *Lb. fermentum* + *Lb. plantarum* + *Lb. rhamnosus.* Prior to inoculation with *L. lactis* the sorghum powder was left untreated (non-sterile; E1ns) or autoclaved (sterilized; E1s). Averaged pH and titratable acidity are indicated.

the respective *ting* samples, there were, however, significant differences ($P < 0.05$) for acceptability of mouth feel, as well as overall acceptability of the *ting* samples (Table 4.6). Based on the results obtained, *ting* prepared from fermentation batch E1ns was the most acceptable amongst the panelists. However, the results also indicated that the overall acceptability of *ting* prepared from sterilized sorghum using starter culture E3 did not differ significantly from *ting* prepared from batch E1ns.

4.4 DISCUSSION

The use of spontaneous fermentation, albeit a simple way to achieve *ting* fermentation, involves a complex microbial process that results in a product of variable quality. It would therefore be desirable to change the fermentation from a wholly natural and unpredictable process to a process that can be manipulated and improved to yield more reliable results. Since lactic acid-fermented porridges have been reported to improve the microbiological stability, as well as nutritional and sensory properties of the product (Chavan and Kadam, 1989; Mugula *et al.*, 2003a; Hammes *et al.*, 2005), an attempt was therefore made to replace the natural fermentation with fermentations conducted with defined starter cultures comprising of different lactic acid bacterial isolates.

The sorghum powder used in the present study was found to be contaminated with Gram-negative bacteria and bacterial spores at levels that are in agreement with previously reported contamination levels of sorghum powder before fermentation (Nout, 1991; Kunene *et al.*, 1999). The bacterial populations were reduced to below the detection limit (< 2.00 cfu/g) in fermented porridge samples prepared from sorghum powder that had been sterilized by autoclaving prior to inoculation with different LAB starter cultures. Despite the non-sterile character of the fermentation, similar results were obtained for fermented porridge prepared from untreated sorghum powder inoculated with *L. lactis* only. In contrast, for fermented porridge samples prepared by natural fermentation, the bacterial spore count was reduced (< 2.00 cfu/g), but both the aerobic plate count (7.9 log cfu/g) and Gram-negative count (5.01 log cfu/g) was increased compared to sorghum powder. Indeed, cooking of the fermented porridge for 30 min was required to reduce the bacterial populations to below the detection limit. However, in all of the fermented porridge samples, lactic acid bacteria were dominant (9.21-9.83 log cfu/g), reinforcing results obtained by Rombouts and Nout (1995) who reported LABC of 9.5-9.7 cfu/g in fermented sorghum.

Table 4.6 Acceptability of the sensory characteristics of *ting* produced, using different starter cultures, based on average hedonic ratings

Fermentation batch[1]	Sensory attribute scores[2]						
	Aroma	Appearance	Sourness	Mouth feel	Taste	Aftertaste	Overall acceptance
NF	5.0 (±2.68)c	6.0 (±2.01)c	5.0 (±2.23)b	5.7 (±1.90)bc	5.3 (±2.27)	5.5 (±2.18)	5.4 (±2.31)c
E1ns	6.7 (±1.99)a	6.8 (±1.60)a	6.4 (±2.11)a	6.4 (±1.85)a	6.2 (±2.30)	6.3 (±1.82)	6.5 (±2.08)a
E1s	5.7 (±2.31)b	6.5 (±1.66)abc	5.3 (±2.21)b	6.1 (±2.03)abc	5.7 (±2.19)	5.8 (±1.83)	5.8 (±2.07)bc
E2	6.1 (±2.20)ab	6.3 (±1.76)bc	5.0 (±2.50)b	5.7(±2.03)c	5.7 (±2.13)	6.0 (±2.09)	5.8 (±2.40)bc
E3	6.2 (±1.94)ab	6.6 (±1.40)ab	5.6 (±2.38)b	6.4 (±1.65)a	6.1(±2.13)	6.0 (±1.91)	6.2 (±1.99)ab
E4	6.0 (±2.00)ab	6.2(±1.82)bc	5.5 (±2.28)b	6.3(±1.76)ab	5.5 (±2.36)	5.8 (±1.90)	5.7 (±2.27)bc

[1] See Table 4.1 for inoculum information.
[2] Sensory attributes of the various *ting* preparations are indicated on a Hedonic scale (1 = Dislike extremely, 9 = Like extremely). The standard deviation of each score is indicated in parentheses. Average score values in the same column that differ significantly ($P < 0.05$) are indicated with different letters (a, b or c).

Table 4.7 Pearson's product-moment correlation coefficients (r)[a] for pH, titratable acidity and acceptability of sensory attributes associated with *ting* produced using different starter cultures

	Titratable acidity	Aroma	Appearance	Sourness	Mouth feel	Taste	Aftertaste	Overall acceptance
pH	(-0.8484) $P = 0.032$	(-0.4899) $P = 0.324$	(-0.4174) $P = 0.410$	(-0.7944) $P = 0.059$	(-0.36030) $P = 0.483$	(-0.4390) $P = 0.384$	(-0.5468) $P = 0.262$	(-0.5788) $P = 0.229$
Titratable acidity		(0.4967) $P = 0.316$	(0.5857) $P = 0.222$	(0.8389) $P = 0.037$	(0.4642) $P = 0.354$	(0.4628) $P = 0.355$	(0.5546) $P = 0.253$	(0.6003) $P = 0.208$
Aroma			(0.8257) $P = 0.043$	(0.7779) $P = 0.069$	(0.6746) $P = 0.142$	(0.8620) $P = 0.027$	(0.9705) $P = 0.01$	(0.9026) $P = 0.014$
Appearance				(0.8091) $P = 0.051$	(0.7139) $P = 0.111$	(0.9602) $P = 0.002$	(0.8744) $P = 0.023$	(0.9558) $P = 0.003$
Sourness					(0.8188) $P = 0.046$	(0.7801) $P = 0.067$	(0.7736) $P = 0.071$	(0.8776) $P = 0.022$
Mouth feel						(0.6880) $P = 0.131$	(0.5670) $P = 0.241$	(0.7337) $P = 0.097$
Taste							(0.9075) $P = 0.012$	(0.9815) $P = 0.001$
Aftertaste								(0.9368) $P = 0.006$

[a] Correlation coefficients (r) are given in brackets. Significant correlations are indicated by $P < 0.05$.

The results presented here indicated that not only does natural fermentation appear to be ineffective in reducing different bacterial populations, but also that addition of *L. lactis* to untreated sorghum slurry is responsible for reducing the bacterial populations effectively during fermentation. Reductions in bacterial populations in LAB-based fermentations have been attributed to a decrease in pH of the fermented gruel (Mante *et al*., 2003) and to the production of organic acids by LAB (Messens and De Vuyst, 2002) and possibly other inhibitory substances (De Vuyst and Vandamme, 1994). Specifically, rapid acidification has been reported to be desirable to not only enhance the sensory characteristics of traditional fermented foods, but also to inhibit growth of pathogens and spoilage bacteria (Kingamkono *et al*., 1995; Mugula *et al*., 2003a; Ammor and Mayo, 2007). In this regard, it is important to note that the lowest pH (3.3) and highest levels of lactic acid (1.44%) were produced the fastest in untreated sorghum inoculated with *L. lactis* (t = 24 h). In contrast, the decrease in pH observed in naturally fermented sorghum was slow and gradual, reaching a pH of 4.6 after 24 h and a final pH of 3.75 at the end of fermentation (t = 54 h) (Fig. 4.1). Due to the changing conditions brought about by the fermentation processes, the bacterial populations in the fermented porridge samples are likely not to have been the same compared to the sorghum powder. Thus, it is possible that the gradual decrease in pH observed during natural fermentation might have selected for and supported the proliferation of acid-tolerant bacteria, thereby accounting for the increase in the aerobic plate count and Gram-negative count. Moreover, the above-mentioned fermentation processes were both found to result in a decrease in bacterial spore counts to below the detection limit (< 2.00 log cfu/g). Previous studies on the survival of spores at low pH have reported a decrease in the counts of *B. cereus* in media with pH values below 5 (Wong and Chen, 1988).

With the exception of *B. cereus* and *E. coli* biotype I, which were both detected in sorghum powder and in naturally fermented porridge samples only, none of the bacterial pathogens tested for were detected in any of the samples analyzed. Both *B. cereus* (Kramer and Gilbert, 1989) and members of the *Enterobacteriaceae* family (Gassem, 1999; Nout, 1991; Mohammed *et al*., 1991) are common contaminants of agricultural commodities, including sorghum crops, and their presence in the samples could have resulted from contamination of the sorghum powder during manufacture and packaging. The absence of *B. cereus* in fermented porridge samples from inoculated fermentations is in agreement with a related study of Kingamkono *et al*. (1994), who also reported an absence of *B. cereus* in lactic acid-fermenting cereal gruels after 24 h. A number of investigations have reported on the survival

of *Enterobacteriaceae* in some traditional African fermented food. Although some of these have reported inhibition of the bacteria by LAB (Nout, 1991; Kunene *et al*., 1999; Mugula *et al*., 2003a), others have reported either a reduction in their numbers (Kingamkono *et al*., 1995) or an increase in the number of coliforms and *E. coli*, especially when non-LAB starter cultures were used (Gran *et al*., 2002; 2003). The survival and growth of *Enterobacteriaceae* at the end of the fermentation process has been ascribed to acid resistance of some strains and/or the presence of microenvironments in the food matrix that support the growth of these bacteria (Arnold and Kasper, 1995; Tomicka *et al*., 1997). Furthermore, the ability of members of *Enterobacteriaceae* to attach to surfaces also results in an increased acid resistance of these bacteria (Rowbury, 1995). In the present study, the *Enterobacteriaceae* identified included *Escherichia* spp., *Klebsiella* spp. and *Enterobacter* spp.

The infestation of sorghum powder by a variety of moulds is in agreement with previous reports (Rabie and Lübben, 1984; Saubois *et al*., 1999; Lefyedi *et al*., 2005). Fungi such as *P. sorghina*, *Alternaria* spp. and *Cladosporium* spp., identified in the present study, may pose possible health risks due to the production of mycotoxins (Rabie *et al*., 1975; Mazur and Kim, 2006). Moreover, elevated levels of moulds in raw materials used for food preparation may render the food unpalatable (Lefyedi *et al*., 2005). However, different LAB species such as *L. lactis* (Roy, 1996), as well as *Lb. plantarum*, *Lb. rhamnosus* and *Lb. paracasei* have been reported to inhibit the growth of certain moulds (Corsetti *et al.*, 1998; Meroth *et al.*, 2003). Isolates of these species produce combinations of lactic acid, acetic acid, phenyllactic acid (PLA), hydrogen peroxide and other low molecular weight compounds that inhibit mold growth (Batish *et al.*, 1997; Lavermicocca *et al.*, 2003). Indeed, the naturally occurring moulds appeared unable to compete under the conditions prevailing in fermenting sorghum inoculated with *L. lactis* as their viable numbers were below the detection limit (< 2.00 log cfu/g), whilst only *Mucor* spp. and *Penicillium* spp. were detected in fermented porridge samples prepared by natural fermentation. This implies that there is less of a risk of mycotoxins being formed during the fermentation process, but the risk of raw materials being contaminated with mycotoxins can, however, not be excluded.

In addition to LAB, yeasts have also been reported to be common in a wide range of traditional African food and beverage fermentations (Olasupo *et al*., 1997; Mugula *et al*., 2003b; Kebede *et al*., 2007). They have been reported to make a useful contribution to the improvement of flavour and acceptability of fermented cereal gruels (Akinrele, 1970; Odunfa

and Adeyele, 1985; Brauman *et al*., 1996; Mugula *et al*., 2003a). In the present study, yeasts were detected in both sorghum powder (2.83 log cfu/g) and in fermented sorghum slurries prepared from untreated sorghum inoculated without or with *L. lactis* (4.23 and 5.41 log cfu/g, respectively). The yeast isolates, *C. laurentii* and *C. humicola*, identified in this study, have been isolated previously from acidic fermentations of cereal substrates (Nout, 1991; Nago *et al.*, 1998; De Vuyst and Neysens, 2005). A co-metabolism between yeasts and lactic acid bacteria has been suggested, whereby the bacteria provide the acid environment, which selects for the growth of yeasts, and the yeasts provide vitamins and other growth factors to the bacteria (Alexander, 1971; Gobetti *et al*., 1994; Steinkraus, 1996). However, despite low levels of yeasts being present in fermented sorghum slurries (<2.00 log cfu/g) prepared from sterilized sorghum powder inoculated with different LAB starter cultures (E1, E2, E3, and E4), the LAB were nevertheless able to proliferate to levels comparable with that determined in fermented sorghum slurries prepared from untreated sorghum powder inoculated without and with *L. lactis* (Table 4.3). Moreover, the lactic acid produced in these fermentations (0.72-0.95%) was comparable to that produced during natural fermentation (0.84%) (Fig. 4.1B). These results suggest that the role of yeasts during lactic acid fermentation is negligible (Martineau and Henick-Kling, 1995), but does not exclude a role in flavour development.

The results from sensory analysis of cooked fermented *ting* porridges prepared from untreated sorghum without or with *L. lactis*, respectively, showed that *ting* from the latter fermentation batch (E1ns) was rated by the panelists as being more acceptable than *ting* prepared from natural fermentation (NF). For *ting* production using untreated sorghum, it therefore appears that the contribution to the fermentation process by *L. lactis* is important. Not only does the presence of this species inhibit growth of fungi and bacteria (Table 4.3), possibly through an accelerated decrease in pH of the fermenting gruel and/or the production of organic acids, but it also exerts a positive influence on the sensory properties of *ting*. This is evidenced by *ting* prepared from *L. lactis*-inoculated untreated sorghum powder being awarded the highest sensory attribute scores. Although *L. lactis* produces diacetyl and acetyladehyde, which are responsible for pleasant flavour in fermented foods (Hugenholtz *et al*., 2000; De Vos and Hugenholtz, 2004), it is possible that yeasts present in the fermenting gruel may also contribute to flavour acceptability. This is evidenced by a significant difference ($P < 0.05$) with regards to the acceptability of the aroma of *ting* prepared from fermentation batches using untreated (E1ns) or sterilized (E1s) sorghum powder inoculated with *L. lactis* (Table 4.6). Conversely, *ting* prepared by natural fermentation earned the lowest attribute scores and

was the least acceptable of all the fermented *ting* porridges. The presence of microorganisms other than lactic acid bacteria during fermentation, especially fungi, may account for the unpalatabilty of this *ting*, as evidenced by low attribute scores for especially aroma, mouth-feel, taste and aftertaste.

The sensory attribute scores awarded to the cooked fermented *ting* porridges prepared from sterilized sorghum powder inoculated with starter cultures E1, E2, E3 and E4 (Table 4.2) fell in between the scores for the ting prepared from untreated sorghum inoculated without (least preferred) or with (most preferred) *L. lactis*. Even so, it is interesting to note that the sensory attribute scores for *ting* prepared from fermentation batch E3 always came second and its overall acceptability amongst panelist did not differ significantly ($P < 0.05$) from that of fermentation batch E1ns, which was the most acceptable (Table 4.6). Since both *L. lactis* and *Lb. plantarum* showed inhibitory reactions against *Lb. fermentum* and *L. rhamnosus* (Table 4.5), it is tempting to speculate that these two LAB strains may contribute only slightly to the sensory characteristics of *ting*. It thus follows that the contribution of *Lb. plantarum*, in addition to *L. lactis* mentioned above, is important.

Traditional natural fermentations in the alcoholic beverage industry have been replaced with defined inocula, high-quality raw materials, strict control of fermentation and better treatment of the final product, thereby resulting in diversification of the market. Traditional African fermented foods, including *ting*, have a long way to go before they reach a similar stage of development. Nevertheless, this is the first report on the use of defined starter cultures for *ting* fermentations. Of the different strains used, *L. lactis* was identified as the most promising candidate *ting* starter culture. This species can be used either in co-culture with *Lb. plantarum* for use in the fermentation of sterilized sorghum or as a monoculture to inoculate untreated sorghum, thus yielding *ting* with better sensory properties and higher microbiological quality than that prepared by natural fermentation. By setting these initial conditions, defined inocula can be considered a means whereby *ting* of more reliable and better quality can be produced and it may possibly be exploited to produce different varieties of *ting*.

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CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS

The objective of this study was essentially to generate new scientific knowledge regarding the microbial diversity and dynamics during sorghum fermentations, using a systematic approach that combined both culture-dependent and culture-independent techniques. Other aims included evaluating microbial populations present in sorghum before, during and after preparation of *ting* in order to elucidate the role of LAB strains with regards to microbiological safety and acceptability of sensory characteristics of *ting*. This research represents the first comprehensive study investigating sorghum fermentations used to produce *ting*. The information generated is significant not only from an academic perspective, but is also relevant for conservation of indigenous knowledge, which may help to bridge the gap between universities and communities by attempting to upgrade the status of indigenous foods.

One of the specific aims of the study was to characterize six representative LAB isolates from spontaneous sorghum fermentations, using a polyphasic approach that encompassed both classical microbiological and DNA-based methods. Sequencing of genes encoding *pheS* clearly assigned the LAB to *Lb. plantarum*, *Lb. rhamnosus* and *Lb. fermentum*. Although sequencing of the 16S rRNA-encoding genes gave similar results, *Lb. plantarum* could not be clearly distinguished from its close phylogenetic neighbours, *Lb. paraplantarum* and *Lb. pentosus*. Likewise, 16S rRNA gene sequencing failed to distinguish *Lb. rhamnosus* from its close relative, *Lb. casei*. These results indicate that a polyphasic approach, which includes both biochemical and DNA-based techniques, should be followed for robust genotypic and phenotypic characterization of LAB obtained from spontaneous sorghum fermentations.

Another aim of this study was to systematically and effectively describe the microbial populations during spontaneous fermentations of two sorghum types at 20, 25 and 30°C using culture-independent PCR-DGGE, as well as culture-dependent methods aided by DNA-based techniques (sequencing of genes encoding 16S rRNA and *pheS*). *Lb. curvatus* was not detected in culture media, but it was predominant in DGGE gels. This is probably due to these bacteria having entered a viable but non-cultivable state, which is characterized by metabolically active cells that do not produce colonies on both selective and non-selective media (Giraffa and Neviani, 2001). This demonstrates one of the virtues of culture-independent approaches over culture-dependent methods (Muyzer and Smalla, 1998; Giraffa and Neviani, 2001). In contrast, *Lb. fermentum*, *Lb. plantarum*, *Lb. rhamnosus*, *E. faecalis* and *E. mundtii* were all isolated in culture media, but they were absent in DGGE gels. This

might be due to either the bacteria occurring in numbers below the detection limits of PCR-DGGE (Muyzer and Smalla, 1998), and/or due to biases introduced by DNA extraction and PCR (Ercolini, 2004; De Vero *et al*., 2006; Camu *et al*., 2007). Indeed, related studies showed that PCR-DGGE was unable to detect some bacterial species that were isolated on culture media (Meroth *et al*., 2003; Ercolini *et al*., 2004). Therefore, the combined approach used in this study was effective in revealing the microbial diversity and dynamics during spontaneous sorghum fermentations.

Other aims of this study included assessing bacterial, yeast and mould populations found in sorghum before, during and after preparation of *ting*, and clarifying the role of selected LAB strains with regards to their contribution to the microbiological safety and acceptability of sensory characteristics of *ting*. Commercial sorghum powder was shown to habour microorganisms such as LAB, *Bacillus* spp., *Enterobacteriaceae*, yeasts and moulds. Natural fermentation alone did not completelely reduce the numbers of pathogens such as *Enterobacteriaceae* members and *Bacillus* spp. below the detection limit, despite the low pH. This may be due to the presence of acid-resistant strains or microenvironments within the fermented sorghum slurries that allowed these bacteria to survive. However, the addition of a *L. lactis* starter culture to untreated sorghum reduced the numbers of *B. cereus*, *Enterobacteriaceae* and moulds to undetectable levels, illustrating the potential that this strain has in improving the microbiological safety of *ting*. Indeed, the addition of *L. lactis* to untreated sorghum resulted in *ting* that was most preferred by panellists. This may be due to the ability of *L. lactis* to control fermentation, as illustrated by its dominance over other LAB in DGGE gels and its ability to reduce the growth of other LAB in antimicrobial studies. For this reason, *L. lactis* may be used either as a monoculture to inoculate untreated sorghum, or in co-culture with *Lb. plantarum* for use in the fermentation of sterilized sorghum, thus yielding *ting* with better sensory properties than that prepared by natural fermentation. These LAB may possibly be exploited to produce different varieties of *ting* in future.

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Appendix A - Current contents of the order *Lactobacillales*, showing all valid species in the genera associated with food technology.

Genus	Species	Author	Phylogenetic group[1]	Metabolism[2]
Lactobacillus	*acetotolerans*	Entani *et al*., 1986	*Lb. delbrueckii*	B
Lactobacillus	*acidifarinae*	Vancanneyt *et al*., 2005	*Lb. buchneri*	C
Lactobacillus	*acidipiscis*	Tanasupawat *et al*., 2000	*Lb. salivarius*	B
Lactobacillus	*acidophilus*	(Moro, 1900) Hansen and Mocquot, 1970	*Lb. delbrueckii*	A
Lactobacillus	*agilis*	Weiss *et al*., 1982	*Lb. salivarius*	B
Lactobacillus	*algidus*	Kato *et al*., 2000	*Lb. salivarius*-single species	B
Lactobacillus	*alimentarius*	(Ex Reuter, 1970) Reuter, 1983	*Lb. alimentarius-Lb. farciminis*	B
Lactobacillus	*amylolyticus*	Bohak *et al*., 1999	*Lb. delbrueckii*	A
Lactobacillus	*amylophilus*	Nakamura and Crowell, 1981	*Lb. delbrueckii*	A
Lactobacillus	*amylotrophicus*	Naser *et al*., 2006	*Lb. delbrueckii*	A
Lactobacillus	*amylovorus*	Nakamura, 1981	*Lb. delbrueckii*	A
Lactobacillus	*animalis*	Dent and Williams, 1983	*Lb. salivarius*	A
Lactobacillus	*antri*	Roos *et al*., 2005	*Lb. reuteri*	C
Lactobacillus	*apodemi*	Osawa *et al*., 2006	*Lb. salivarius*	B
Lactobacillus	*arizonensis*	Swezey *et al*., 2000[a]		
Lactobacillus	*aviaries*	Fujisawa *et al*., 1985	*Lb. salivarius*	A
Lactobacillus	*aviarius* subsp. *araffinosus*	Fujisawa *et al*., 1986	*Lb. salivarius*	A
Lactobacillus	*aviarius* subsp. *aviaries*	Fujisawa *et al*. ,1985	*Lb. salivarius*	A
Lactobacillus	*bavaricus*	Stetter and Stetter, 1980		
Lactobacillus	*bifermentans*	Kandler *et al*., 1983	*Lb. coryneformis*	B
Lactobacillus	*brevis*	(Orla-Jensen, 1919) Bergey *et al*., 1934	*Lb. brevis*	C
Lactobacillus	*buchneri*	(Henneberg, 1903) Bergey *et al*., 1923	*Lb. buchneri*	C
Lactobacillus	*casei*	(Orla-Jensen, 1916) Hansen and Lessel, 1971	*Lb. casei*	B
Lactobacillus	*catenaformis*	(Eggert, 1935) Moore and Holdeman, 1970		A
Lactobacillus	*coleohominis*	Nikolaitchouk *et al*., 2001	*Lb. reuteri*	B
Lactobacillus	*collinoides*	Carr and Davis, 1972		C
Lactobacillus	*composti*	Endo and Okada, 2007	*Lb. coryneformis*	B
Lactobacillus	*concavus*	Tong and Dong, 2005	*P. dextrinicus*	A
Lactobacillus	*coryniformis* subsp. *coryniformis*	Abo-Elnaga and Kandler, 1965	*Lb. coryniformis*	B
Lactobacillus	*coryniformis* subsp. *torquens*	Abo-Elnaga and Kandler, 1965	*Lb. coryniformis*	B
Lactobacillus	*crispatus*	(Brygoo and Aladame, 1953) Moore and Holdeman, 1970	*Lb. delbrueckii*	A
Lactobacillus	*curvatus*	(Troili-Petersson, 1903) Abo-Elnaga and Kandler, 1965; Klein *et al*., 1996	*Lb. sakei*	B
Lactobacillus	*curavtus*	(Troili-Petersson, 1903) Abo-Elnaga and Kandler, 1965 emend. Klein *et al*., 1996	*Lb. sakei*	B
Lactobacillus	*delbrueckii* subsp. *bulgaricus*	(Orla-Jensen, 1919) Weiss *et al*., 1984	*Lb. delbrueckii*	A
Lactobacillus	*delbrueckii* subsp. *delbrueckii*	(Leichmann, 1896) Beijerinck, 1901	*Lb. delbrueckii*	A
Lactobacillus	*delbrueckii* subsp. *indicus*	Dellaglio *et al*., 2005	*Lb. delbrueckii*	A
Lactobacillus	*delbrueckii* subsp. *lactis*	(Orla-Jensen, 1919) Weiss *et al*., 1984	*Lb. delbrueckii*	A
Lactobacillus	*diolivorans*	Krooneman *et al*., 2002	*Lb. buchneri*	C
Lactobacillus	*equi*	Morotomi *et al*., 2002	*Lb. salivarius*	A
Lactobacillus	*farciminis*	(Ex Reuter, 1970) Reuter, 1983	*Lb. alimentarius-Lb. farciminis*	A
Lactobacillus	*farraginis*	Endo and Okada, 2007	*Lb. buchneri*	B
Lactobacillus	*fermentum*	Beijerinck, 1901 emend. Dellaglio *et al*., 2004	*Lb. reuteri*	C
Lactobacillus	*fornicalis*	Dicks *et al*., 2000	*Lb. delbrueckii*	B
Lactobacillus	*fructivorans*	Charlton *et al*., 1934	*Lb. fructivorans*	C
Lactobacillus	*frumenti*	Müller *et al*., 2000	*Lb. reuteri*	C
Lactobacillus	*fuchuensis*	Sakala *et al*., 2002	*Lb. sakei*	B
Lactobacillus	*gallinarum*	Fujisawa *et al*., 1992	*Lb. delbrueckii*	A
Lactobacillus	*gasseri*	Lauer and Kandler, 1980	*Lb. delbrueckii*	A
Lactobacillus	*gastricus*	Roos *et al*., 2005	*Lb. reuteri*	C
Lactobacillus	*graminis*	Beck *et al*., 1989	*Lb. sakei*	B
Lactobacillus	*hammesii*	Valcheva *et al*., 2005	*Lb. brevis*	B
Lactobacillus	*hamsteri*	Mitsuoka and Fujisawa, 1988	*Lb. delbrueckii*	B
Lactobacillus	*harbinensis*	Miyamoto *et al*., 2006	*Lb. perolens*	B
Lactobacillus	*helveticus*	(Orla-Jensen, 1919) Bergey *et al*., 1925	*Lb. delbrueckii*	A
Lactobacillus	*hilgardii*	Douglas and a Cruess, 1936	*Lb. buchneri*	C
Lactobacillus	*homohiochii*	Kitahara *et al*., 1957	*Lb. fructivorans*	B
Lactobacillus	*iners*	Falsen *et al*., 1999	*Lb. delbrueckii*	A
Lactobacillus	*ingluviei*	Baele *et al*., 2003	*Lb. reuteri*	C
Lactobacillus	*intestinalis*	(ex Hemme, 1974) Fujisawa *et al*., 1990	*Lb. delbrueckii*	B
Lactobacillus	*jensenii*	Gasser *et al*., 1970	*Lb. delbrueckii*	B
Lactobacillus	*johnsonii*	Fujisawa *et al*., 1992	*Lb. delbrueckii*	A
Lactobacillus	*kalixensis*	Roos *et al*., 2005	*Lb. delbrueckii*	A
Lactobacillus	*kefiranofaciens* subsp. *kefiranofaciens*	Fujisawa et *al*., 1988	*Lb. delbrueckii*	A
Lactobacillus	*kefiranofaciens* subsp. *kefirgranum*	(Takizawa *et al*., 1994) Vancanneyt *et al*., 2004	*Lb. delbrueckii*	A
Lactobacillus	*kefiri*	Kandler and Kunath, 1983	*Lb. buchneri*	C
Lactobacillus	*kimchii*	Yoon *et al*., 2000	*Lb. alimentarius-Lb. farciminis*	B
Lactobacillus	*kitasatonis*	Mukai *et al*., 2003	*Lb. delbrueckii*	B
Lactobacillus	*kunkeei*	Edwards *et al*., 1998	Single species	C
Lactobacillus	*lindneri*	Back *et al*., 1997	*Lb. fructivorans*	C
Lactobacillus	*malefermentans*	Farrow *et al*., 1989	Single species	C

Genus	Species	Author	Phylogenetic group[1]	Metabolism[2]
Lactobacillus	*mali*	Carr and Davies, 1970, Kaneuchi *et al*., 1988	*Lb. salivarius*	A
Lactobacillus	*manihotivorans*	Morlon-Guyet *et al.*, 1998	Couple3	A
Lactobacillus	*mindensis*	Ehrmann *et al.*, 2003	*Lb. alimentarius-Lb. farciminis*	A
Lactobacillus	*mucosae*	Roos *et al.*, 2000	*Lb. reuteri*	C
Lactobacillus	*murinus*	Hemme *et al.*, 1982	*Lb. salivarius*	B
Lactobacillus	*nagelii*	Edwards *et al*., 2000	*Lb. salivarius*	A
Lactobacillus	*namurensis*	Scheirlinck *et al*., 2007	*Lb. buchneri*	C
Lactobacillus	*oris*	Farrow and Collins, 1988	*Lb. reuteri*	C
Lactobacillus	*panis*	Wiese *et al*., 1996	*Lb. reuteri*	C
Lactobacillus	*nantensis*	Valcheva *et al.*, 2006	*Lb. alimentarius-Lb. farciminis*	B
Lactobacillus	*pantheris*	Liu and Dong, 2002	Single species	A
Lactobacillus	*parabrevis*	Vancanneyt *et al.*, 2006	*Lb. brevis*	C
Lactobacillus	*parabuchneri*	Farrow *et al*., 1989	*Lb. buchneri*	C
Lactobacillus	*paracasei* subsp. *paracasei*	Collins *et al*., 1989	*Lb. casei*	B
Lactobacillus	*paracasei* subsp. *tolerans*	Collins *et al*., 1989	*Lb. casei*	B
Lactobacillus	*paracollinoides*	Suzuki *et al*., 2004	*Lb. perolens*	C
Lactobacillus	*parafarraginis*	Endo and Okada, 2007	*Lb. buchneri*	B
Lactobacillus	*parakefiri*	Takizawa *et al*., 1994	*Lb. buchneri*	C
Lactobacillus	*paralimentarius*	Cai *et al*., 1999	*Lb. alimentarius-Lb. farciminis*	B
Lactobacillus	*paraplantarum*	Curk *et al.*, 1996	*Lb. plantarum*	B
Lactobacillus	*pentosus*	Zanoni *et al*., 1997	*Lb. plantarum*	B
Lactobacillus	*perolens*	Back *et al*., 2000	*Lb. perolens*	B
Lactobacillus	*plantarum*	(Orla-Jensen, 1919) Bergey *et al*., 1923	*Lb. plantarum*	B
Lactobacillus	*platarum* subsp. *argentoratensis*	Bringel *et al*., 2005	*Lb. plantarum*	B
Lactobacillus	*pontis*	Vogel *et al*., 1994	*Lb. reuteri*	C
Lactobacillus	*pscittasi*	Lawson *et al*., 2001	*Lb. delbrueckii*	C
Lactobacillus	*rennini*	Chenoll *et al*., 2006	*Lb. coryneformis*	B
Lactobacillus	*reuteri*	Kandler *et al*., 1982	*Lb. reuteri*	C
Lactobacillus	*rhamnosus*	(Hansen, 1968) Collins *et al*., 1989	*Lb. casei*	B
Lactobacillus	*rogosae*	Holdeman and Moore, 1974	Strain not available	
Lactobacillus	*rossiae*	Corsetti *et al*., 2005	Couple1	C
Lactobacillus	*ruminis*	Sharpe *et al*., 1973	*Lb. salivarius*	A
Lactobacillus	*saerimneri*	Pedersen and Roos, 2004	*Lb. salivarius*	A
Lactobacillus	*sakei* subsp. *carnosus*	Torriani *et al*., 1996	*Lb. sakei*	B
Lactobacillus	*sakei* subsp. *sakei*	Katagiri *et al*., 1934 emend. Klein *et al*., 1996	*Lb. sakei*	B
Lactobacillus	*salivarius*	Rogosa *et al*., 1953, Li *et al*., 2006	*Lb. salivarius*	A
Lactobacillus	*sanfranciscensis*	Weiss and Schillinger, 1984	*Lb. fructivorans*	C
Lactobacillus	*satsumensis*	Endo and Okada, 2005	*Lb. salivarius*	A
Lactobacillus	*secaliphilus*	Ehrmann *et al*., 2007	*Lb. reuteri*	B
Lactobacillus	*sharpeae*	Weiss *et al.*, 1982	Single species	A
Lactobacillus	*siliginis*	Aslam *et al*., 2006	Couple1	C
Lactobacillus	*sobrius*	Kostantinov *et al*., 2006	*Lb. delbrueckii*	B
Lactobacillus	*spicheri*	Meroth *et al.*, 2004	*Lb. buchneri*	B
Lactobacillus	*suebicus*	Kleymans *et al*., 1989	Couple2	C
Lactobacillus	*tucceti*		*Lb. alimentarius-Lb. farciminis*	A
Lactobacillus	*ultunensis*	Roos *et al*., 2005	*Lb. delbrueckii*	A
Lactobacillus	*vaccinostercus*	(Kozaki and Okada, 1983) Okada *et al*., 1979	Couple2	C
Lactobacillus	*vaginalis*	Embley *et al*., 1989	*Lb. reuteri*	C
Lactobacillus	*versmoldensis*	Kröckel *et al*., 2003	*Lb. alimentarius-Lb. farciminis*	A
Lactobacillus	*vini*	Rodas *et al*., 2006	*Lb. salivarius*	B
Lactobacillus	*vitulinus*	Sharpe *et al*., 1973		A
Lactobacillus	*zeae*	Dicks *et al*., 1996	*Lb. casei*	B
Lactobacillus	*zymae*	Vancanneyt *et al*., 2005	*Lb. buchneri*	C
Paralactobacillus	*selangonsis*	Leisner *et al.*, 2000	ND	ND
Pediococcus	*acidilactici*	Lindner, 1887	ND	ND
Pedicoccus	*cellicola*	Zhang *et al.*, 2005	ND	ND
Pediococcus	*halophilus*	Mees, 1934	ND	ND
Pediococcus	*inopinatus*	Back, 1988	ND	ND
Pediococcus	*parvulus*	Gunther and White, 1961	ND	ND
Pediococcus	*pentosaceus*	Mees, 1934	ND	ND
Pediococcus	*stilesii*	Franz *et al*., 2006	ND	ND
Pediococcus	*urinaeequi*	(ex Mees, 1934) Garvie, 1988	ND	ND

[1,2] The phylogenetic groups and metabolism of *Lactobacillus* genera are from Felis and Dellaglio, 2007. A: obligately homofermentative; B: facultatively heterofermentative, and C: obligately heterofermentative. [a]*Lactobacillus arizonensis* is a later heterotypic synonym of *Lactobacillus plantarum* (Kostinek *et al*., 2005).

Appendix B - Current contents of the order *Lactobacillales*, showing all valid species other than *Lactacillaceae* family.

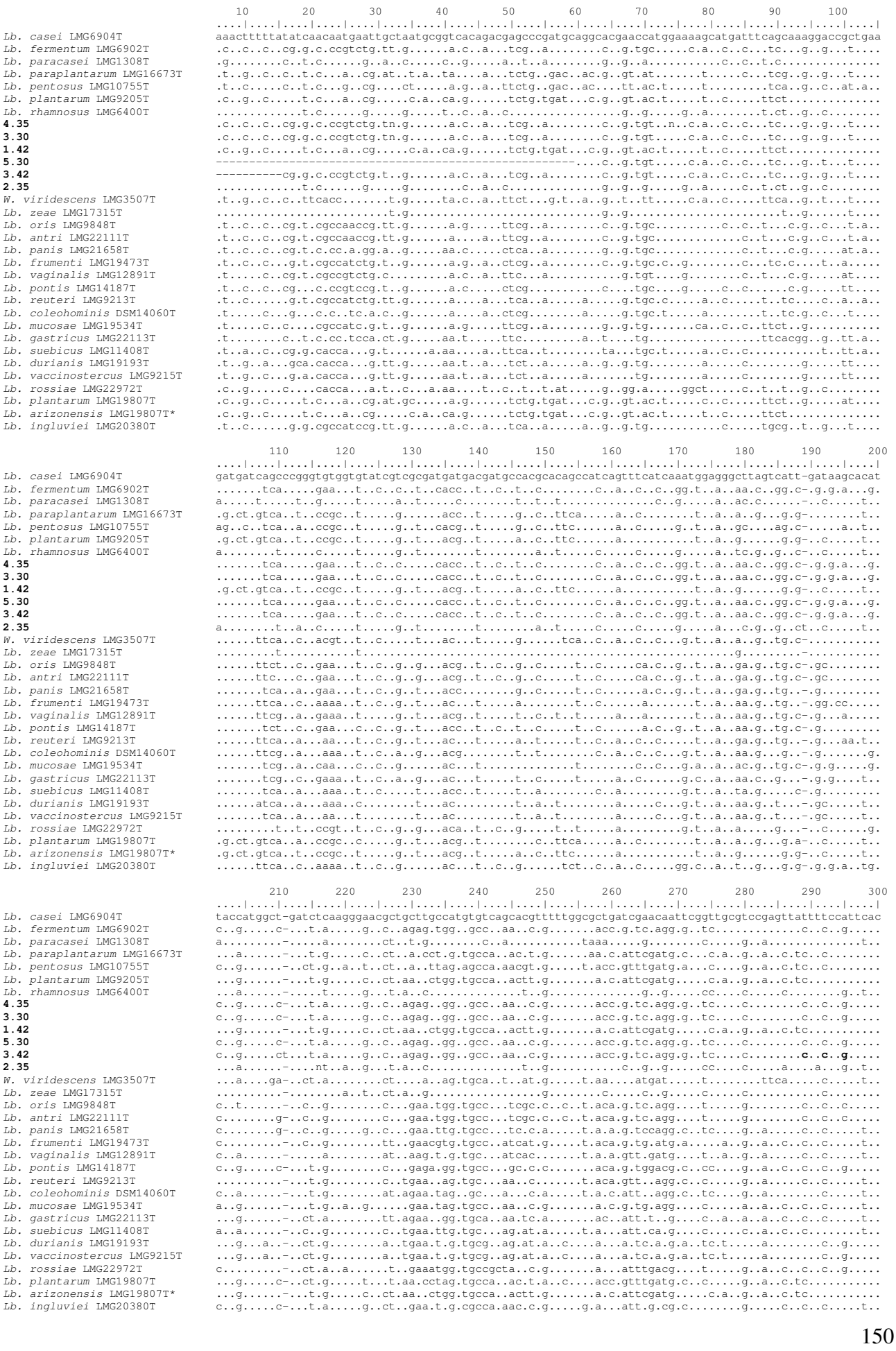
Family	Genus	Species	Author
Aerococcaceae	*Aerococcus*	*sanguinicola*	Lawson *et al*., 2001
	Aerococcus	*urinae*	Aguirre and Collins, 1992
	Aerococcus	*urinaeequi*	(Garvie, 1988) Felis *et al*., 2005
	Aerococcus	*urinaehominis*	Lawson *et al*., 2001
	Aerococcus	*viridans*	Williams *et al*., 1953
	Abiotrophia	*defectiva*	(Bouvet *et al*., 1989) Kawamura *et al*., 1995
	Abiotrophia	*adiacens*	(Bouvet *et al*., 1989) Kawamura *et al*., 1995
	Abiotrophia	*balaenopterae*	Lawson *et al*., 1999
	Abiotrophia	*elegans*	Roggenkamp *et al*., 1999
	Dolosicoccus	*paucivorans*	Collins *et al*., 1999
	Eremococcus	*coleocora*	Collins *et al*., 1999
	Facklamia	*hominis*	Collins *et al*., 1997
	Facklamia	*Ignava*	Collins *et al*., 1998
	Facklamia	*languida*	Lawson *et al*., 1999
	Facklamia	*miroungae*	Hoyles *et al*., 2001
	Facklamia	*sourekii*	Collins *et al*., 1999
	Facklamia	*tabacinasalis*	Collins *et al*., 1999
	Globicatella	*sanguinis*	Collins *et al*., 1995
	Globicatella	*salfidifaciens*	Vandamme *et al*., 2001
	Ignavigranum	*ruoffiae*	Collins *et al*., 1999
Carnobacteriaceae	*Agitococcus*	*lubricus*	Franzmann and Skermann, 1981
	Alkalibacterium	*olivapovliticus*	Ntougias and Russel, 2001
	Alkalibacterium	*iburiense*	Nakajima *et al*., 2005
	Alkalibacterium	*psychrotolerans*	Yumoto *et al*., 2004
	Allofustis	*seminis*	Collins *et al*., 2003
	Alloiococcus	*otitis*	Aguirre and Collins, 1992
	Desemzia	*incerta*	(Steinhaus, 1941) Stackebrandt *et al*., 1999
	Dolosigranulum	*pigrum*	Aguirre *et al*., 1994
	Granulicatella	*adiacens*	(Bouvet *et al*., 1989) Collins and Lawson, 2000
	Granulicatella	*balaenopterae*	(Lawson *et al*., 1999) Collins and Lawson, 2000
	Isobaculum	*melis*	Collins *et al*., 2002
	Lactosphaera	*pasteurii*	(Schink, 1985) Janssen *et al*., 1995
	Marinilactibacillus	*psychrotolerans*	Ishikawa *et al*., 2003
	Tricohococcus	*collinsii*	Liu *et al*., 2002
	Tricohococcus	*flocculiformis*	Scheff *et al*., 1984
	Tricohococcus	*pasteurii*	(Schink, 1985) Liu *et al*., 2002
	Tricohococcus	*patagoniensis*	Pikuta *et al*., 2006
	Carnobacterium	*alterfunditum*	Franzmann *et al*., 1993
	Carnobacterium	*divergens*	(Holzapfel and Gerber, 1984) Collins *et al*., 1987
	Carnobacterium	*funditum*	Franzmann *et al*., 1993
	Carnobacterium	*gallinarum*	Collins *et al*., 1987
	Carnobacterium	*inhibens*	Jöborn *et al*., 1999
	Carnobacterium	*maltomaticun*	(Miller *et al*., 1974) Mora *et al*., 2003
	Carnobacterium	*mobile*	Collins *et al*., 1987
	Carnobacterium	*piscicola*	(Hiu *et al*., 1984) Collins *et al*., 1987
	Carnobacterium	*pleistoceneum*	Pikuta *et al*., 2005
	Carnobacterium	*viridans*	Holley *et al*., 2002
Leuconostocaceae	*Leuconostoc*	*amelibiosum*	Schillinger *et al*., 1989
	Leuconostoc	*argentinum*	Dicks *et al*., 1993
	Leuconostoc	*carnosum*	Shaw and Harding, 1989
	Leuconostoc	*fallax*	Martinez-Murcia and Collins, 1992
	Leuconostoc	*ficulneums*	Antunes *et al*., 2002
	Leuconostoc	*fructosum*	Antunes *et al*., 2002
	Leuconostoc	*gelidum*	Shaw and Harding, 1989
	Leuconostoc	*gasicomitatum*	Björkroth *et al*., 2001
	Leuconostoc	*inhae*	Kim *et al*., 2003
	Leuconostoc	*kimchii*	Kim *et al*., 2000
	Leuconostoc	*mesemteroides* subsp. *cremoris*	(Knudsen and Sörensen, 1929) Garvie, 1983
	Leuconostoc	*mesemteroides* subsp. *dextranicum*	(Beijerinck, 1912) Garvie, 1983
	Leuconostoc	*paramesenteroides*	Garvie, 1967
	Leuconostoc	*pseudoficulneum*	Chambel *et al*., 2006
	Leuconostoc	*pseudomesenteroides*	Farrow *et al*., 1989
	Oenococcus	*kitaharae*	Endo and Okada, 2006
	Oenococcus	*oeni*	(Garvie, 1967) Dicks *et al*., 1995
	Weissella	*cibaria*	Björkroth *et al*., 2002
	Weissella	*confusa*	(Holzapfel and Knadler, 1969) Collins *et al*., 1994
	Weissella	*halotolerans*	(Kandler *et al*., 1983) Collins *et al*., 1994
	Weissella	*hellenica*	Collins *et al*., 1994
	Weissella	*kandleri*	(Holzapfel and van Wyk, 1983) Collins *et al*. ,1994
	Weissella	*kimchii*	Choi *et al*., 2002
	Weissella	*koreensis*	Lee *et al*., 2002
	Weissella	*minor*	(Kandler *et al*., 1983) Collins *et al*., 1994
	Weissella	*paramesenteroides*	(Garvie, 1967) Collins *et al*., 1994
	Weissella	*soli*	Magnusson *et al*., 2002
	Weissella	*viridescens*	(Niven and Evans, 1957) Collins *et al*., 1994

Family	Genus	Species	Author
Enterococcaceae	*Enterococcus*	*aquimarinus*	Svec *et al*., 2005
	Enterococcus	*asini*	De Vaux *et al*., 1998
	Enterococcus	*avium*	(ex Nowlan and Deibel, 1967) Collins *et al*., 1984
	Enterococcus	*caccae*	Carvalho *et al*., 2006
	Enterococcus	*canintestini*	Naser *et al*., 2005
	Enterococcus	*asini*	De Vaux *et al*., 1998
	Enterococcus	*avium*	(ex Nowlan and Deibel, 1967) Collins *et al*., 1984
	Enterococcus	*caccae*	Carvalho *et al*., 2006
	Enterococcus	*canintestini*	Naser *et al*., 2005
	Enterococcus	*canis*	De Graef *et al*., 2003
	Enterococcus	*cecorum*	(Devriese *et al*., 1983) Williams *et al*., 1989
	Enterococcus	*columbae*	Devriese *et al*., 1993
	Enterococcus	*devriesei*	Svec *et al*., 2005
	Enterococcus	*dispar*	Collins *et al*., 1991
	Enterococcus	*durans*	(ex Sherman and Wing, 1973) Collins *et al*., 1984)
	Enterococcus	*faecalis*	(Andrewes and Horder, 1906) Schleifer and Kilpper-Bälz, 1984
	Enterococcus	*faecalis*	(Andrewes and Horder, 1906) Schleifer and Kilpper-Bälz, 1984
	Enterococcus	*faecium*	(Orla-Jensen, 1919) Schleifer and Kilpper-Bälz, 1984
	Enterococcus	*flavescens*	Pompei *et al*., 1992
	Enteroccus	*gallinarum*	(Bridge and Sneath, 1982) Collins *et al*., 1984
	Enterococcus	*gilvus*	Tyrell *et al*., 2002
	Enterococcus	*haemoperoxidus*	Svec *et al*., 2001
	Enterococcus	*moraviensis*	Svec *et al*., 2001
	Enterococcus	*mundtii*	Collins *et al*., 1986
	Enterococcus	*pallens*	Tyrell *et al*., 2002
	Enterococcus	*phoeniculicola*	Law-Brown and Meyers, 2003
	Enterococcus	*hermanniensis*	Koort *et al*., 2004
	Enterococcus	*hirae*	Farrow and Collins, 1985
	Enterococcus	*italicus*	Fortina *et al*., 2004
	Enterococcus	*malodoratus*	(ex Pette, 1955) Collins *et al*., 1984
	Enterococcus	*porcinus*	Teixerira *et al*., 2001
	Enterococcus	*pseudoavium*	Collins *et al*., 1989
	Enterococcus	*raffinosus*	Collins *et al*., 1989
	Enterococcus	*saccharolyticus*	(Farrow *et al*., 1985) Rodrigues and Collins, 1991
	Enterococcus	*seriolicida*	Kusuda *et al*., 1991
	Enterococcus	*saccharominimus*	Vancanneyt *et al*., 2004
	Enterococcus	*silesiacus*	Svec *et al*., 2006
	Enterococcus	*solitarius*	Collins *et al*., 1989
	Enterococcus	*sulfurous*	Martinez-Murcia and Collins, 1991
	Enterococcus	*termitis*	Svec *et al*., 2006
	Enterococcus	*villorum*	Vancanneyt *et al*., 2001
	Atopobacter	*phocae*	Lawson *et al*., 2000
	Mellissococcus	*plutonius*	Bailey and Collins, 1983
	Tetragenococcus	*halophilus*	(Mees, 1934) Collins *et al*., 1993
	Tetragenococcus	*muriaticus*	Satomi *et al*., 1997
	Tetragenococcus	*koreensis*	Lee *et al*., 2005
	Vagococcus	*elongatus*	Lawson *et al*., 2007
	Vagococcus	*fessus*	Hoyles *et al*., 2000
	Vagococcus	*fluvialis*	Collins *et al*., 1990
	Vagococcus	*lutrae*	Lawson *et al*., 1999
	Vagococcus	*salmoninarum*	Wallbanks *et al*., 1990
Streptococcaceae	*Streptococcus*	*adjacens*	Bouvet *et al*., 1989
	Streptococcus	*agalactiae*	Lehmann and Neumann, 1896
	Streptococcus	*alactolyticus*	Farrow *et al*., 1985
	Streptococcus	*alactolyticus*	Farrow *et al*., 1985
	Streptococcus	*anginosus*	(Andrews and Horder, 1906) Smith and Sherman, 1938
	Streptococcus	*australis*	Willcox *et al*., 2001
	Streptococcus	*bovis*	Orla-Jensen, 1919
	Streptococcus	*canis*	Devriese *et al*., 1986
	Streptococcus	*caprinus*	Brooker *et al*,. 1996
	Streptococcus	*castoreus*	Lawson *et al*,. 2005
	Streptococcus	*cecorum*	Devriese *et al*., 1983)
	Streptococcus	*constellatus*	(Prévot, 1924) Holdeman and Moore, 1974
	Streptococcus	*constellatus* subsp. *constellatus*	(Prévot, 1924) Holdeman and Moore, 1974
	Streptococcus	*constellatus* subsp. *pharyngis*	Whiley *et al*., 1999
	Streptococcus	*criceti*	Coykendall, 1977
	Streptococcus	*cristatus*	Handley *et al*., 1991
	Streptococcus	*defectivus*	Bouvet *et al*., 1989
	Streptococcus	*devriesei*	Collins *et al*., 2004
	Streptococcus	*didelphis*	Rurangirwa *et al*., 2000
	Streptococcus	*difficilis*	Eldar *et al*., 1995
	Streptococcus	*adjacens*	Bouvet *et al*., 1989
	Streptococcus	*durans*	(ex Sherman and Wing, 1937) Knight *et al*., 1984
	Streptococcus	*dysgalactiae*	(ex Diernhofer, 1932) Garvie *et al*., 1983
	Streptococcus	*dysgalactiae* subsp. *dysgalactiae*	(ex Diernhofer, 1932) Garvie *et al*., 1983
	Streptococcus	*dysgalactiae* subsp. *equisimilis*	Vandamme *et al*., 1996
	Streptococcus	*entericus*	Vela *et al*., 2002
	Streptococcus	*equi*	Sand and Jensen, 1888
	Streptococcus	*equi* subsp. *equi*	Sand and Jensen, 1888
	Streptococcus	*equi* subsp. *ruminatorum*	Fernández *et al*., 2004

Family	Genus	Species	Author
Streptococcaceae	*Streptococcus*	*equi* subsp. *zooepidermicus*	(ex Frost and Englebrecht, 1936) Farrow and Collins, 1985
	Streptococcus	*equinus*	Andrewes and Horder, 1906
	Streptococcus	*faecalis*	Andrewes and Horder, 1906
	Streptococcus	*faecium*	Orla-Jensen, 1919
	Streptococcus	*ferus*	(ex Coykendall, 1977) Coykendall, 1983
	Streptococcus	*gallinaceus*	Collins *et al*., 2002
	Streptococcus	*gallinarum*	Bridge and Sneath, 1982
	Streptococcus	*gallolyticus*	Osawa *et al*., 1996
	Streptococcus	*gallolyticus* subsp. *gallolyticus*	Osawa *et al*., 1996
	Streptococcus	*garvieae*	Collins *et al*., 1984
	Streptococcus	*gordonii*	Kilian *et al*., 1989
	Streptococcus	*halichoeri*	Lawson *et al*., 2004
	Streptococcus	*hansenii*	Holdeman and Moore, 1974
	Streptococcus	*hyointestinalis*	Devriesse *et al*., 1988
	Streptococcus	*hyovaginalis*	Devriesse *et al*., 1997
	Streptococcus	*infantarius*	Schlegel *et al*., 2000
	Streptococcus	*infantarius* subsp. *coli*	Schlegel *et al*., 2003
	Streptococcus	*infantarius* subsp. *infantarius*	Schlegel *et al*., 2003
	Streptococcus	*infantis*	Kawamura *et al*., 1998
	Streptococcus	*iniae*	Pier and Madin, 1976
	Streptococcus	*intermedius*	Prévot, 1925
	Streptococcus	*intestinalis*	Robinson *et al*., 1988
	Streptococcus	*lutetiensis*	Poyart *et al*., 2002
	Streptococcus	*macacae*	Beighton *et al*., 1984
	Streptococcus	*macedonicus*	Tsakalidou *et al*., 1998
	Streptococcus	*marimammalium*	Lawson *et al*., 2005
	Streptococcus	*massiliensis*	Glazunova *et al*., 2006
	Streptococcus	*minor*	Vancanneyt *et al*., 2004
	Streptococcus	*mitis*	Andrewes and Horder, 1906
	Streptococcus	*morbillorum*	(Prévot, 1933) Holdeman and Moore, 1974
	Streptococcus	*mutans*	Coykendall, 1977
	Streptococcus	*oligofermentans*	Tong *et al*., 2003
	Streptococcus	*oralis*	Bridge and Sneath, 1982
	Streptococcus	*orisratti*	Zhu *et al*., 2000
	Streptococcus	*ovis*	Collins *et al*., 2001
	Streptococcus	*parasanguinis*	Whiley *et al*., 1990
	Streptococcus	*parauberis*	Williams and Collins, 1990
	Streptococcus	*parvulus*	(Weinberg *et al*., 1937) Cato, 1983
	Streptococcus	*pasteurianus*	Poyart *et al*., 2002
	Streptococcus	*peroris*	Kawamura *et al*., 1998
	Streptococcus	*plantarum*	Collins *et al*., 1984
	Streptococcus	*pleomorphus*	Barnes *et al*., 1979
	Streptococcus	*pluranimalium*	Devriese *et al*., 1999
	Streptococcus	*pneumoniae*	(Klein, 1884) Chester, 1901
	Streptococcus	*porcinus*	Collins *et al*., 1985
	Streptococcus	*pseudopneumoniase*	Arbique *et al*., 2005
	Streptococcus	*pseudoporcinus*	Bekal *et al*., 2007
	Streptococcus	*pyogenes*	Rosenbach, 1884
	Streptococcus	*raffinolactis*	Orla-Jensen and Hansen, 1932
	Streptococcus	*ratti*	Coykendall, 1977
	Streptococcus	*saccharolyticus*	Farrow *et al*., 1985
	Streptococcus	*salivarius*	Andrewes and Horder, 1906
	Streptococcus	*salivarius* subsp. *salivarius*	Andrewes and Horder, 1906
	Streptococcus	*salivarius* subsp. *thermophilus*	(Orla-Jensen, 1919) Farrow and Collins, 1984
	Streptococcus	*sanguinis*	White and Niven, 1946
	Streptococcus	*shiloi*	Eldar *et al*., 1995
	Streptococcus	*sinensis*	Woo *et al*., 2002
	Streptococcus	*sobrinus*	(ex Coykendall, 1974) Coykendall, 1983
	Streptococcus	*suis*	(ex Elliot, 1966) Kilper-Bälz and Schleifer 1987
	Streptococcus	*thermophilus**	Orla-Jensen, 1919
	Streptococcus	*thoraltensis*	Devriese *et al*., 1997
	Streptococcus	*uberis*	Diernhofer, 1932
	Streptococcus	*urinalis*	Collins *et al*., 2000
	Streptococcus	*vestibularis*	Whiley and Hardie, 1988
	Streptococcus	*waius*	Flint *et al*., 1999
	Lactococcus	*garvieae*	(Collins *et al*., 1984) Schleifer *et al*., 1986
	Lactococcus	*lactis* subsp. *cremoris**	(Orla-Jensen, 1919) Schleifer *et al*., 1986
	Lactococcus	*lactis* subsp. *hordniae*	(ex Latorre-Guzman *et al*., 1977) Schleifer *et al*., 1986
	Lactococcus	*lactis* subsp. *lactis**	(Lister, 1873) Schleifer *et al*., 1986
	Lactococcus	*piscium*	Williams *et al*., 1990
	Lactococcus	*plantarum*	(Collins *et al*., 1984) Schleifer *et al*., 1986
Incertae sedis	*Acetoanaerobium*	*noterae*	Sleat *et al*., 1985
	Oscillospira	*guilliermondii*	Chatton and Perad, 1913

* These species are widely used in industry as starter cultures.

Appendix C - Multiple *pheS* gene sequence alignment for LAB isolated from natural sorghum fermentations and type strains (Chapter 3). Sequences for isolates from sorghum slurries are indicated in bold.



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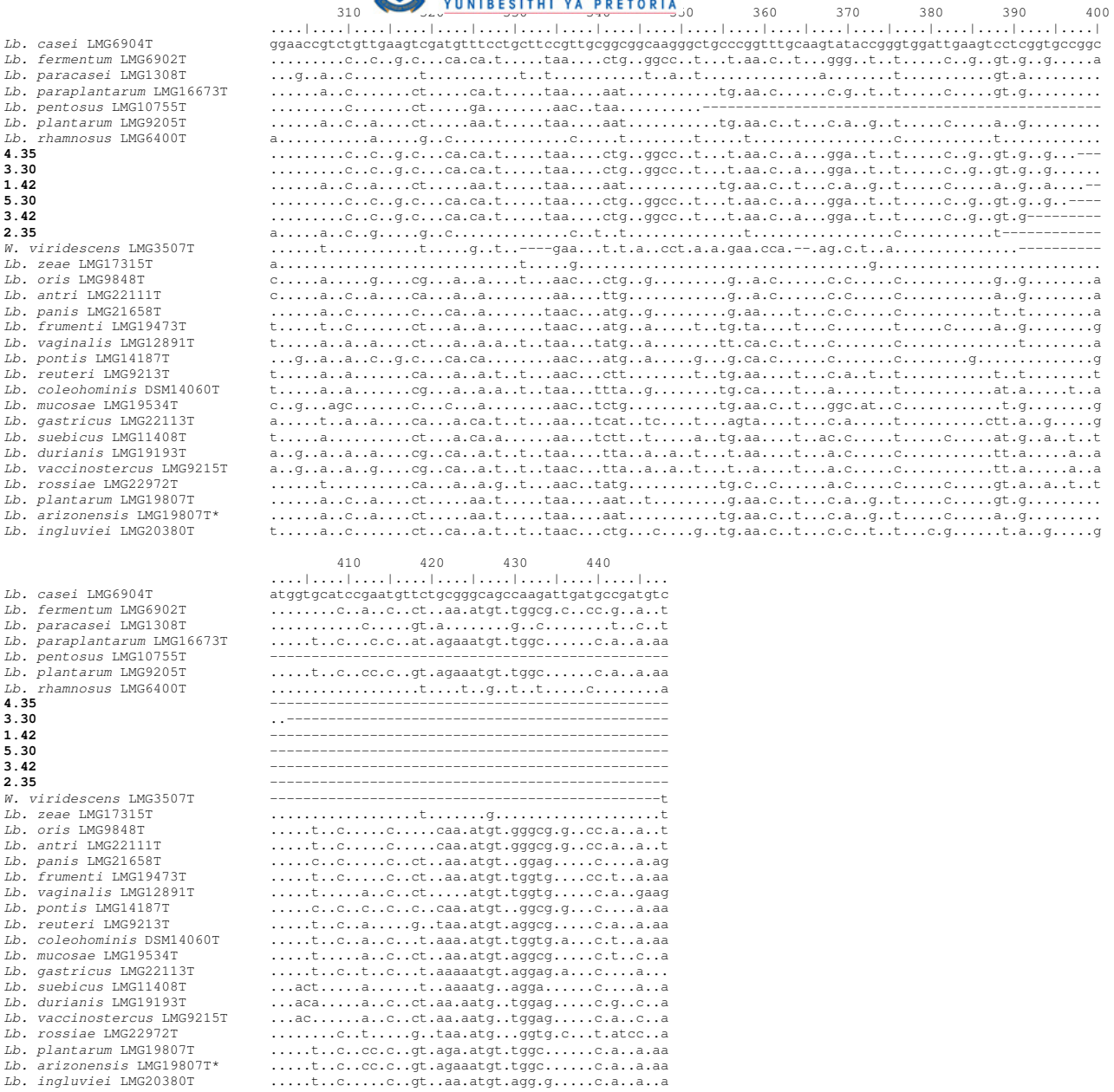
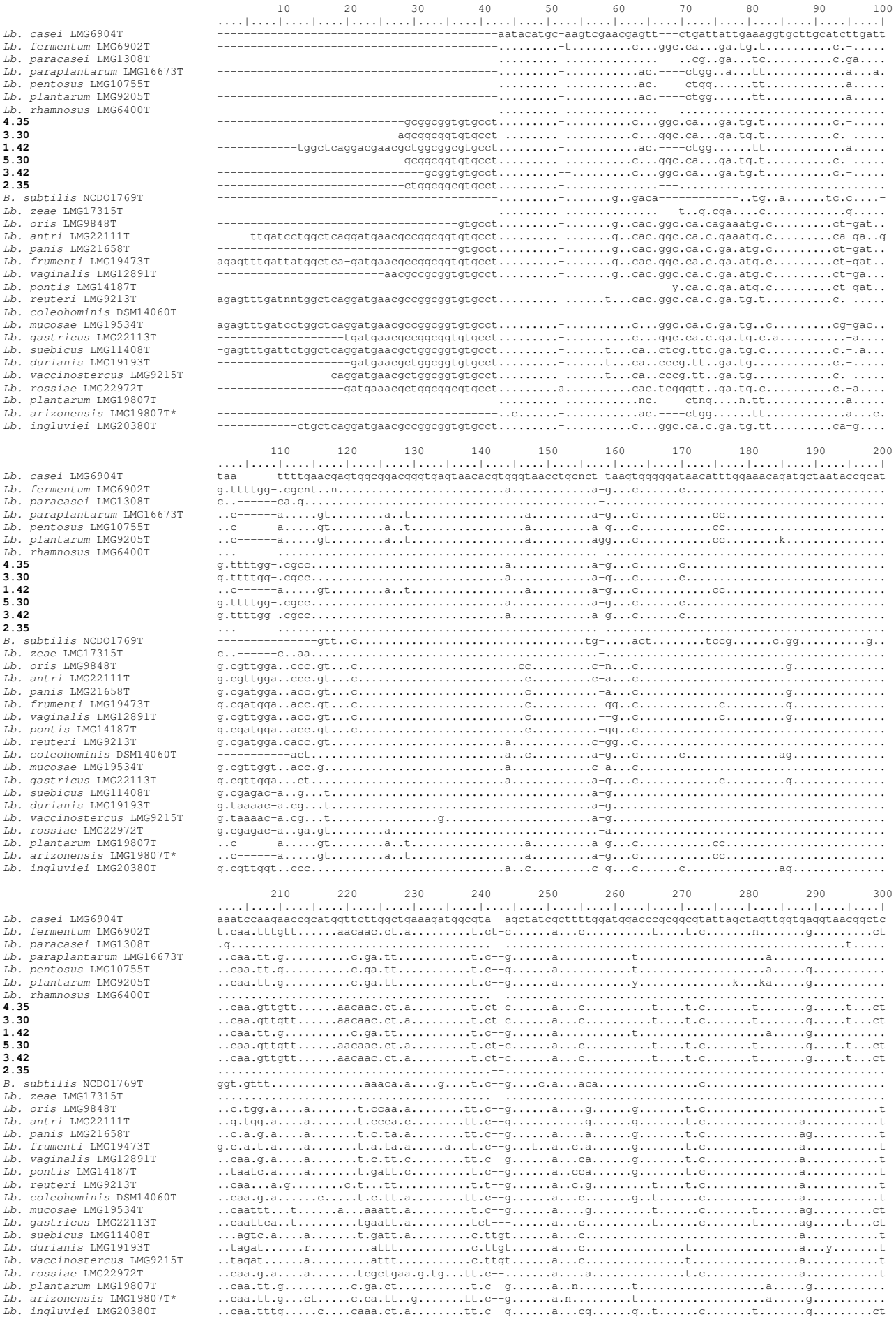


Appendix D - Multiple 16S rRNA gene sequence alignment for LAB isolated from natural sorghum fermentations and type strains (Chapter 3). Sequences for isolates from sorghum slurries are indicated in bold.



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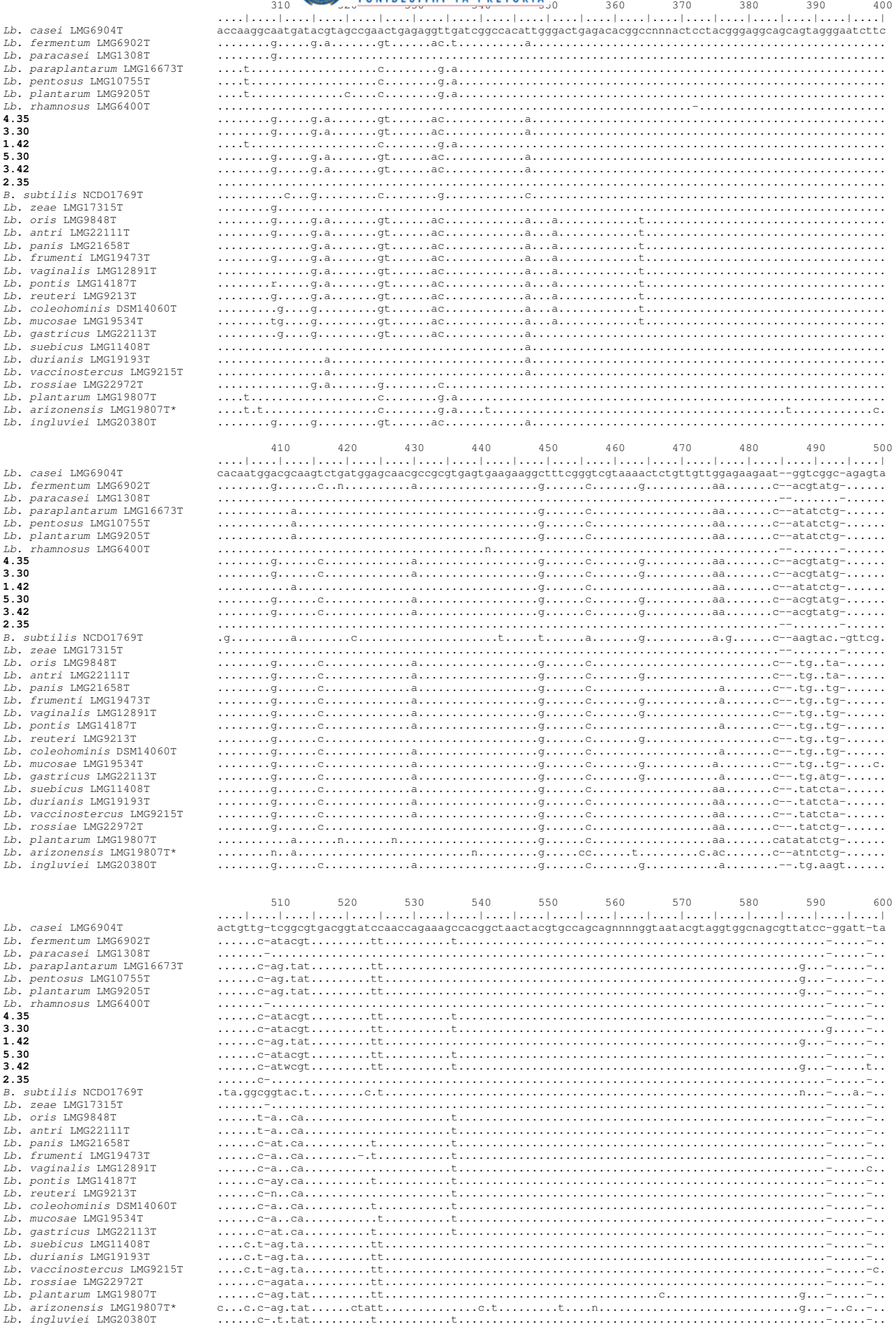

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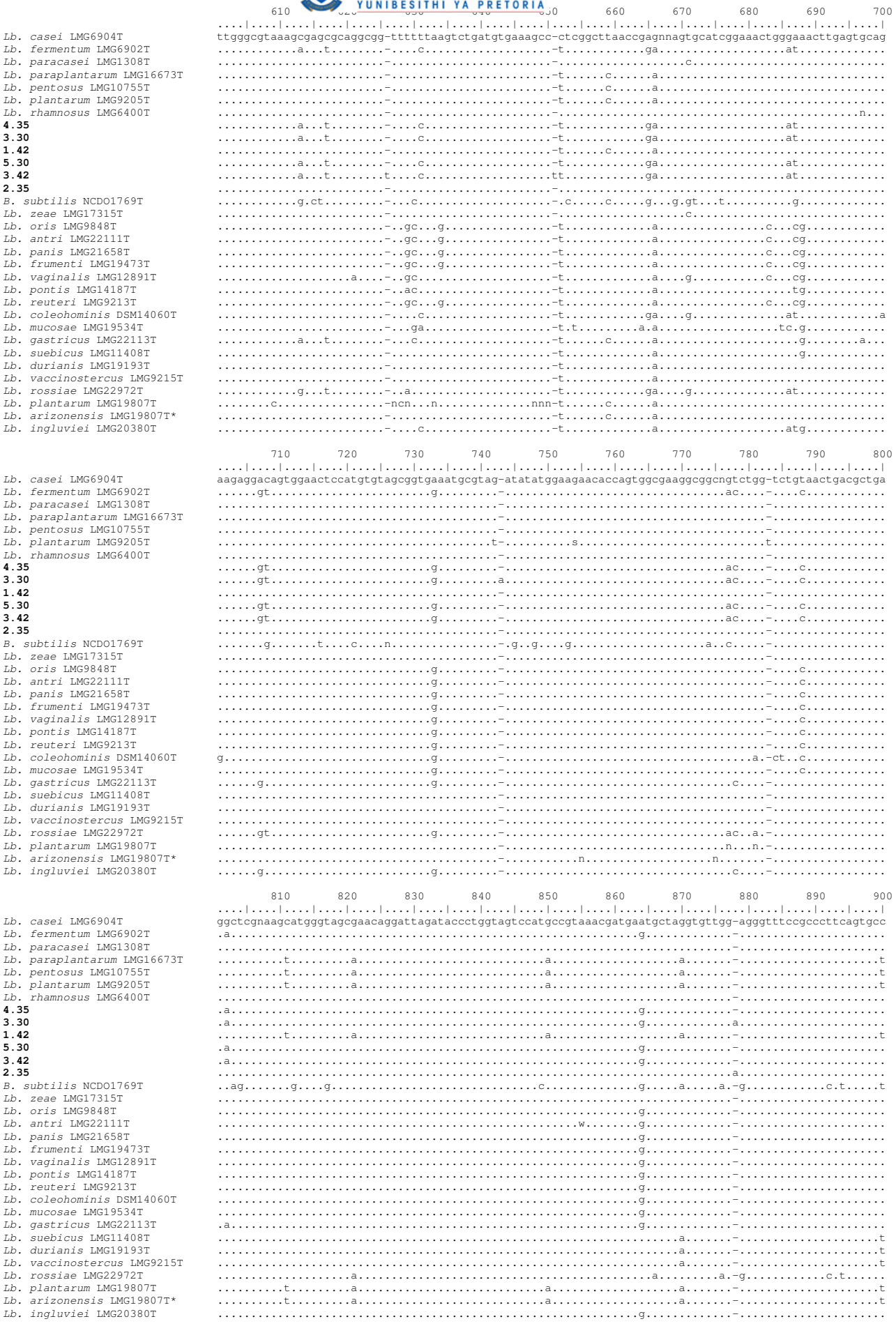

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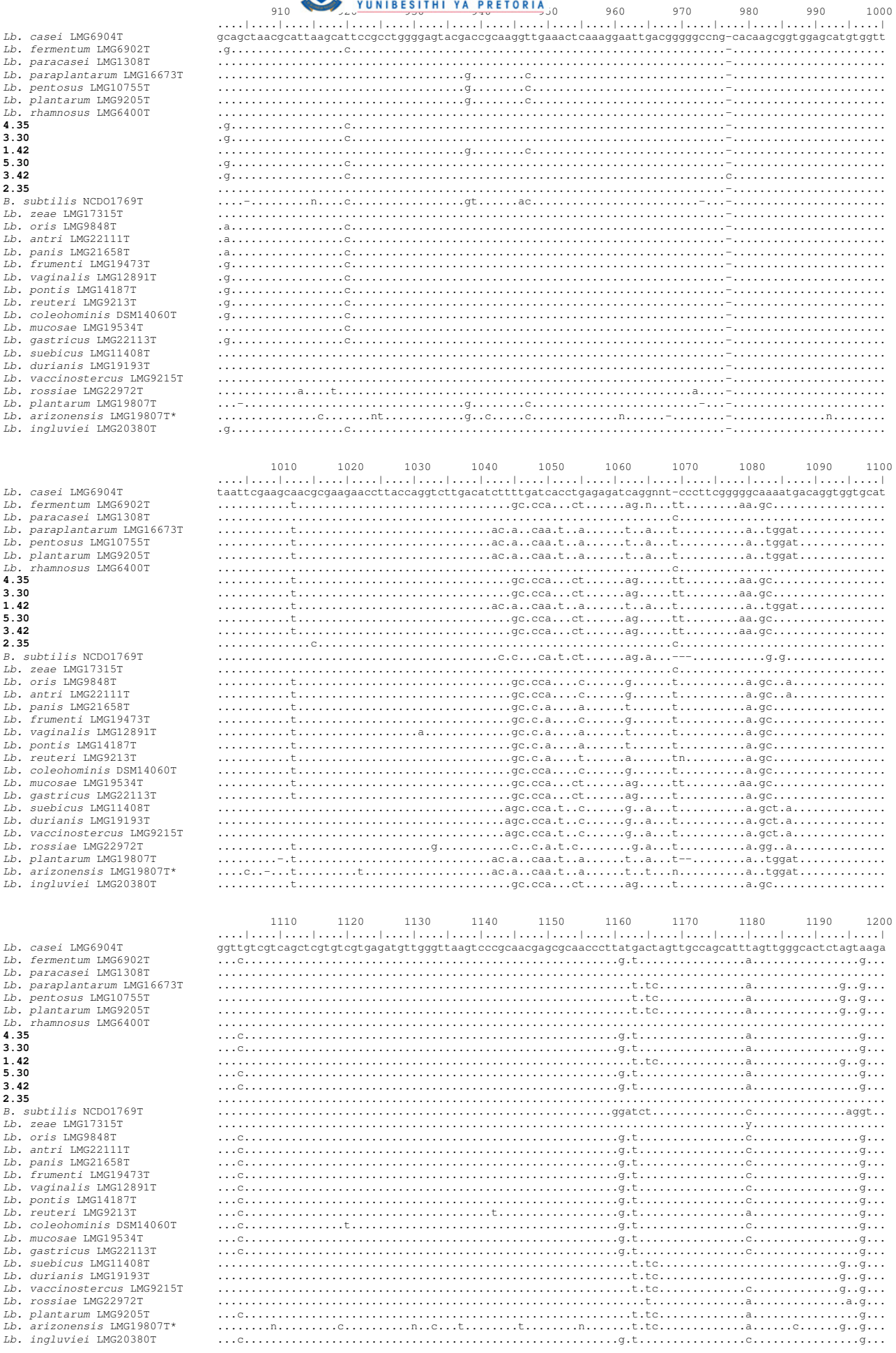

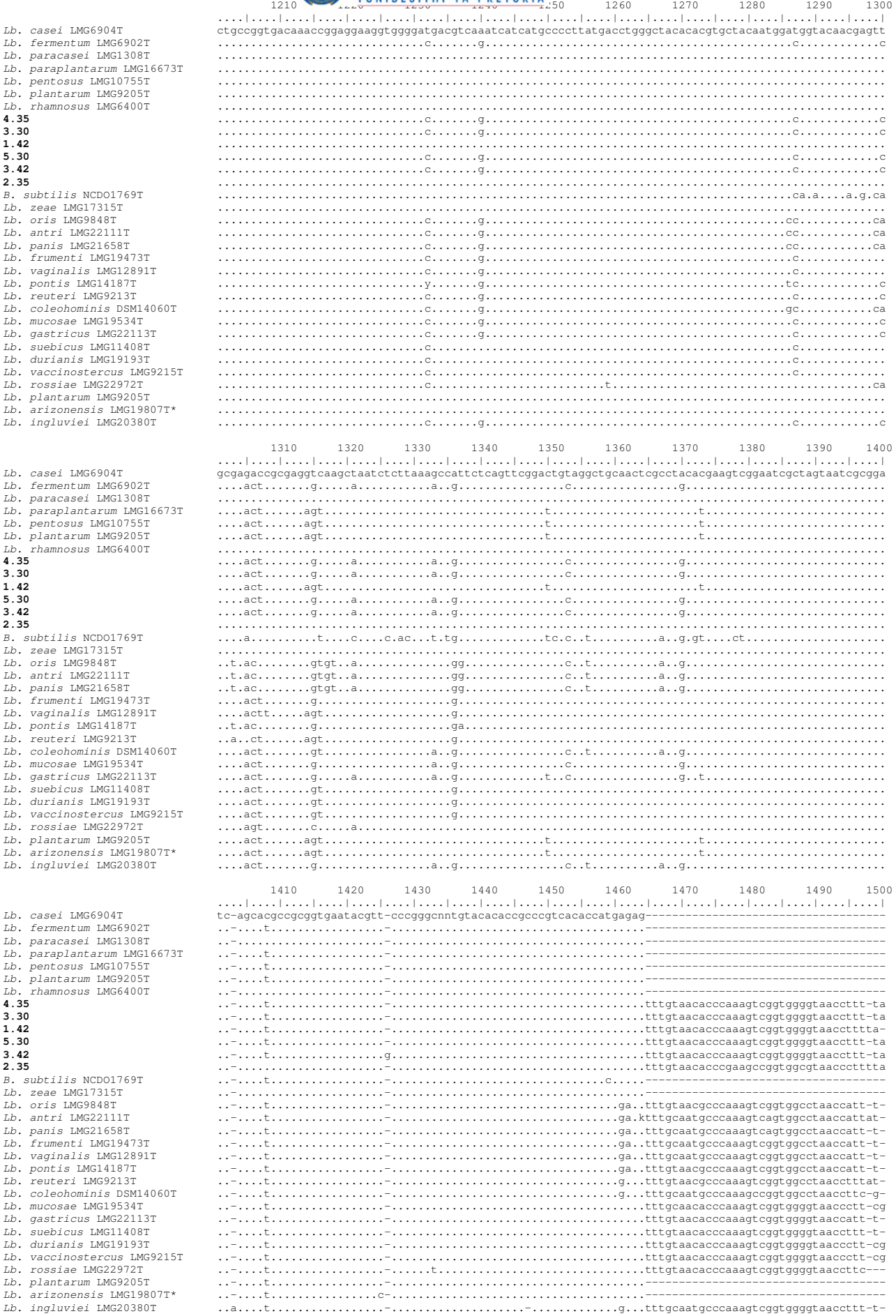
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Lb. fermentum LMG6902T .....c.....g.....c.....c
Lb. paracasei LMG1308T
Lb. paraplantarum LMG16673T
Lb. pentosus LMG10755T
Lb. plantarum LMG9205T
Lb. rhamnosus LMG6400T
4.35 .....c.....g.....c.....c
3.30 .....c.....g.....c.....c
1.42
5.30 .....c.....g.....c.....c
3.42 .....c.....g.....c.....c
2.35
B. subtilis NCDO1769T .....ca.a....a.g.ca
Lb. zeae LMG17315T
Lb. oris LMG9848T .....c.....g.....cc.....ca
Lb. antri LMG22111T .....c.....g.....cc.....ca
Lb. panis LMG21658T .....c.....g.....cc.....ca
Lb. frumenti LMG19473T .....c.....g.....c.....
Lb. vaginalis LMG12891T .....c.....g.....c.....
Lb. pontis LMG14187T .....y.....g.....tc.....c
Lb. reuteri LMG9213T .....c.....g.....c.....c
Lb. coleohominis DSM14060T .....c.....g.....gc.....ca
Lb. mucosae LMG19534T .....c.....g.....c.....c
Lb. gastricus LMG22113T .....c.....g.....c.....c
Lb. suebicus LMG11408T .....c.....c.....
Lb. durianis LMG19193T .....c.....c.....
Lb. vaccinostercus LMG9215T .....c.....c.....
Lb. rossiae LMG22972T .....c.....t.....ca
Lb. plantarum LMG9205T
Lb. arizonensis LMG19807T* .....
Lb. ingluviei LMG20380T .....c.....g.....c.....c
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
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Lb. fermentum LMG6902T ...act.....g.....a.....a..g.....c.....g.....
Lb. paracasei LMG1308T
Lb. paraplantarum LMG16673T ...act.....agt.....t.....t.....
Lb. pentosus LMG10755T ...act.....agt.....t.....t.....
Lb. plantarum LMG9205T ...act.....agt.....t.....t.....
Lb. rhamnosus LMG6400T
4.35 ...act.....g.....a.....a..g.....c.....g.....
3.30 ...act.....g.....a.....a..g.....c.....g.....
1.42 ...act.....agt.....t.....t.....
5.30 ...act.....g.....a.....a..g.....c.....g.....
3.42 ...act.....g.....a.....a..g.....c.....g.....
2.35
B. subtilis NCDO1769T ...a.....t....c....c.ac...t.tg.....tc.c..t.....a..g.gt....ct.....
Lb. zeae LMG17315T
Lb. oris LMG9848T ..t.ac.....gtgt..a.....gg.....c..t.....a..g.....
Lb. antri LMG22111T ..t.ac.....gtgt..a.....gg.....c..t.....a..g.....
Lb. panis LMG21658T ..t.ac.....gtgt..a.....gg.....c..t.....a..g.....
Lb. frumenti LMG19473T ...act.....g.....g.....
Lb. vaginalis LMG12891T ...actt.....agt.....g.....
Lb. pontis LMG14187T ..t.ac.....g.....ga.....
Lb. reuteri LMG9213T ..a..ct.....agt.....g.....
Lb. coleohominis DSM14060T ...act.....gt.....a..g.....c..t.....a..g.....
Lb. mucosae LMG19534T ...act.....g.....a..g.....c.....g.....
Lb. gastricus LMG22113T ...act.....g.....a.....a..g.....t..c.....g..t.....
Lb. suebicus LMG11408T ...act.....gt.....g.....
Lb. durianis LMG19193T ...act.....gt.....g.....
Lb. vaccinostercus LMG9215T ...act.....gt.....g.....
Lb. rossiae LMG22972T ...agt.....c.....a.....
Lb. plantarum LMG9205T ...act.....agt.....t.....t.....
Lb. arizonensis LMG19807T* ...act.....agt.....t.....t.....
Lb. ingluviei LMG20380T ...act.....g.....a..g.....c..t.....a..g.....
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
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Lb. paracasei LMG1308T ..-.....-.....----------
Lb. paraplantarum LMG16673T ..-....t.....-.....----------
Lb. pentosus LMG10755T ..-....t.....-.....----------
Lb. plantarum LMG9205T ..-....t.....-.....----------
Lb. rhamnosus LMG6400T ..-.....-.....----------
4.35 ..-....t.....-.....tttgtaacacccaaagtcggtggggtaacctttta
3.30 ..-....t.....-.....tttgtaacacccaaagtcggtggggtaacctttta
1.42 ..-....t.....-.....tttgtaacacccaaagtcggtggggtaacctttta
5.30 ..-....t.....-.....tttgtaacacccaaagtcggtggggtaacctttta
3.42 ..-....t.....g.....tttgtaacacccaaagtcggtggggtaacctttta
2.35 ..-.....-.....tttgtaacacccgaagccggtggcgtaacccttta
B. subtilis NCDO1769T ..-....t.....-.....c.....----------
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Lb. oris LMG9848T ..-....t.....-.....ga..tttgtaacgcccaaagtcggtggcctaaccatt-t
Lb. antri LMG22111T ..-....t.....-.....ga.ktttgcaatgcccaaagtcagtggcctaaccattat
Lb. panis LMG21658T ..-....t.....-.....ga..tttgcaatgcccaaagtcagtggcctaaccatt-t
Lb. frumenti LMG19473T ..-....t.....-.....ga..tttgcaatgcccaaagtcggtggcctaaccatt-t
Lb. vaginalis LMG12891T ..-....t.....-.....ga..tttgcaatgcccaaagtcggtggcctaaccatt-t
Lb. pontis LMG14187T ..-....t.....-.....ga..tttgtaacgcccaaagtcggtggcctaaccatt-t
Lb. reuteri LMG9213T ..-....t.....-.....g...tttgtaacgcccaaagtcggtggcctaacctttat
Lb. coleohominis DSM14060T ..-....t.....-.....g...tttgcaatgcccaaagccggtggcctaaccttc-g
Lb. mucosae LMG19534T ..-....t.....-.....tttgcaacacccaaagtcggtggggtaaccctt-cg
Lb. gastricus LMG22113T ..-....t.....-.....tttgtaacacccaaagtcggtggggtaaccatt-t
Lb. suebicus LMG11408T ..-....t.....-.....tttgtaacacccaaagtcggtggggtaaccttt-t
Lb. durianis LMG19193T ..-....t.....-.....tttgtaacacccaaagtcggtggggtaaccctt-cg
Lb. vaccinostercus LMG9215T ..-....t.....-.....tttgtaacacccaaagtcggtggggtaaccctt-cg
Lb. rossiae LMG22972T ..-....t.....-.....t.....tttgtaacacccaaagtcggtggggtaaccttc---
Lb. plantarum LMG9205T ..-....t.....-.....----------
Lb. arizonensis LMG19807T* ..-....t.....c-.....----------
Lb. ingluviei LMG20380T ..a....t.....-.....-.....g...tttgcaatgcccaaagtcggtggggtaaccttt-t

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                                1510      1520      1530      1540      1550      1560      1570      1580      1590
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
Lb. casei LMG6904T              ----------------------------------------------------------------------------------------
Lb. fermentum LMG6902T          ----------------------------------------------------------------------------------------
Lb. paracasei LMG1308T          ----------------------------------------------------------------------------------------
Lb. paraplantarum LMG16673T     ----------------------------------------------------------------------------------------
Lb. pentosus LMG10755T          ----------------------------------------------------------------------------------------
Lb. plantarum LMG9205T          ----------------------------------------------------------------------------------------
Lb. rhamnosus LMG6400T          ----------------------------------------------------------------------------------------
4.35                            ggagccag-ccgcctaaggtgga-----------------------------------------------------------------
3.30                            ggagccatgccgcctaaggtggacag--------------------------------------------------------------
1.42                            -ggaaccagccgcctaaggtgggacagatgattagggtgaagtcgtaacaaggtagccgtaggagaacctgcggct-----------------
5.30                            ggagccagccgccctaaggtgacaga---------------------------------------------------------------
3.42                            ggagccatgccgcctaaggtggacaga--------------------------------------------------------------
2.35                            gggagcgagccgtctaaggg---------------------------------------------------------------------
B. subtilis NCDO1769T           ----------------------------------------------------------------------------------------
Lb. zeae LMG17315T              ----------------------------------------------------------------------------------------
Lb. oris LMG9848T               -ggagggagccgcctaaggcgggacagatgactggggtgaagtcgtaacaaggtagccgaggaggaacct-----------------------
Lb. antri LMG22111T             -ggagggasctgcctaaggcagggcagatgactggggtgaagtcgt-------------------------------------------
Lb. panis LMG21658T             -ggagggagctgcctaaggcaggacagatgactggggtgaagtcgtaa-----------------------------------------
Lb. frumenti LMG19473T          -ggagggaaccgcctaaggcagggcagatgactggggtgaagtcgtaacaaggtagccgtaggagaacctgcggctggatcacct-------
Lb. vaginalis LMG12891T         -ggagggaaccgcttaaggcagggcagatgactggggtgaagtcgtaacaagg-agctgtaggagaacctgcggctggatcacctcctt---
Lb. pontis LMG14187T            -ggagggagccgcctaaggcgggacagatgactggggtgaagtcgtaacaaggtagccgtaggagaacc------------------------
Lb. reuteri LMG9213T            -ggagggagccgcctaaggcgggacagatgactggggtgaagtcgtaacaaggtagccgtaggagagcctgcggctggatcacctcctttnt
Lb. coleohominis DSM14060T      -ggaaggagccgtctaaggcagg-----------------------------------------------------------------
Lb. mucosae LMG19534T           gggagctagccgcctaaggtggggcagatgattagggtgaagtcgtaacaaggtagccgtaggagaacctgcggctggatcacctcct----
Lb. gastricus LMG22113T         -ggagccagccgcctaaggtgggacagatgattagggtgaagtcgtaacaaggtagccgtaggagaacctgcggctggatcacctcctttct
Lb. suebicus LMG11408T          aggagccagccgcctaaggtgggacagatgattagggtgaagtcgtaacaaggtagccgtaggagaacctgcggctggatcacctct-----
Lb. durianis LMG19193T          gggagccagccgcctaaggtgggacagatgattagggtgaagtcgtaacaaggtagccgtaggagaacc------------------------
Lb. vaccinostercus LMG9215T     gggagccagccgcctaaggtgggacagatgattagggtgaagtcgtaacaaggtagccgtaggag-------------------------
Lb. rossiae LMG22972T           gggagccaaccgcctaaggtgggacagatgattagggtg--------------------------------------------------
Lb. plantarum LMG9205T          ----------------------------------------------------------------------------------------
Lb. arizonensis LMG19807T*      ----------------------------------------------------------------------------------------
Lb. ingluviei LMG20380T         -ggagccagccg-ctaaggcagggcagatgactgggacaa-------------------------------------------------
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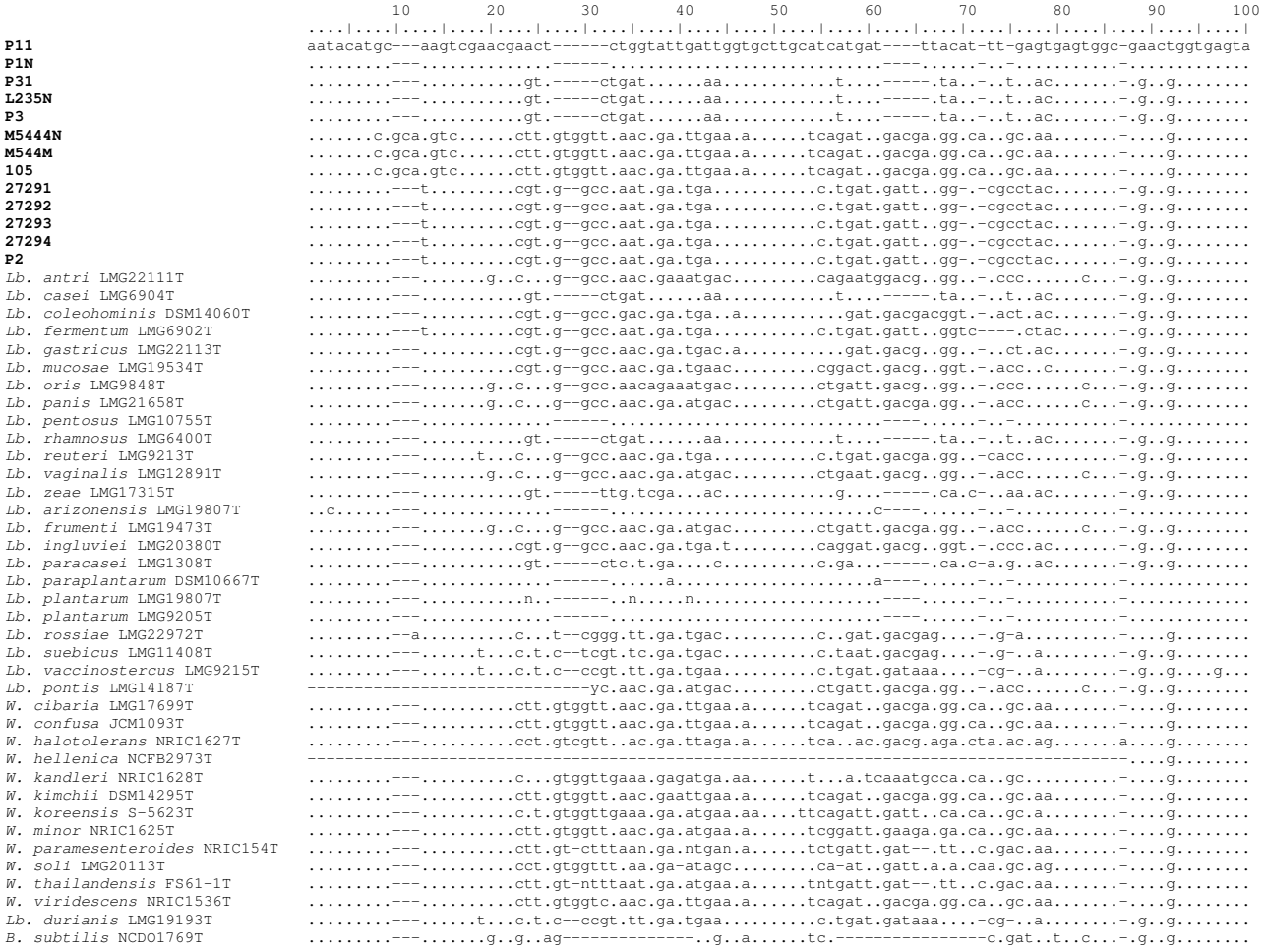
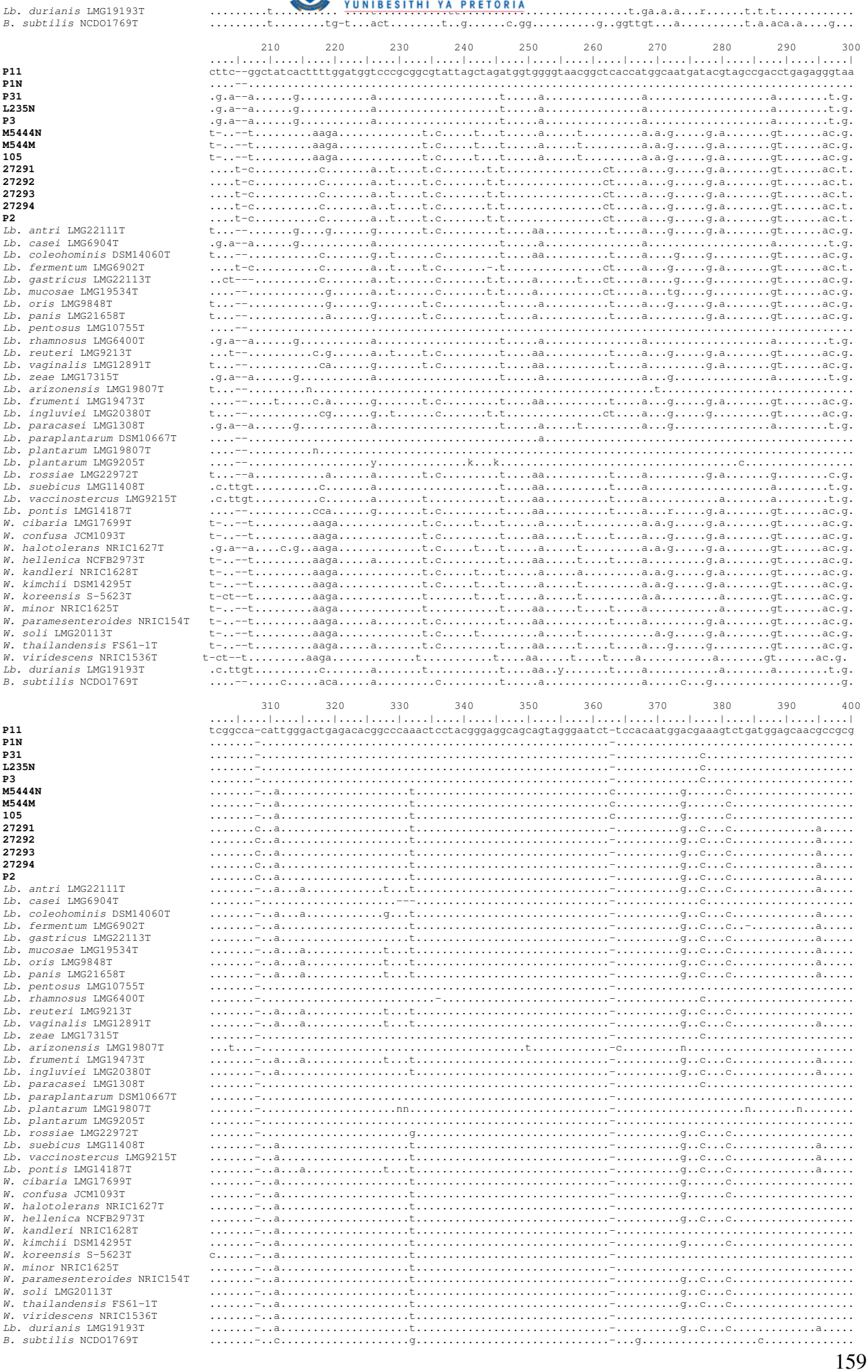
Appendix E - Multiple 16S rRNA gene sequence alignment for *Lactobacillus* and *Weissella* species isolated from natural sorghum fermentations and type strains (Chapter 4). Sequences for isolates from sorghum slurries are indicated in bold.

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B. subtilis NCDO1769T
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P11
P1N
P31
L235N
P3
M5444N
M544M
105
27291
27292
27293
27294
P2
Lb. antri LMG22111T
Lb. casei LMG6904T
Lb. coleohominis DSM14060T
Lb. fermentum LMG6902T
Lb. gastricus LMG22113T
Lb. mucosae LMG19534T
Lb. oris LMG9848T
Lb. panis LMG21658T
Lb. pentosus LMG10755T
Lb. rhamnosus LMG6400T
Lb. reuteri LMG9213T
Lb. vaginalis LMG12891T
Lb. zeae LMG17315T
Lb. arizonensis LMG19807T
Lb. frumenti LMG19473T
Lb. ingluviei LMG20380T
Lb. paracasei LMG1308T
Lb. paraplantarum DSM10667T
Lb. plantarum LMG19807T
Lb. plantarum LMG9205T
Lb. rossiae LMG22972T
Lb. suebicus LMG11408T
Lb. vaccinostercus LMG9215T
Lb. pontis LMG14187T
W. cibaria LMG17699T
W. confusa JCM1093T
W. halotolerans NRIC1627T
W. hellenica NCFB2973T
W. kandleri NRIC1628T
W. kimchii DSM14295T
W. koreensis S-5623T
W. minor NRIC1625T
W. paramesenteroides NRIC154T
W. soli LMG20113T
W. thailandensis FS61-1T
W. viridescens NRIC1536T
Lb. durianis LMG19193T
B. subtilis NCDO1769T
310 320 330 340 350 360 370 380 390 400
P11
P1N
P31
L235N
P3
M5444N
M544M
105
27291
27292
27293
27294
P2
Lb. antri LMG22111T
Lb. casei LMG6904T
Lb. coleohominis DSM14060T
Lb. fermentum LMG6902T
Lb. gastricus LMG22113T
Lb. mucosae LMG19534T
Lb. oris LMG9848T
Lb. panis LMG21658T
Lb. pentosus LMG10755T
Lb. rhamnosus LMG6400T
Lb. reuteri LMG9213T
Lb. vaginalis LMG12891T
Lb. zeae LMG17315T
Lb. arizonensis LMG19807T
Lb. frumenti LMG19473T
Lb. ingluviei LMG20380T
Lb. paracasei LMG1308T
Lb. paraplantarum DSM10667T
Lb. plantarum LMG19807T
Lb. plantarum LMG9205T
Lb. rossiae LMG22972T
Lb. suebicus LMG11408T
Lb. vaccinostercus LMG9215T
Lb. pontis LMG14187T
W. cibaria LMG17699T
W. confusa JCM1093T
W. halotolerans NRIC1627T
W. hellenica NCFB2973T
W. kandleri NRIC1628T
W. kimchii DSM14295T
W. koreensis S-5623T
W. minor NRIC1625T
W. paramesenteroides NRIC154T
W. soli LMG20113T
W. thailandensis FS61-1T
W. viridescens NRIC1536T
Lb. durianis LMG19193T
B. subtilis NCDO1769T

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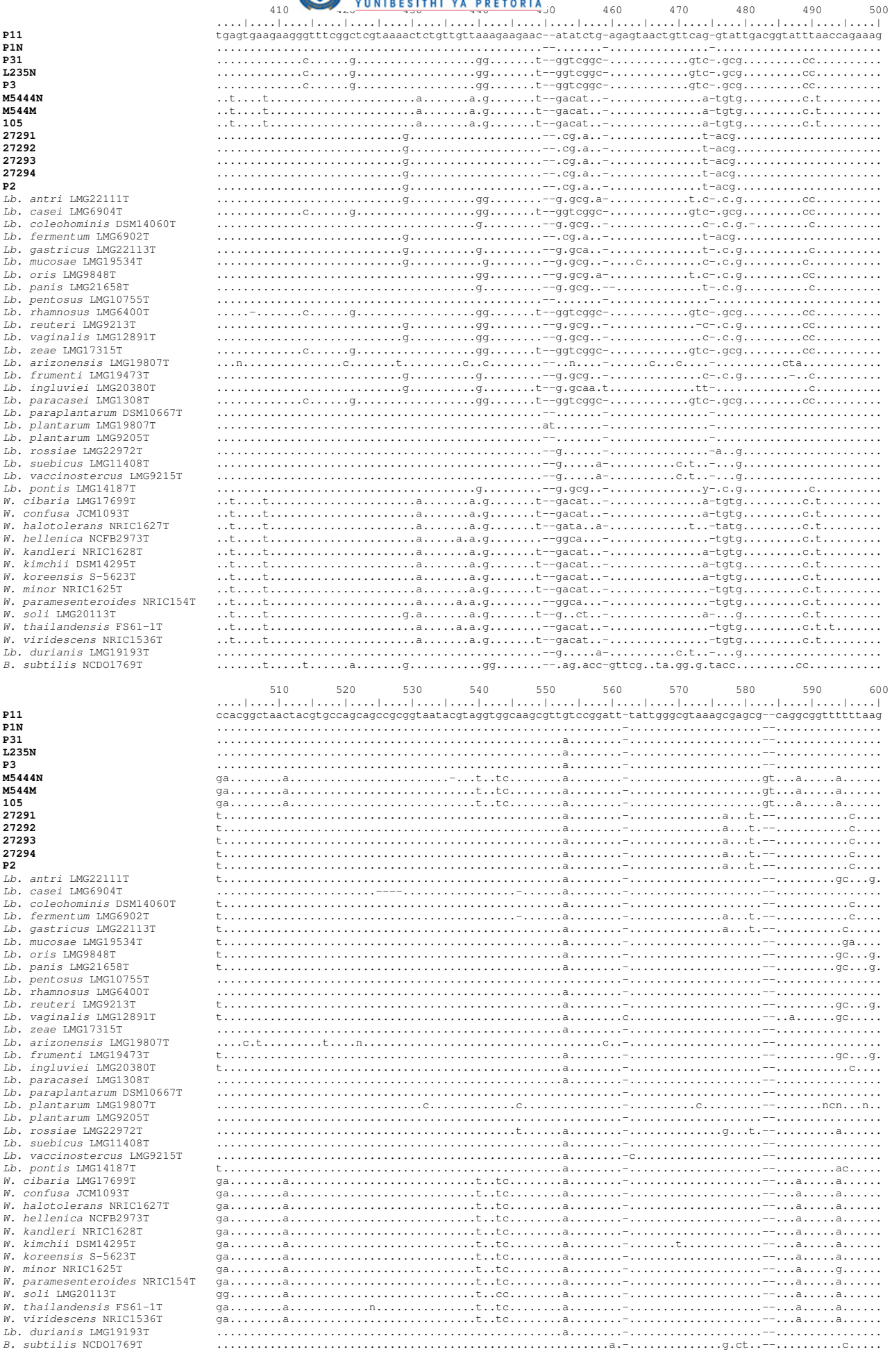

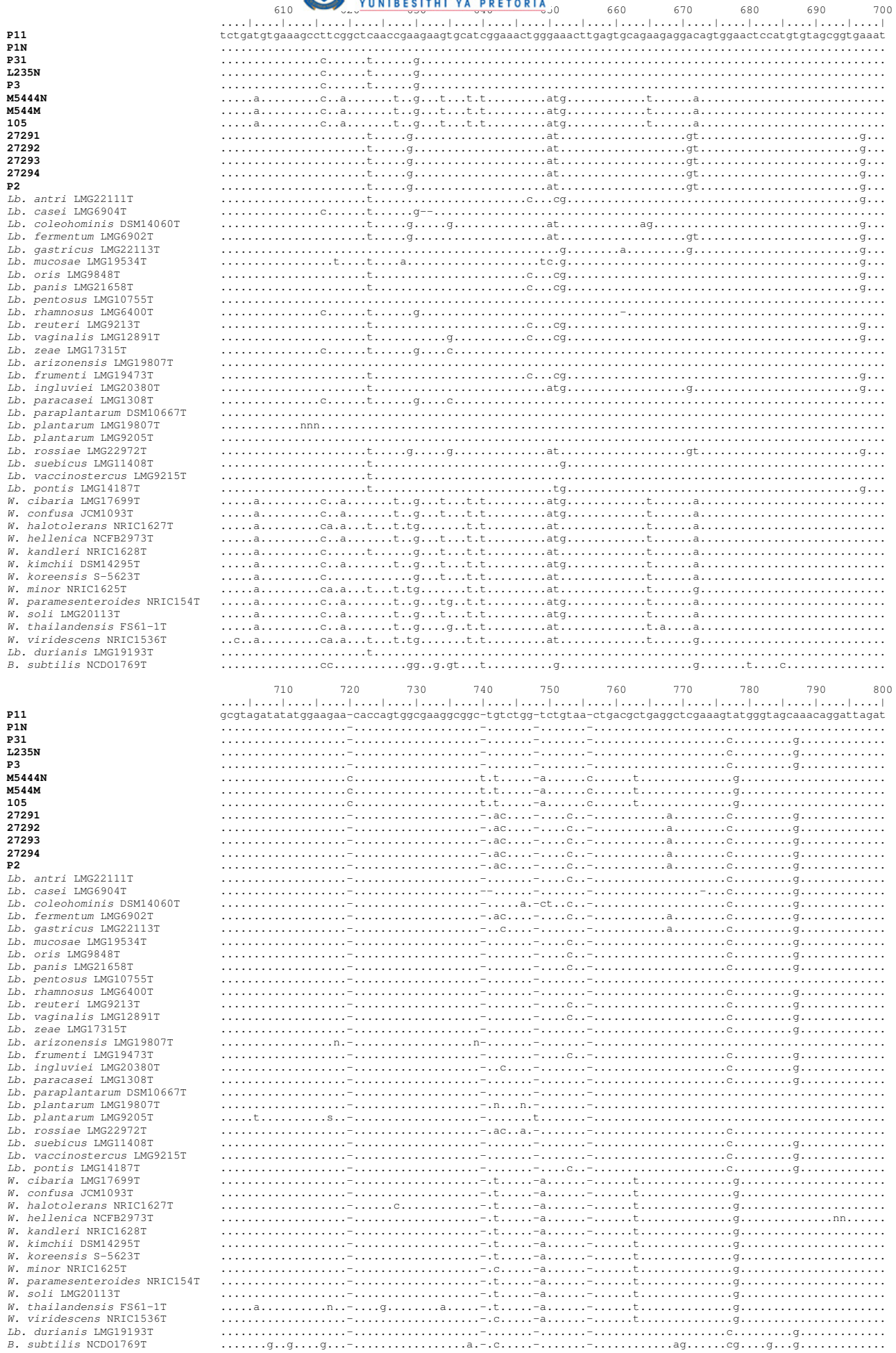
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710 720 730 740 750 760 770 780 790 800
P11 gcgtagatatatggaagaa-caccagtggcgaaggcggc-tgtctgg-tctgtaa-ctgacgctgaggctcgaaagtatgggtagcaaacaggattagat

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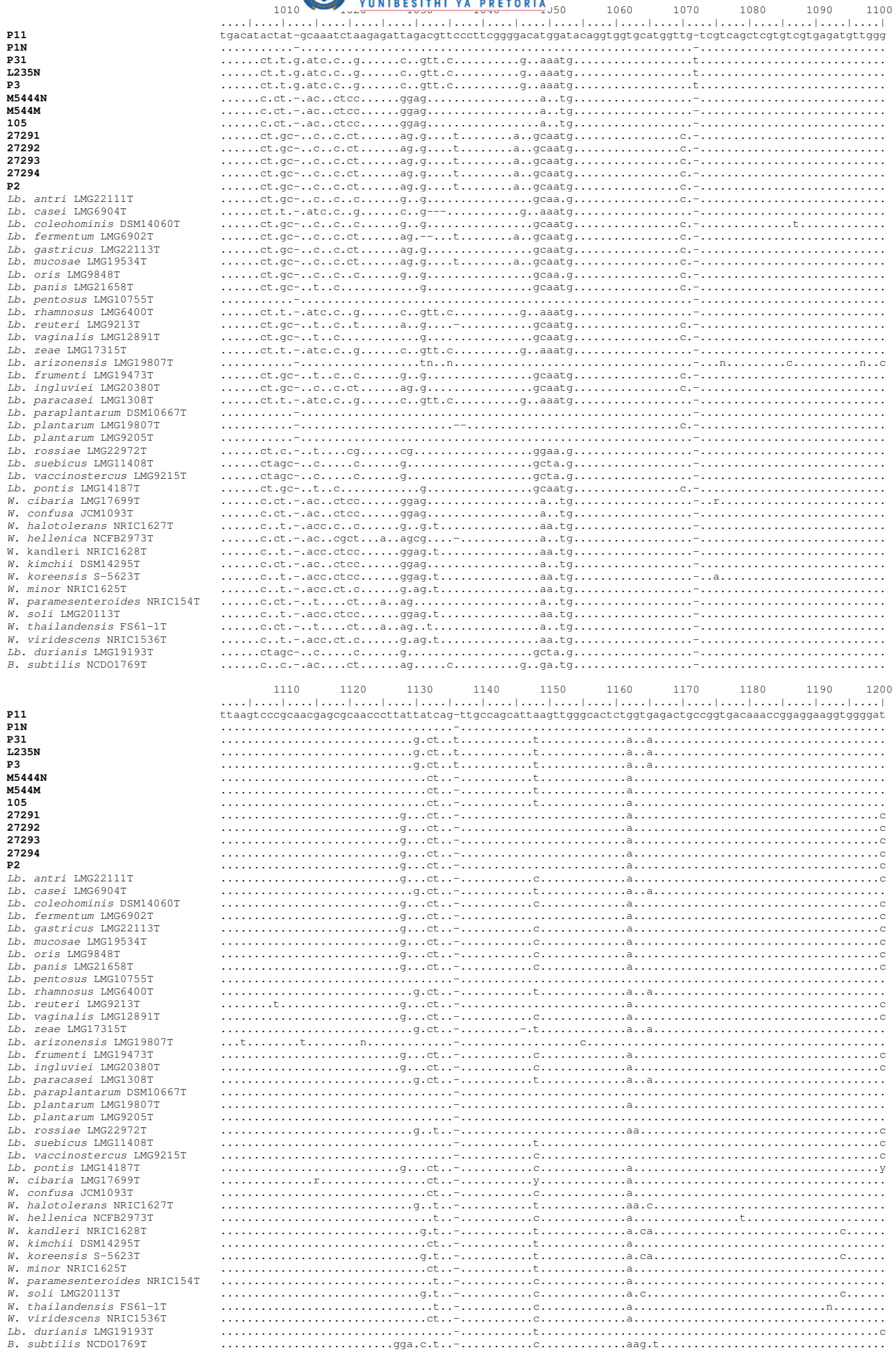

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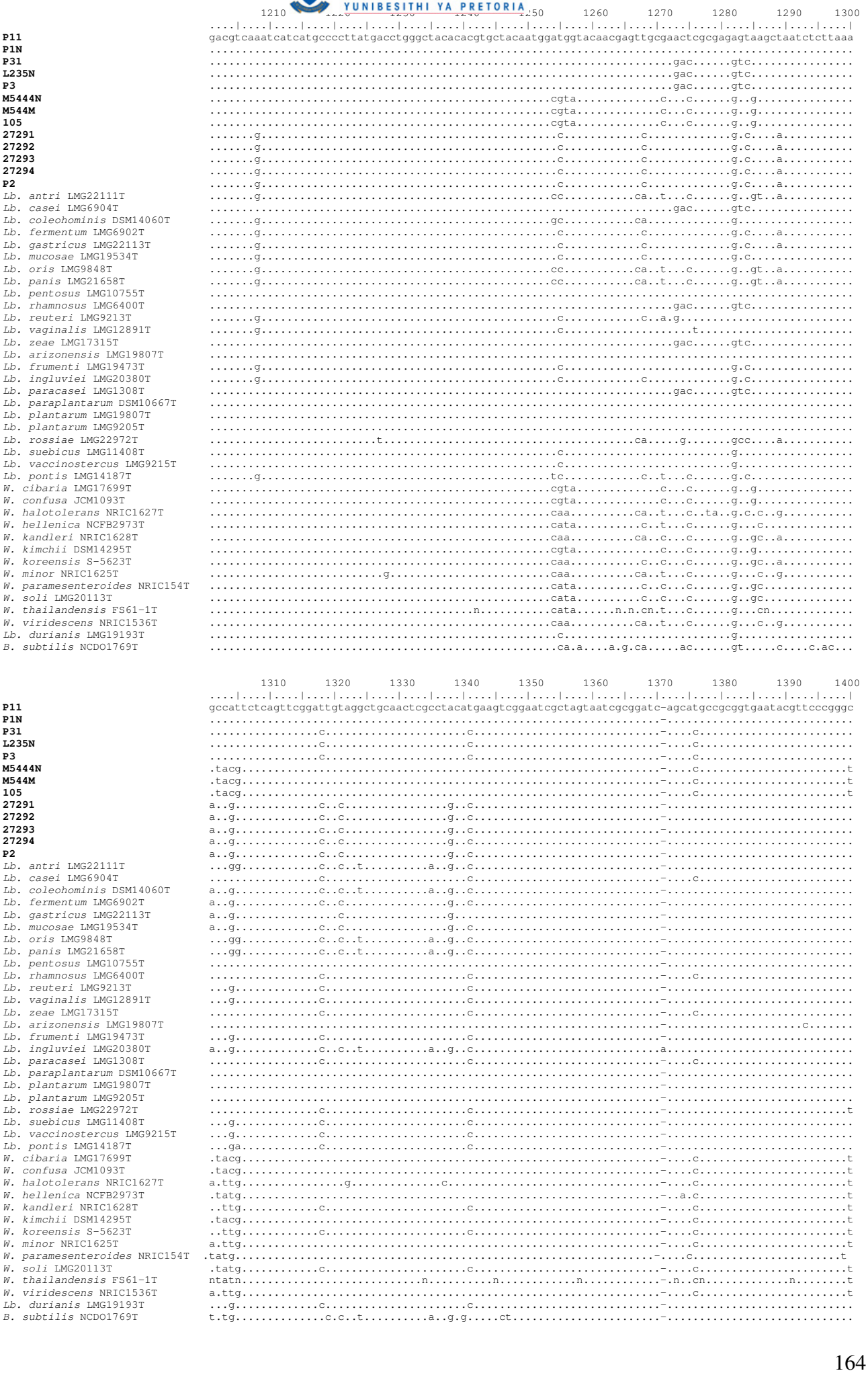

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                                        1410      1420      1430
                                        ....|....|....|....|....|....|...
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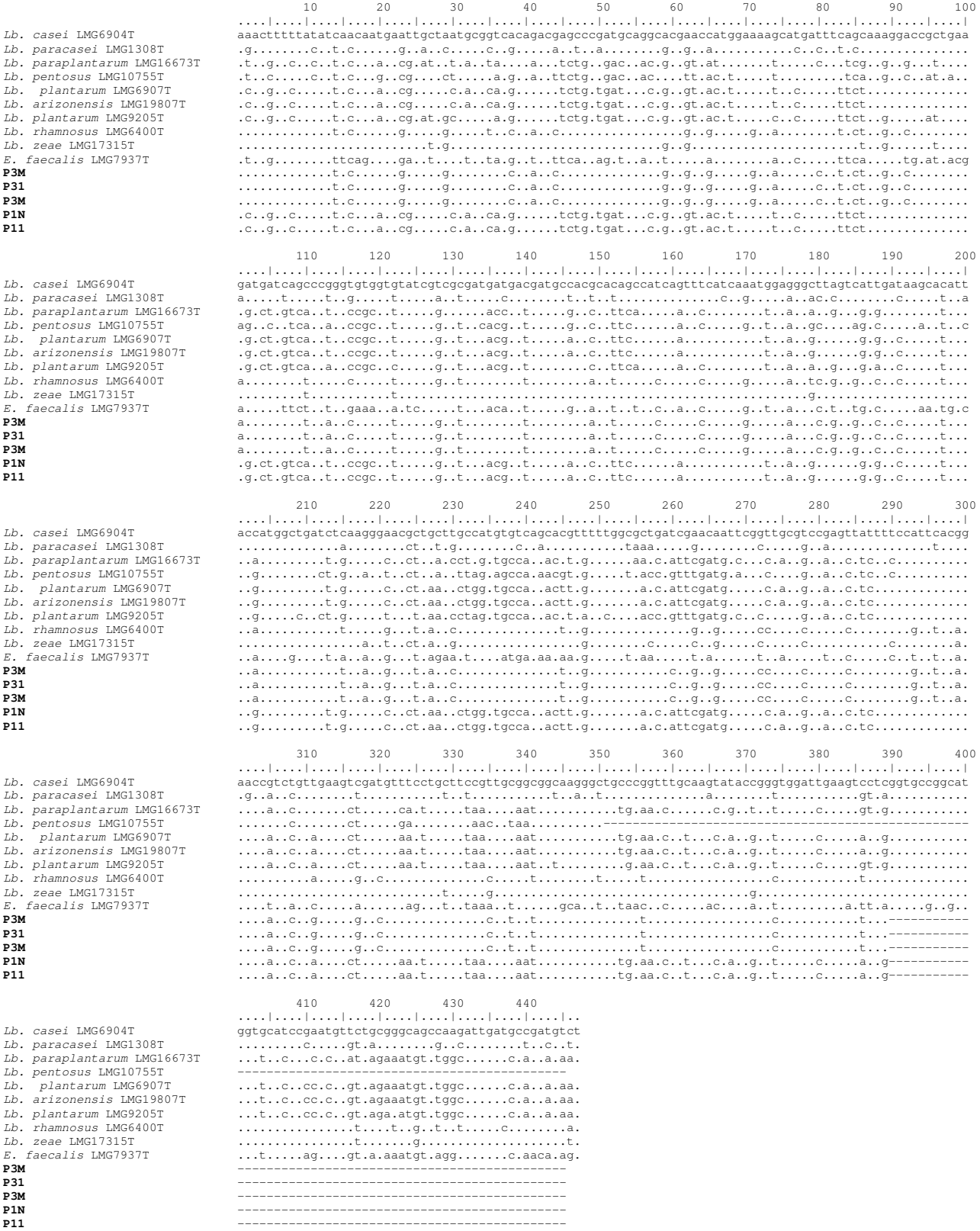
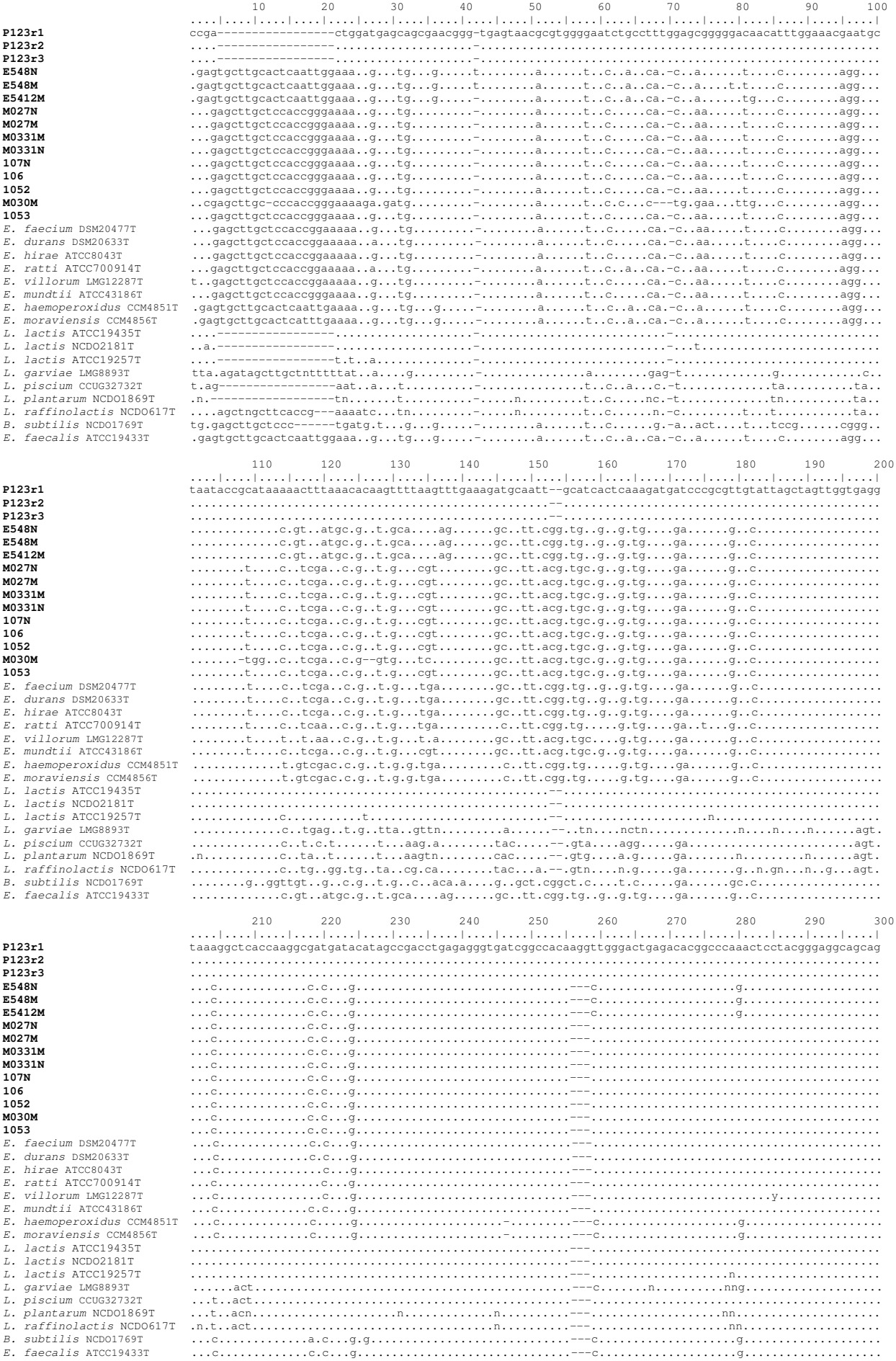
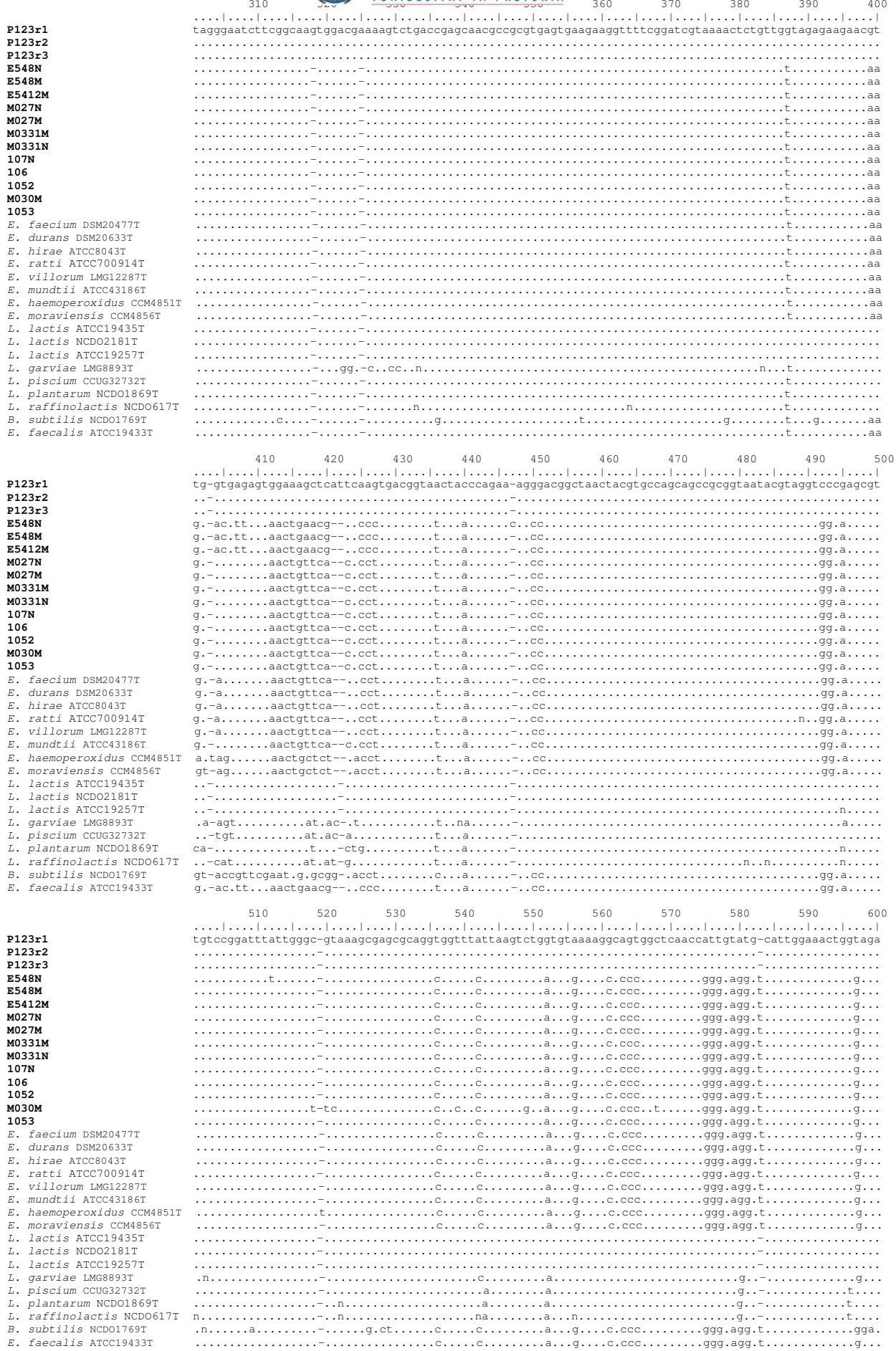
P1N                                     ................-....--..........
P31                                     ................-....--..........
L235N                                   ................-....--..........
P3                                      ................-....--..........
M5444N                                  ................t...gact.........
M544M                                   ................t...gact.........
105                                     ................t...gact.........
27291                                   ................-....--..........
27292                                   ................-....--..........
27293                                   ................-....--..........
27294                                   ................-....--..........
P2                                      ................-....--..........
Lb. antri LMG22111T                     ................-....--.......ga.
Lb. casei LMG6904T                      --...............-....--..........
Lb. coleohominis DSM14060T              ................-....--.......g..
Lb. fermentum LMG6902T                  ................-....--..........
Lb. gastricus LMG22113T                 ................-....--..........
Lb. mucosae LMG19534T                   ................-....--..........
Lb. oris LMG9848T                       ................-....--.......ga.
Lb. panis LMG21658T                     ................-....--.......ga.
Lb. pentosus LMG10755T                  ................-....--..........
Lb. rhamnosus LMG6400T                  ................-....--..........
Lb. reuteri LMG9213T                    ................-....--.......g..
Lb. vaginalis LMG12891T                 ................-....--.......ga.
Lb. zeae LMG17315T                      ................-....--..........
Lb. arizonensis LMG19807T               ................-....--..........
Lb. frumenti LMG19473T                  ................-....--.......ga.
Lb. ingluviei LMG20380T                 .............-..-....--.......g..
Lb. paracasei LMG1308T                  ................-....--..........
Lb. paraplantarum DSM10667T             ................-....--..........
Lb. plantarum LMG19807T                 ................-....--..........
Lb. plantarum LMG9205T                  ................-....--..........
Lb. rossiae LMG22972T                   ................-....--..........
Lb. suebicus LMG11408T                  ................-....--..........
Lb. vaccinostercus LMG9215T             ................-....--..........
Lb. pontis LMG14187T                    ................-....--.......ga.
W. cibaria LMG17699T                    ................-....--..........
W. confusa JCM1093T                     ................-....--..........
W. halotolerans NRIC1627T               ................-....--..........
W. hellenica NCFB2973T                  ................-....--..........
W. kandleri NRIC1628T                   ................-....--..........
W. kimchii DSM14295T                    ................-....--..........
W. koreensis S-5623T                    ................-....--..........
W. minor NRIC1625T                      ................-....--..........
W. paramesenteroides NRIC154T           ................-....--..........
W. soli LMG20113T                       ................-....--..........
W. thailandensis FS61-1T                ................-....--......----
W. viridescens NRIC1536T                ................-....--..........
Lb. durianis LMG19193T                  ................-....--..........
B. subtilis NCDO1769T                   ................-....--.....c....
```

Appendix F - Multiple *pheS* gene sequence alignment for *Lactobacillus* species isolated from spontaneous sorghum fermentations and type strains (Chapter 4). Sequences for isolates from fermented sorghum slurries are indicated in bold.



Appendix G - Multiple 16S rRNA gene sequence alignment for *Lactococcus* and *Enterococcus* species isolated from spontaneous sorghum fermentations and type strains (Chapter 4). Sequences for isolates from sorghum slurries are indicated in bold.

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P123r2
P123r3
E548N
E548M
E5412M
M027N
M027M
M0331M
M0331N
107N
106
1052
M030M
1053
E. faecium DSM20477T
E. durans DSM20633T
E. hirae ATCC8043T
E. ratti ATCC700914T
E. villorum LMG12287T
E. mundtii ATCC43186T
E. haemoperoxidus CCM4851T
E. moraviensis CCM4856T
L. lactis ATCC19435T
L. lactis NCDO2181T
L. lactis ATCC19257T
L. garviae LMG8893T
L. piscium CCUG32732T
L. plantarum NCDO1869T
L. raffinolactis NCDO617T
B. subtilis NCDO1769T
E. faecalis ATCC19433T
410 420 430 440 450 460 470 480 490 500
P123r1 tg-gtgagagtggaaagctcattcaagtgacggtaactacccagaa-agggacggctaactacgtgccagcagccgcggtaatacgtaggtcccgagcgt
P123r2
P123r3
E548N
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E. moraviensis CCM4856T
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L. lactis ATCC19257T
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L. piscium CCUG32732T
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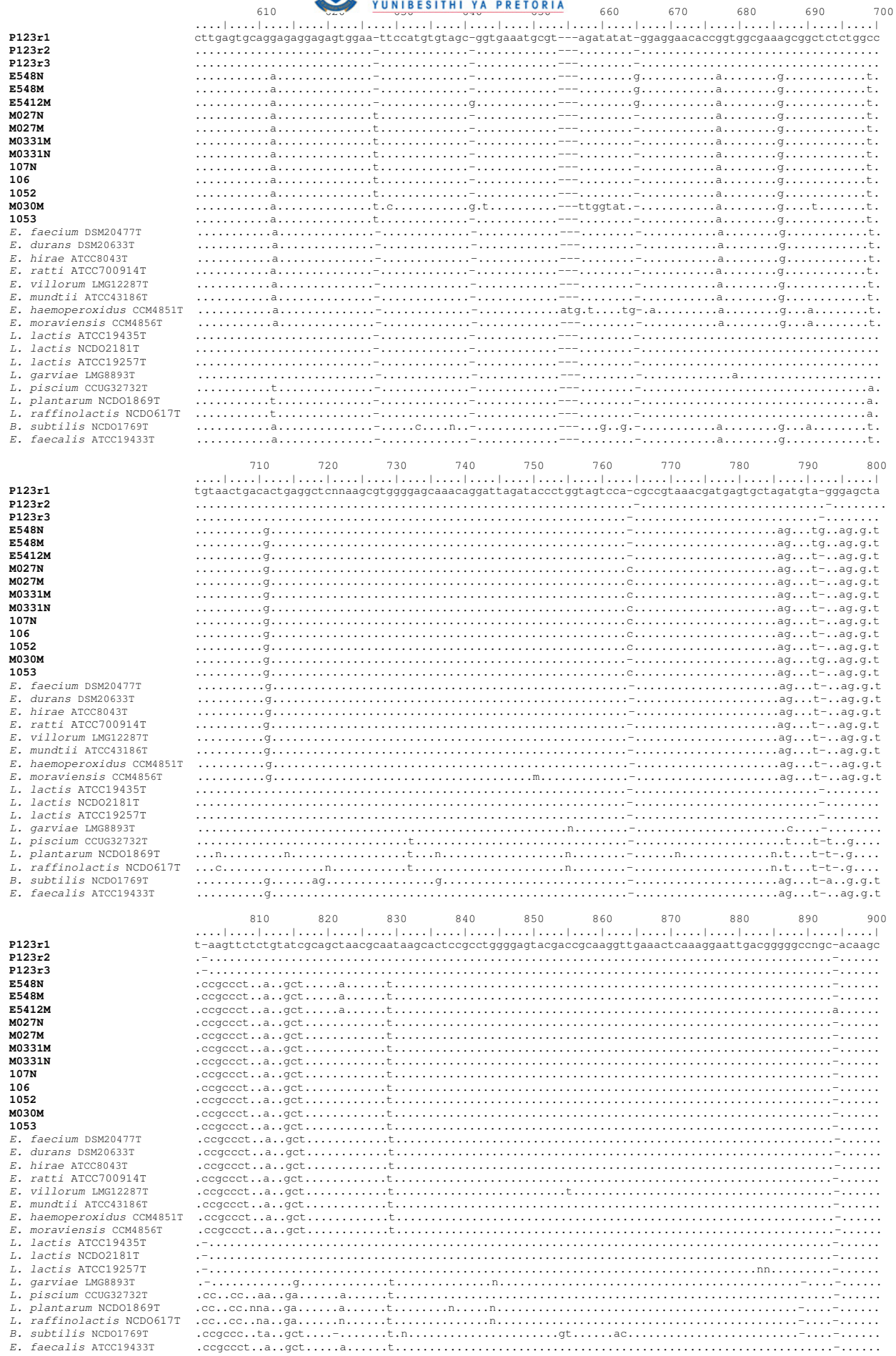
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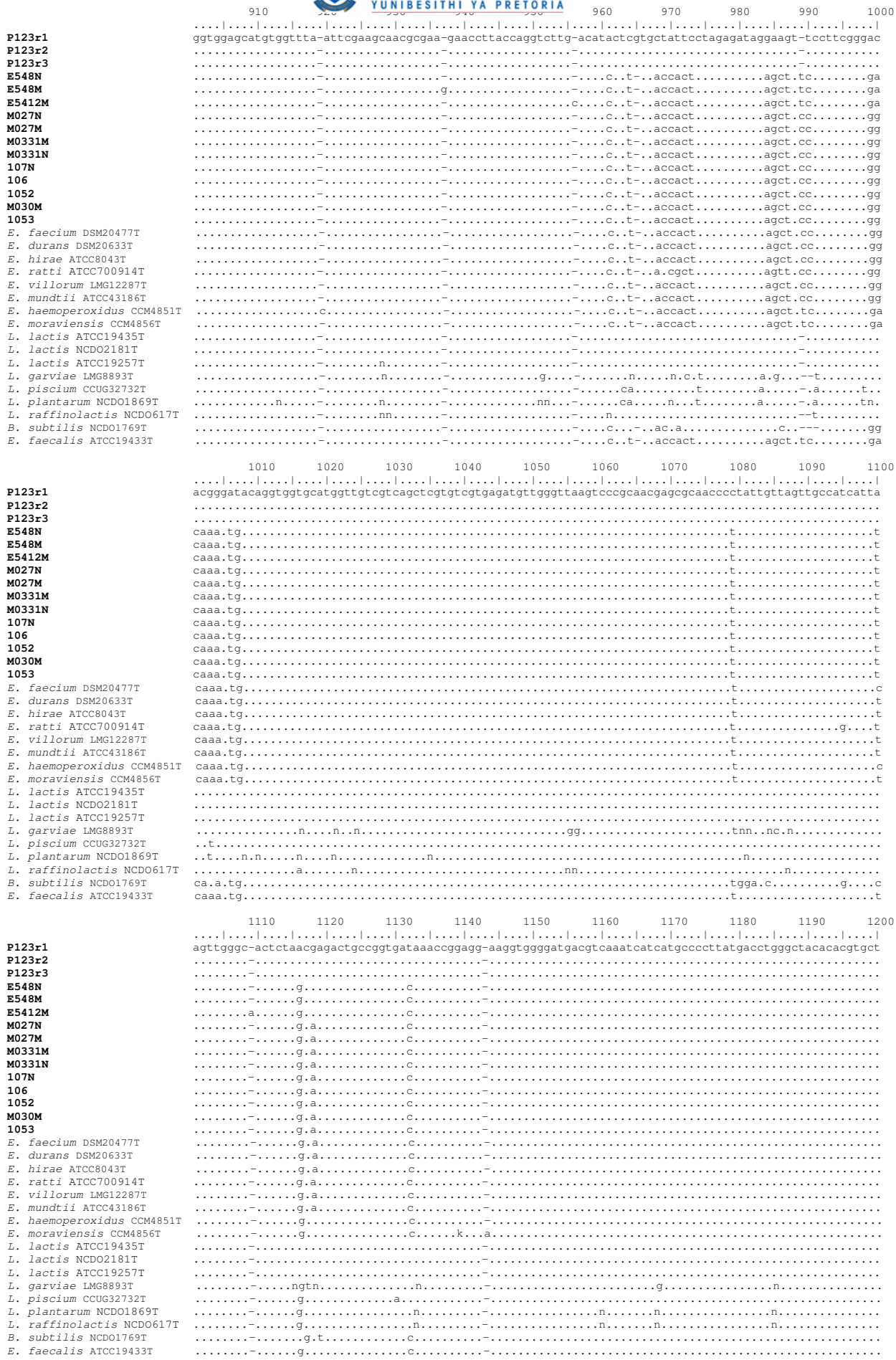

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L. lactis NCDO2181T             ............................................................................................
L. lactis ATCC19257T            ............................................................................................
L. garviae LMG8893T             ........................c.ac.c.c..g.g.gc.................................c..........n....g........
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L. raffinolactis NCDO617T       .......t................a..g.........ca..............g...a.....................................
B. subtilis NCDO1769T           ........ca.a....a.g.ca....a..c.c..g...a...c....c.ac...t.tg.............c.c..t..........a..g.g.....c
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L. raffinolactis NCDO617T       ..........n..n.....................................n..........n...........a.....t.t.a.....a...
B. subtilis NCDO1769T           t.........................t.....................................................a.....t.t.ac....----
E. faecalis ATCC19433T          ..........................................................................a.....t.t.ac........

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P123r2                          ..........
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M027N                           .c...gagg.
M027M                           .c...gagg.
M0331M                          .c...gagg.
M0331N                          .c...gagg.
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M030M                           .c..------
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L. lactis ATCC19257T            ..........
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L. plantarum NCDO1869T          cc...gag..
L. raffinolactis NCDO617T       cc...gag..
B. subtilis NCDO1769T           ----------
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Appendix H - References

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