IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM SOUTH AFRICAN FORTIFIED WINES

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

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SUMMARY

During the primary fermentation of wine, grape must is fermented to ethanol, CO_2 and flavour compounds by *Saccharomyces cerevisiae*. During the secondary fermentation malolactic bacteria converts L-malic acid to L-lactic acid and CO_2 and increases the wine pH by as much as 0.03 units. Only certain species are capable of performing malolactic fermentation (MLF) and include *Oenococcus oeni* and members of the genera *Lactobacillus* and *Pediococcus*. Malolactic fermentation often takes place in the presence of 8% (v/v) or higher ethanol, a pH of 3.2-3.8 and SO_2 levels of 30-50 mg l^{-1}. In wines with a pH of 3.75 and higher *Pediococcus* spp. often dominate and increases the wine pH to unacceptable levels. However, MLF can be of benefit to high acid wines, especially wines produced in cold viticultural regions.

Fortification of wines with alcohol before primary fermentation is completed prevents the yeast from utilizing all sugars. The high level of sugars that remain renders the wine sweet and fruity, but also susceptible to bacterial spoilage. The high level of alcohol usually prevents microbial spoilage in these wines, but certain strains of *Lactobacillus hilgardii*, *Lactobacillus fructivorans*, *Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus mali* and *Lactobacillus buchneri* are able to tolerate alcohol levels as high as 18-20% (v/v). These strains are also known for their high acid tolerance (pH 3-4). It is interesting to note that these species are all obligately heterofermentative, i.e. classified as *Lactobacillus* phenotypic Group III.

Thirty-two strains, all Gram-positive, catalase negative rods, were isolated from a sweet South African fortified wine during its submerged-culture flor sherry production process; 27 strains were isolated at the onset of this process and five strains from the mid-production phase. No bacteria were isolated from the final phase of the production process. Thirty strains were also isolated from a spoiled bottle of the same wine.

Concluded from the morphology of the cells, their gram-reaction and catalase activity, all strains were identified as members of the genus *Lactobacillus*. Further separation was made based on results obtained by CO_2 production from glucose and gluconate fermentation. Based on these reactions, 35 strains were classified as facultative heterofermentative (Group II), and 27 as obligately heterofermentative (Group III). Identification to species level was performed by numerical analysis of total soluble cell protein patterns (SDS-PAGE) and 16S rRNA sequencing.

Based on results obtained by numerical analysis of total soluble cell protein patterns, the Group II lactobacilli were identified as members of the species *Lactobacillus plantarum*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus zeae* and *Lactobacillus casei* subsp. *alactosus*. Sequencing of the 16S rRNA of representative strains from the different protein profile clusters confirmed the identity of the *Lact. plantarum* and *Lact. casei* strains.

The Group III lactobacilli grouped into four phenotypic clusters according to numerical analysis of total soluble cell protein patterns. None of the wine strains were phenotypically closely related to the reference strains included in this study. However, 16S rRNA sequencing analysis indicated that the strains from one of the phenotypic groups are members of *Lact. buchneri*. Strains from the remaining three phenotypic groups were identified as *Lactobacillus vermiforme*.

Lact. casei was the only species isolated from the production-phase fortified wine. The apparent absence of strains of *Lact. casei* from the final phase in production indicates that the species is not a potentially serious spoilage organism in the fortified wines studied. Strains of *Lact. buchneri, Lact. casei, Lact. zeae, Lact. plantarum* and *Lact. vermiforme* were isolated from the spoiled fortified wine. This is the first report of the presence of *Lact. casei, Lact. zeae* and *Lact. plantarum* in spoiled fortified wines, their low numbers suggesting a minor role as spoilage organisms.

One of the *Lact. plantarum* strains produced a bacteriocin with a broad spectrum of antimicrobial activity, also active against malolactic bacteria. It might thus very well be that this strain prevented the growth of other bacteria in the wine. This finding merits further research.

OPSOMMING

Tydens die primêre fermentasie van wyn word mos deur *Saccharomyces cerevisiae* na etanol, CO_2 en geurkomponente gefermenteer. Tydens 'n sekondêre fermentasie skakel appel-melksuurbakterieë L-appelsuur na L-melksuur en CO_2 om en verhoog die wyn se pH met soveel as 0.03 eenhede. Slegs sekere spesies kan appel-melksuurgisting (AMG) teweegbring en sluit in *Oenococcus oeni* en lede van die genera *Lactobacillus* en *Pediococcus*. Appel-melksuurgisting vind dikwels plaas in die die teenwoordigheid van 8% (v/v) of selfs hoër alkoholvlakke, 'n pH van 3.2-3.8 en SO_2-vlakke van 30-50 mg l^{-1}. In wyn met 'n pH van 3.75 en hoër mag *Pediococcus* spp. domineer en die wyn se pH tot onaanvaarbare hoë vlakke laat styg. Appel-melksuurgisting mag egter tot voordeel wees vir wyne met 'n hoe suurinhoud, veral wyne wat in koue streke geproduseer word.

Fortifisering van wyn met alkohol voordat primêre fermentasie voltooi is verhoed dat die wyngis die suikers volledig fermenteer. Die hoë vlakke van suikers wat oorbly maak die wyn soet en vrugtig, maar ook vatbaar vir bakteriese bederf. Die hoë alkoholvlakke verhoed gewoonlik mikrobiese bederf, maar sommige stamme van *Lactobacillus hilgardii, Lactobacillus fructivorans, Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus mali* en *Lactobacillus buchneri* kan alkoholvlakke van tot so veel as 18-20% (v/v) weerstaan. Hierdie stamme is ook bekend vir hul hoë suurtoleransie (pH 3-4). Interessant genoeg is al hierdie spesies obligaat heterofermentatief en sorteer onder fenotipiese Groep III van *Lactobacillus*.

Twee-en-dertig stamme, almal Gram-positiewe katalase negatiewe stafies, is van 'n Suid-Afrikaanse soet gefortifiseerde wyn geïsoleer wat volgens die ondergedompelde-kultuur flor sjerrie produksieproses gemaak is. Sewe-en-twintig stamme was voor die aanvang van die fermentasieproses geisoleer en vyf stamme tydens die middel van die fermentasieproses. Geen bakterieë is van die finale fase van die produksieproses geïsoleer nie. Dertig stamme is van 'n bederfde bottel van dieselfde tipe wyn geisoleer.

Afgelei van die morfologie van die selle, hul gram-reaksie en katalase aktiwiteit, behoort al die stamme tot die genus *Lactobacillus*. Verdere onderskeid is gemaak gebasseer op CO_2 produksie vanaf die fermentasie van glukose en glukonaat. Gebasseer op hierdie reaksies, is 35 stamme as fakultatief heterofermentatief (Groep II) en 27 as verpligtend heterofermentatief (Groep III) geklassifiseer. Identifikasie tot op spesievlak is m.b.v. numeriese analise van

totale oplosbare selproteien-bandpatrone (SDS-PAGE) en 16S rRNA-volgordebepaling gemaak.

Gegrond op die resultate verkry met numeriese analise van totale oplosbare selproteien bandpatrone, is die Groep III lactobacilli as lede van die spesies *Lactobacillus plantarum, Lactobacillus casei* subsp. *casei, Lactobacillus zeae* en *Lactobacillus casei* subsp. *alactosus* geklassifiseer. Nukleotiedopeenvolgorde van die 16S rRNA van verteenwoordigende stamme van die onderskeie proteïenprofiel-groepe het die identiteit van die *Lact. plantarum*- en *Lact. casei*-stamme bevestig.

Die Groep III lactobacilli het, na aanleiding van numeriese analise van totale oplosbare selproteïen-bandpatrone in vier fenotipiese groepe gesorteer. Nie een van die wynstamme is fenotipies naverwant aan die verwysingsstamme wat in hierdie studie ingesluit is nie. Resultate verkry met 16S rRNA-volgordebepaling het egter getoon dat stamme van een van die fenotipiese groepe wel lid is van *Lact. buchneri*. Stamme van die oorblywende drie fenotipiese groepe is as *Lactobacillus vermiforme* geïdentifiseer.

Lact. casei was die enigste spesie wat van die produksie-fase van die gefortifiseerde wyn geisoleer is. Die skeinbare afwesigheid van *Lact. casei* van die finale fase van produksie dui daarop dat die spesie nie 'n potensiële bederforganisme van die betrokke gefortifiseerde wyn is nie. Stamme van *Lact. buchneri, Lact. casei, Lact. zeae, Lact. plantarum* en *Lact. vermiforme* is van die bederfde gefortifiseerde wyn geïsoleer. Hierdie is die eerste verslag van die teenwoordigheid van *Lact. casei, Lact. zeae* en *Lact. plantarum* in bederfde gefortifiseerde wyne. Die relatief lae selgetalle van hierdie twee spesies suggereer dat hulle 'n klein rol as bederforganismes speel.

Een van die *Lact. plantarum*-stamme het 'n bakteriosien met 'n breë spektrum van antimikrobiese aktiwiteit, ook aktief teen appel-melksuurbakterieë, geproduseer. Dit mag dus wel wees dat hierdie stam die groei van ander bakterieë in die wyn inhibeer. Hierdie bevinding regverdig verdere navorsing.

BIOGRAPHICAL SKETCH

Amalia Lilian Stratiotis was born on the 23rd of September 1973 in Germiston. She matriculated from Germiston High School in 1991 and thereafter enrolled at the University of the Witwatersrand. In 1994, she obtained her B.Sc degree, with Microbiology and Zoology as majors. She then obtained her B.Sc.Hons. degree in 1995.

PREFACE

The literature review includes a brief discussion of the lactic acid bacteria, the taxonomic methods used to classify lactic acid bacteria, with special emphasis placed on the classification methods and taxonomic status of species within the genus *Lactobacillus*. The types of fortified wines and their characteristics are also discussed, in addition to the mechanisms of alcohol tolerance of the spoilage bacteria.

The paper "Identification of *Lactobacillus* spp. isolated from a South-African fortified wine by numerical analysis of total soluble cell protein patters and 16S rRNA sequencing" has been prepared for publication in Journal of Applied Microbiology.

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CONTENTS

1. INTRODUCTION

Lactic acid bacteria are Gram-positive cocci or rods, which are divided into the genera *Lactobacillus*, *Carnobacterium*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weissella* (Kandler and Weiss, 1986; Holzapfel and Wood 1995; Vandamme *et al.* 1996). The natural habitat of these bacteria is plants, as evident from the many species isolated from vegetables, fruit, silage, dough, wine, beer and other traditional products fermented from plant material (Nissen *et al.* 1991). However, an even larger number of species have been isolated from buttermilk, cheese, yoghurt, fermented meats, sewage and the genital, intestinal and respiratory tracts of man and animals (Hammes *et al.* 1991; Vandenbergh 1993).

Since biblical times, lactic acid bacteria have been inadvertently utilized in food and beverage fermentations (Nissen *et al.* 1991). The genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* are used as starter cultures in many fermented products. The lactic acid, acetic acid, formic acid, fatty acids, acetoin, diacetyl and 2,3-butanediol produced by these bacteria contributes to aroma and flavour development (De Vuyst and Vandamme 1994; Holzapfel *et al.* 1995). Apart from these organoleptic changes, many strains produce H_2O_2, bacteriocins (antimicrobial proteins or peptides), antibiotics and other low molecular weight antimicrobial compounds (e.g. reuterin-like) which act as *in situ* food preservatives (De Vuyst and Vandamme 1994).

Lactic acid bacteria, especially malolactic strains which convert L-malic acid to L-lactic acid, play an important role in wine fermentations. During the primary fermentation of wine, grape must is fermented to ethanol, carbon dioxide and flavour compounds by *Saccharomyces cerevisiae* (Davis *et al.* 1985; Wibowo *et al.* 1985). In a secondary fermentation by *Lactobacillus, Leuconostoc* and *Pediococcus* spp., the residual sugars are fermented and L-malic acid is decarboxylated to L(+)-lactic acid. Although the wine is considered to be microbiologically stable after malolactic fermentation (MLF), the pH of the wine may increase with as much as 0.03 units (Kunkee 1967; Rankine and Bridson 1971; Davis *et al.* 1985). Wines produced in cold regions, i.e. Germany, France and the Eastern United States, have a high acid

content and may benefit from deacidification by MLF. However, wines from warmer viticultural regions, i.e. South Africa, California and Australia, have a lower acidity and a further increase in pH could result in a flat, insipid wine with undesirable sensory characteristics (Davis *et al.* 1985; Wibowo *et al.* 1985) and subsequent growth of spoilage bacteria such as *Pediococcus* and *Lactobacillus* spp. (Rankine and Bridson 1971). Apart from this, red wines often show a colour reduction of 30% when fermented with *Leuconostoc* spp. (Vetsch and Lüthi 1964). In some cases high concentrations of histamine were reported after MLF, especially in wines of higher pH (Davis *et al.* 1985; Wibowo *et al.* 1985; Radler 1986). These claims are, however, questionable since malolactic bacteria do not produce histamine in wine (Buteau *et al.* 1984).

Despite the many contradictory views about MLF in wine, a number of studies have indicated that wines fermented with malolactic bacteria increases the sensory quality of wine (Giannakopoulous *et al.* 1984; McDaniel *et al.* 1987; Rodriques *et al.* 1990). According to Chalfan *et al.* (1977) carefully selected strains of malolactic bacteria could even improve the quality of high pH and low acidity wines.

Little is known about the bacterial population in fortified wines. The alcohol levels in these wines prevents the growth of most malolactic bacteria. However, *Lactobacillus fructivorans, Lactobacillus hilgardii, Lactobacillus brevis* and *Lactobacillus buchneri* tolerate alcohol levels as high as 20%, v/v (Fornachon *et al.* 1949; Farrow *et al.* 1983; Hecker and Völker 1990) and they should be able to survive the conditions in most fortified wines, depending on the method of production. Most fortified wines are produced by adding distilled alcohol after alcoholic fermentation (Goswell 1986). Some of the wines have undergone complete fermentation prior to fortification (flor sherry), whereas others have had their fermentation halted by fortification, i.e. sweet dessert wines (Goswell 1986). The high level of sugars that remain in these wines may become a source of energy for microbial growth and spoilage (Goswell 1986).

The only other bacteria that have been associated with the spoilage of fortified wines are members of the genus *Bacillus* and include the species *Bacillus coagulans, Bacillus circulans, Bacillus macerans, Bacillus pumilis, Bacillus sphaericus, Bacillus panothenticus* and *Bacillus subtilis* (Gini and Vaughan 1962). *Saccharomyces cerevisiae* and *Saccharomyces bayanus*

normally survive in wines with a content of 18% (v/v) ethanol, whereas the yeast *Saccharomyces bisporus* var. *bisporus* has been isolated from sherries containing 22% (v/v) alcohol. Most yeasts tolerate up to 15% (v/v) ethanol (Hammond 1975).

Bacterial spoilage of fortified wines in the pre- and post-production phases are responsible for huge financial losses in the South African wine industry. Little is known about the bacterial species present in fortified wines or the conditions leading to bacterial spoilage. This research was aimed at identifying the *Lactobacillus* spp. from two of the most popular South African fortified wines, generally referred to as sherry.

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A noble wine your Sherry
Which with wondrous stealth
Changes drunkards dim to merry,
Human ills to vigorous health
And grasping greed to kinder wealth.
With it, dark doubt to calm gives way,
And puny fears to valour bright,
It is the sun of youthful day
Which melts the ice of age-cold night.

(Prof. José Mariá de Ortega Morejón)

2. TAXONOMY OF *LACTOBACILLUS* SPP. ISOLATED FROM WINE AND THEIR ROLE IN THE SPOILAGE OF FORTIFIED WINES

TAXONOMY OF *LACTOBACILLUS* SPP. ISOLATED FROM WINE AND THEIR ROLE IN THE SPOILAGE OF FORTIFIED WINES

INTRODUCTION

The genus *Lactobacillus* is a member of the *Clostridium-Bacillus* subdivision of the Gram-positive eubacteria (De Vuyst and Vandamme 1994), which also includes the genera *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus, Carnobacterium* (Holzapfel and Wood 1995), *Oenococcus* (Dicks *et al.* 1995a), *Enterococcus, Tetragenococcus, Vagococcus* and *Weissella* (Vandamme *et al.* 1996). The genus *Bifidobacterium*, previously included with the other genera of lactic acid bacteria, is now considered to be a member of the more ancient group of the second subdivision of the Gram-positive bacteria, the *Actinomycetes* (Pot *et al.* 1994). Included in the latter subdivision are the genera *Brevibacterium, Corynebacterium, Microbacterium* and *Propionibacterium*, all with a DNA base composition higher than 50 mol% G+C (Holzapfel and Wood 1995).

The genus *Lactobacillus* consists of 56 species, of which five are divided into at least two subspecies (Vandamme *et al.* 1996). The genus was first proposed by Beijerinck (1901), who based his description on morphology and physiology. The information below was taken from Kandler and Weiss (1986).

Morphology

Like all other lactic acid bacteria, *Lactobacillus* spp. are non-motile and non-sporeforming. The cells are rod-shaped, with bipolar bodies or internal granules visible in some of the strains. Some strains have a barred appearance when stained with methylene blue (Kandler and Weiss 1986). On agar media, the colonies are usually small (2-5 mm), smooth and convex, with entire margins, opaque (no pigment) and glistening, and in rare cases they may be yellowish or reddish, or produce rough colonies (Kandler and Weiss 1986).

Habitat

Like typical lactic acid bacteria, lactobacilli are microaerophilic or anaerobic, and aciduric or acidophilic (Kandler and Weiss 1986). They prefer growing in nutritionally rich habitats with a pH range between 4.5 and 6.4 and at mesophilic to slightly thermophilic temperatures (Kandler and Weiss 1986). The diverse habitats of *Lactobacillus* spp. include the oral cavity, intestinal tract and vagina of humans, environmental sources such as plants, soil and water, fermented food products, manure and silage (Stiles and Holzapfel 1997). *Lactobacillus* spp. have also been associated with spoiled foods, e.g. milk, fermented beverages, fruit, meat and meat products (Stiles and Holzapfel 1997).

Metabolism

All species are catalase-negative due to the absence of cytochromes (Kandler and Weiss 1986; Hammes *et al.* 1992). However, some species produce catalase when grown in media supplemented with blood (Aguirre and Collins 1993) or produce a pseudocatalase when grown in medium with a low sugar content (Vandamme *et al.* 1996).

Lactobacillus spp. have the same fastidious nutritional requirements than most other species in the family *Lactobacteriaceae*, i.e. they are chemo-organotrophic and grow only in complex media with fermentable carbohydrates as an energy source and adequate concentrations of amino acids, peptides, nucleic acid derivatives and vitamins (Kandler and Weiss 1986; Aguirre and Collins 1993). Most strains exhibit slight proteolytic activity due to cell wall-bound or cell wall-released proteases and peptidases (Kandler and Weiss 1986). Some species have a weak lipolytic activity. Starch degradation is observed in only a few species, e.g. *Lactobacillus amylophilus* and *Lactobacillus amylovorans* (Kandler and Weiss 1986). Nitrate is not reduced, gelatin not liquefied and cesein not digested. Indole and H_2S are not produced (Kandler and Weiss 1986). Some *Lactobacillus* spp. produce exopolysaccharides (dextrans) with the assistance of dextran sucrases (Kandler and Weiss 1986).

Hexoses are fermented via two main fermentation pathways, *viz.* the Embden-Meyerhof (glycolysis) pathway and the 6-phosphogluconate/phosphoketolase pathway. Three metabolic categories have been defined based on differences in hexose and pentose fermentations (Vandamme *et al.* 1996). The first category includes the group I lactobacilli, which are obligately homofermentative, meaning that sugars can only be fermented via the Embden-Meyerhof pathway. This results in lactate being the major end product; more than 85% (Kandler and Weiss 1986; Hammes *et al.* 1992). The second category includes the group III lactobacilli, also referred to as the obligately heterofermentative species, i.e. sugars are only fermented via the 6-phosphogluconate pathway to produce equal amounts of lactic acid, CO_2 and ethanol, or acetic acid (Kandler and Weiss 1986). The third category includes *Lactobacillus* spp. with both the enzymes FDP aldolase and phosphoketolase. These species are homofermentative with regard to hexoses and heterofermentative with regard to pentoses and are classified as facultatively heterofermentative (Kandler and Weiss 1986). Heterofermentative species produce lactate, acetate, ethanol, carbon-dioxide, formate or succinate in equimolar amounts (Kandler and Weiss 1986; Hammes *et al.* 1992). Examples of *Lactobacillus* spp. from the three metabolic groups are listed in Table 1.

Homolactic fermentation may become heterofermentative, depending on how pyruvate is utilized (Kandler and Weiss 1986). Pyruvate, which is intermediately formed in both homolactic and heterolactic pathways, may either be converted to acetic acid or to diacetyl and its derivatives, or with hexose limitation, homolactic fermentation may become a heterofermentation with acetic acid, ethanol and formic acids as main products (Kandler and Weiss 1986).

Several *Lactobacillus* spp. are also able to degrade organic acids, such as citric, tartaric and malic acids, via oxaloacetic acid and pyruvate, to produce CO_2 and lactic acid or acetic acid (Kandler and Weiss 1986). Many lactobacilli are also able to split L-malic acid to L-lactic acid and CO_2. This process, well known in the wine industry, is known as malolactic fermentation - although, technically, no fermentation is involved (Kandler and Weiss 1986).

Table 1 Major divisions within the genus *Lactobacillus* based on phenotypic characteristics (Pot *et al.* 1994; Hammes and Vogel 1995; Vandamme *et al.* 1996)

Group 1	Group 2	Group 3
Obligate homofermenters	Facultative heterofermenters	Obligate heterofermenters
Lact. acidophilus	*Lact. acetotolerans*	*Lact. brevis*
Lact. amylophilus	*Lact. agilis*	*Lact. buchneri*
Lact. amylovorus	*Lact. alimentarius*	*Lact. collinoides*
Lact. aviarius subsp. *araffinosus*	*Lact. bifermentans*	*Lact. fermentum*
Lact. aviarius subsp. *aviarius*	*Lact. casei*	*Lact. fructivorans*
Lact. crispatus	*Lact. coryniformis* subsp. *coryniformis*	*Lact. fructosus**
Lact. delbrueckii subsp. *bulgaricus*	*Lact. coryniformis* subsp. *torquens*	*Lact. hilgardii*
Lact. delbrueckii subsp. *delbrueckii*	*Lact. curvatus*	*Lact. kefir*
Lact. delbrueckii subsp. *lactis*	*Lact. graminis*	*Lact. malefermentans*
Lact. farciminis	*Lact. hamsteri*	*Lact. oris*
Lact. gallinarum	*Lact. homohiochii*	*Lact. panis*†
Lact. gasseri	*Lact. intestinalis*	*Lact. parabuchneri*
Lact. helveticus	*Lact. murinus*	*Lact. parakefir*†
Lact. jensenii	*Lact. paracasei* subsp. *paracasei*	*Lact. pontis*†
Lact. johnsonii	*Lact. paracasei* subsp. *tolerans*	*Lact. reuteri*
Lact. kefiranofaciens	*Lact. paraplantarum*†	*Lact. sanfrancisco*
Lact. kefirgranum†	*Lact. pentosus*	*Lact. suebicus*
Lact. mali	*Lact. plantarum*	*Lact. vaccinostercus*
Lact. ruminis	*Lact. rhamnosus*	*Lact. vaginalis*
Lact. salivarius subsp. *salicinus*	*Lact. sake*	
Lact. salivarius subsp. *salivarius*		
Lact. sharpeae		

**Lact. fructosus* classified with the *Leuconostoc* group of lactic acid bacteria

†Species added since the review by Pot *et al.* (1994)

The lactic acid produced from sugar fermentations may be of two configurations, i.e. the L- or the D-configuration, depending on the stereospecificity of the lactic acid dehydrogenase present in the cells. When both types of lactic acid are produced, racemate may be formed (Kandler and Weiss 1986).

A number of metabolic compounds are produced, most of which are beneficial in the production of food products and beverages (Table 2). Of all lactic acid bacteria *Lactobacillus* spp. are probably the best represented in fermented food and beverages (Table 3).

Although *Lactobacillus* spp. are generally regarded as safe, a number of studies have been published in which strains of *Lactobacillus acidophilus* and *Lactobacillus plantarum* are linked to human infections (Biocca and Reitano 1943; Sharpe *et al.* 1973; Bayer *et al.* 1978; Shinar *et al.* 1984; Allison and Galloway 1988; Friedland *et al.* 1990; Aguirre and Collins 1993). In all of these cases, the patients were either immunocompromised or received antibiotics over an extended period.

TAXONOMIC METHODS USED

A number of different taxonomic methods are used to identify *Lactobacillus* spp. Despite this, a number of misclassifications are still being made and many descrepencies have been pointed out. The Subcommittee on *Bifidobacterium, Lactobacillus* and Related Organisms of the International Committee on Systematic Bacteriology (ICSB) of the International Union of Microbiological Societies (IUMS) is currently compiling a set of rules as guideline for the designation of new species and the confirmation of existing *Lactobacillus* spp. (Prof. L.M.T Dicks, personal communication). The definition of any newly described species should at least consist of a number of strains with a representative strain (type or neotype strain) (Staley and Krieg 1984). It is generally accepted that the type strain has a DNA homology of 70% or higher with the other strains in the same species. Furthermore, the ΔTm values recorded among strains within the same species should not differ with more than 5°C (Staley and Krieg 1984). Tm is the melting temperature of the hybrid as determined by stepwise denaturation, and ΔTm is the difference in

Tm in °C between the homologous and heterologous hybrids formed under standardized conditions (Wayne *et al.* 1987).

To obtain the most accurate classification of an organism, it is important to compare all characteristics of the strain with all known characteristics of as many species as possible. This means that phenotypic, genotypic and genetic classification methods have to be used in an attempt to apply a more polyphasic taxonomic approach. Experience has, however, shown that some characteristics are of greater importance than others (Vandamme *et al.* 1996).

Table 2. Commercial significance of metabolic products of lactic acid bacteria (Holzapfel *et al.* 1995)

Metabolite	Beneficial	Deleterious
Lactic acid	Preservation Sensory improvement Enhancement of digestion and of nutrient uptake	Acidification
Acetic acid	Aroma	Off-taste
Diacetyl/acetoin	Aroma (dairy products)	Off-taste (beer)
CO_2	Preservation	Discolouration Greening
Biogenic amines	-	Health (allergies)
Slime	Stabilization (e.g. yoghurt)	Sensory
Methane thiol. H_2S	Aroma	Sensory (off-taste and odour)
Bacteriocins	Preservation (inhibition of closely related bacteria)	Inhibition of beneficial lactic acid bacteria
Wide-spectrum antimicrobials	Inhibition of pathogens and spoilage microorganisms	Resistance of intestinal microorganisms

Table 3 Lactic acid bacteria used in food fermentations (Aguirre and Collins 1993)

Foods/product	Raw ingredients	Microorganisms
Dairy products:		
Acidophilus milk	Milk	*Lactobacillus acidophilus*
Bulgarian buttermilk	Milk	*Lactobacillus delbrueckii* subsp. *bulgaricus**
Ripened cheeses	Milk curd	Lactobacilli, lactococci
Kefir	Milk	*Lactobacillus lactis, Lactobacillus delbrueckii* subsp. *bulgaricus*
Kummis	Mare's milk	*Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus leichmanii*
Taette	Milk	*Lactococcus lactis*
Yoghurt	Milk	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Meat and fish products:		
Dry sausages	Pork, beef	Pediococci, *Lactobacillus plantarum, Lactobacillus brevis*
Semi-dry sausages	Beef	Pediococci
Burong dalag	Fish, rice	*Leuconostoc mesenteroides, Lactobacillus plantarum*
Izushi	Fish, rice, vegetables	Lactobacilli
Plant products:		
Kenkey	Corn	Lactobacilli
Ogi	Corn	*Lactobacillus plantarum, Lactococcus lactis*
Olives	Green olives	*Lactobacillus plantarum,* pediococci, *Lactobacillus brevis, Leuconostoc mesenteroides*
Pickles	Cucumbers	Pediococci, *Lactobacillus plantarum*
Sauerkraut	Cabbage	*Lactobacillus plantarum, Leuconostoc mesenteroides*
Soy sauce	Soy beans	*Lactobacillus delbrueckii*
Wine	Grapes	*Oenococcus oeni*
Sake	Rice	*Lactobacillus sake, Lactobacillus homohiochi, Lactobacillus collinoides*
Breads:		
San Fransisco sourdough	Wheat flour	*Lactobacillus sanfransisco*
Sour pumpernickel	Wheat flour	*Leuconostoc mesenteroides*
Idli	Rice and bean flour	*Leuconostoc mesenteroides*

* Pot *et al.* (1994)

Phenotypic Methods

Phenotypic methods of classification include methods that are not directed toward DNA or RNA, but toward morphological, physiological and biochemical features. As a whole, all these characteristics provide useful information, which allow us to recognise taxa. Morphological features of a bacterium include both cellular (shape, endospore, Gram-staining, inclusion bodies, flagella) and colonial (form, colour and dimension) characteristics. Physiological and biochemical features include an organism's ability to grow at different temperatures, pH values, salt concentrations or atmospheric conditions, growth in the presence of different chemicals (e.g. antimicrobial agents), the presence and activity of different enzymes, the metabolization of different compounds, etc. (Vandamme *et al.* 1996).

Fermentation

The genus *Lactobacillus* is divided into three fermentation groups: (i) Obligately homofermentative, (ii) facultatively heterofermentative and (iii) obligately heterofermentative. Until the late 1970s, the three fermentation groups were maintained with some major modifications (Rogosa 1970; Rogosa 1974; Sharpe 1979). The increasing number of newly described species resulted in the redefinition of these groups by Kandler and Weiss (1986) and by Hammes *et al.* (1991). In 1995, Hammes and Vogel proposed a new organization of the lactobacilli to reconcile the phenotypic divisions with the phylogenetic data obtained from rRNA sequencing. In this subdivision, the capital letters A, B and C refer to fermentation types, i.e. obligately homofermentative lactobacilli were named group A, facultatively heterofermentative lactobacilli group B and obligately heterofermentative lactobacilli group C. The lowercase letters a, b and c refer in each subdivision to the three phylogenetic subgroups (the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group and the *Leuconostoc* group, respectively). For example, Hammes and Vogel (1995) have allocated the obligately homofermentative lactobacilli to two phylogenetic subgroups, the *Lact. delbruekii* subgroup (group Aa) and the *Lact. casei-Pediococcus* subgroup (group Ab). This is illustrated in Table 4.

Table 4 Subdivision of *Lactobacillus* spp. according to their phenotypic and phylogenetic assignments (Hammes and Vogel 1995)

Phylogenetic group	**Species in fermentation group**		
	A (obligately homofermentative)	**B (facultatively heterofermentative)**	**C (obligately heterofermentative)**
(*Lact. delbruekii* group)	*Lact. acidophilus, Lact. amylophilus, Lact. amylovorus, Lact. crispatus, Lact. delbruekii* subsp. *bulgaricus* ("*Lact. bulgaricus*"), subsp. *delbruekii*, subsp. *lactis* ("*Lact. lactis*"), *Lact. gallinarum, Lact. gasseri, Lact. helveticus* ("*Lact. jugurti*"), *Lact. jensenii, Lact. johnsonii, Lact. kefiranofaciens, Lact. kefirgranum*	*Lact. acetotolerans, Lact. hamsteri*	
(*Lact. casei-Pediococcus* group)	*Lact. aviarius* subsp. *aviarius*, subsp. *araffinosus, Lact. farciminis, Lact. ruminis, Lact. mali* ("*Lact. yamanashiensis*"), *Lact. salivarius* subsp. *salicinus*, subsp. *salivarius, Lact. sharpae, Ped. damnosus, Ped. dextrinicum, Ped. parvulus*	*Lact. agilis, Lact. alimentarius, Lact. casei, Lact. bifermentans, Lact. coryniformis*, subsp. *coryniformis*, subsp. *torquens, Lact. curvatus, Lact. graminis, Lact. homohiochii, Lact. intestinalis, Lact. murinus* ("*Lact. animalis*"), *Lact. paracasei* subsp. *paracasei*, subsp. *tolerans, Lact. pentosus, Lact. plantarum, Lact. rhamnosus, Lact. sake* ("*Lact. bavaricus*"), *Ped. acidilactici, Ped. pentosaceus*	*Lact. brevis, Lact. buchneri, Lact. collinoides, Lact. fermentum* ("*Lact. cellobiosus*"), *Lact. fructivorans* ("*Lact. trichodes*"), *Lact. hilgardii* ("*Lact. vermiforme*"), *Lact. kefir, Lact. malefermentans, Lact. oris, Lact. parabuchneri, Lact. parakefir, Lact. pontis, Lact. reuteri, Lact. suebicus, Lact. sanfrancisco, Lact. vaccinostercus, Lact. vaginalis*
(*Leuconostoc* group)			*Lact. fructosus, W. confusus* ("*Lact. confusus*"), *W. halotolerans* ("*Lact. kandleri*"), *W. minor* ("*Lact. minor*"), *W. viridescens* ("*Lact. viridescens*"), *W. hellenica, W. paramesenteroides* ("*Leuc. paramesenteroides*"), *Leuc. amelibiosum, Leuc. argentinum, Leuc. lactis, Leuc. mesenteroides, Leuc. pseudomesenteroides, Leuc. gelidum, Leuc. carnosum, Leuc. fallax, Oen. oeni*
Other lactobacilli	*Lact. catenaformis, Lact. vitulinus, Lact. rogosae, Atopobium minutum* ("*Lact. minutus*"), *Atopobium rimae* ("*Lact. rimae*"), *Atopobium uli* ("*Lact. uli*"), *Carnobacterium divergens* ("*Lact. divergens*") ("*Lact. carnis*"), *Carnobacterium piscicola* ("*Lact. piscicola*") ("*Lact. maltaromicus*"), *Lactococcus lactis* ("*Lact. hordniae*") ("*Lact. xylosus*")		

Lact. kefirgranum and *Lact. parakefir* have not been included in 16S rRNA sequence analysis.
Lact. = *Lactobacillus* *Ped.* = *Pediococcus* *W.* = *Weissella* *Leuc.* = *Leuconostoc* *Oen.* = =*Oenococcus.*

The three fermentation groups are as follows:

(i) Obligately homofermentative lactobacilli (group A). These lactobacilli degrade hexoses almost exclusively to lactic acid via the Embden-Meyerhof pathway and they cannot utilize pentoses or gluconate. They are divided into two phylogenetic subgroups, the *Lact. delbruekii* subgroup (group Aa) and the *Lact. casei-Pediococcus* subgroup (group Ab). Group Aa consists of industrially important species, e.g. *Lact. delbruekii, Lactobacillus jensenii* and the *Lact. acidophilus*-group. Taxonomy of the industrially and medically important species of *Lact. acidophilus* (Hawley *et al.* 1959; Kandler 1964) had for a long time been in confusion (Gasser 1970; Sharpe 1970) until DNA hybridization studies and standardized SDS-PAGE of whole-cell proteins clarified the taxonomic status of this species. *Lact. acidophilus* spp. cannot be reliably differentiated by simple phenotypic tests (Fujisawa *et al.* 1992).

(ii) Facultatively heterofermentative lactobacilli (group B). These lactobacilli ferment hexoses almost exclusively to lactic acid by the Embden-Meyerhof pathway, or to lactic acid, acetic acid, ethanol, and formic acid under glucose limitation. Pentoses are fermented to lactic acid and acetic acid through an inducible pentose phosphoketolase. Two subgroups were identified. Group Ba (facultatively heterofermentative lactobacilli from the *Lact. delbrueckii* rRNA branch) comprises *Lactobacillus acetotolerans* and *Lactobacillus hamsteri*, which were formerly regarded as obligate homofermenters until recently (Hammes and Vogel 1995). Group Bb comprises the facultatively heterofermentative lactobacilli from the *Lact. casei-Pediococcus* branch (Hammes and Vogel 1995).

(iii) Obligately heterofermentative lactobacilli (group C). The lactobacilli of this group ferment hexoses to lactic acid, acetic acid, ethanol and CO_2 via the phospho-gluconate pathway. Pentoses are fermented to lactic acid and acetic acid. A pentose phosphoketolase is usually involved in both pathways. Hammes and Vogel (1995) have assigned 17 *Lactobacillus* spp. to group Cb (obligately heterofermentative lactobacilli from the *Lact. casei-Pediococcus* branch), and have assigned five *Lactobacillus* spp. to group Cc (obligately heterofermentative lactobacilli

from the *Leuconostoc* branch), four of which have recently been transferred to the genus *Weissella* (Collins *et al.* 1993).

Cell wall composition

A biochemical feature used in the classification of bacteria, especially Gram-positive bacteria, is the determination of their cell wall composition. The peptidoglycan type found in Gram-positive bacteria is quite uniform and does not provide very much information. Gram-negative bacteria, however, contain various peptidoglycan types in their cell walls, which may be genus or species specific (Schleifer and Kandler 1972). Teichoic acid may also be analysed using gas-liquid chromatography (Fischer *et al.* 1980; Fischer *et al.* 1981).

The different murein types of the cell wall have been used to classify lactic acid bacteria (Hammes and Vogel 1995). The Lys-D-Asp type of murein is specific to the homofermentative (group Aa), as well as the facultatively heterofermentative (group Ba) lactobacilli from the *Lact. delbruekii* group. The obligately heterofermentative organisms from the *Leuconostoc* group all have the Lys amino type [Lys-L-Ser-L-Ala_2 or Lys-L-Ala_2 (Dellaglio *et al.* 1995)]. The Lys-D-Asp type or the diaminopimelic-direct type is common to representative species of the *Lact. casei-Pediococcus* group [homofermentative (Ab), facultatively heterofermentative (Bb), and obligately heterofermentative (Cb)].

Cellular fatty acids

The variety of lipids present in bacterial cells is also useful for taxonomic purposes. Polar lipids (the major constituents of the lipid bilayer of bacterial membranes) and sphingophospholipids which are present in a restricted number of taxa (Jones and Krieg 1984) are examples of these. The lipopolysaccharides present in the outer membranes of Gram-negative bacteria (De Weger *et al.* 1987), and fatty acids, which are the constituents of lipids and lipopolysaccharides, have also been used extensively for classification (Suzuki *et al.* 1993). The variability of fatty acid chain

length, double-bond position and substituent groups has proved useful in the characterization of bacterial taxa (Suzuki *et al.* 1993).

The determination of the resolution level of this technique for every isolate is important, as well as the standardization of growth conditions to obtain reproducible results. The method is rapid, simple and has become highly automated for the analysis of large numbers of strains (Vandamme *et al.* 1996). However, the technique is not always reliable. For some genera, the results may allow for differentiation and identification of individual species or even subspecies, while for other genera, different species have identical fatty acid profiles (Welch 1991).

Isoprenoid quinones

The structure of isoprenoid quinones has been used to characterise bacteria at different taxonomic levels (Collins and Jones 1981). These compounds, present in the cytoplasmic membrane of most prokaryotes, play an important role in electron transport, oxidative phosphorylation and active transport (Collins and Jones 1981; Collins 1994).

Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis, which is useful in the field of population genetics, involves the electrophoretic separation of native enzymes, followed by staining for activity, and comparison of their electrophoretic mobilities. Multiple polymorphisms present in a gene influences the rate of mobility of the resulting enzyme (Selander *et al.* 1986).

The electrophoretic mobility of lactate dehydrogenase (LDH) in starch gels (Gasser 1970) or in polyacrylamide gels (Hensel *et al.* 1977) has allowed for the classification of lactic acid bacteria, especially for the discrimination of phenotypically closely related species, as shown with studies performed by Fujisawa *et al.* (1992). Results obtained by Gasser (1970) have shown, however, that this method is not always very discriminatory. A study of the electrophoretic mobility of

LDH of *Lact. jensenii* and *Lact. delbruekii* has shown that these two species are indistinguishable. However, LDH PAGE and Starch gel electrophoresis of D-LDH have shown that the latter two species are different (Kandler and Weiss 1986; Gasser 1970).

Whole-cell protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Whole-cell protein analysis by SDS-PAGE has proven to be highly reliable to identify isolates at species and subspecies levels, provided cultivation and SDS-PAGE conditions are standardized (Hertel *et al.* 1993; Descheemaeker *et al.* 1994; Devriese *et al.* 1995). Strains within a single species have unique cellular protein patterns, which often distinguishes strains at a level DNA hybridization studies cannot do (Vandamme *et al.* 1996).

Computer-assisted numerical analysis is a useful tool for the objective processing of large amounts of data. Various computer programs are available, of which GelCompar (Applied Maths BVBA, Kortrijk, Belgium) and 1DSCAN (Borland International Inc., Scotts Valley, CA, USA) are the most used. Databases of digitized and normalized protein patterns for all known species of lactic acid bacteria have been compiled (Pot and Janssens 1993). The technique has proven useful in the grouping of large numbers of strains (Descheemaeker *et al.* 1994).

Dicks and van Vuuren (1987) have used whole-cell protein analysis to determine the phenotypic relatedness among several heterofermentative *Lactobacillus* strains, resulting in the designation of some species and the placement of others as distinct, separate species. The genotypic relatedness of 74 *Leuconostoc* spp. and one *Pediococcus* sp. has also been clarified with the assistance of numerical analysis of total soluble cell protein patterns (Dicks *et al.* 1990). Holzapfel *et al.* (1997) has also successfully used this method, together with RAPD-PCR, to distinguish between representative species of the *Lact. acidophilus*-group, the *Lact. casei*-group, and between *Lactobacillus fermentum* and *Lactobacillus reuteri*.

Polyamines

Polyamines are used to classify bacteria at and above genus level, as well as at species level (Busse and Auling 1988; Hamana and Matsuzak 1990). Gas chromatography (Yamamoto *et al.* 1983) or high-performance liquid chromatography (Scherer and Kniefel 1983) are used to characterise the molecules and determine variations on a quantitative and qualitative level.

Phenotypic fingerprinting

Recently, commercially available phenotypic fingerprinting systems have become available. These systems consist of a series of dehydrated reagents to which a standardised inoculum is added. The enzymatic activity is recorded after a short incubation. For these tests to be accurate, it is important that they are performed under standardised conditions (Vandamme *et al.* 1996). The API ZYM system (API Laboratory Products Ltd.) has been sucessfully used to identify the enzymatic pattern of strains of *Lact. casei*. The system allows for the detection of enzymes such as esterase, lipase, alkaline phosphatase and α-galactosidase.

Serology

Serological tests are used to determine the serovars of strains, although these tests are often only suitable for specific organisms. Serotyping detects variability in the antigenic constituents of many cell components (Henrikson 1978). Structures such as capsules, cell envelopes, fimbriae, flagella, intracellular molecules, or secretion products such as toxins and enzymes have been subjects for serotyping. Henrikson (1978) has described several different types of serological reactions, such as simple precipitation or agglutination tests and complement fixation tests. Lactate dehydrogenase, and enzymes such as fructose-1,6-disphosphate aldolase (London and Chase 1976), malic enzymes (London and Chase 1971) and glyceraldehyde-3-phosphate dehydrogenase (London and Chase 1983) have been analyzed using quantitative immunological

techniques. From these results, dendrograms and three-dimensional phylogenetic maps are created.

Total chemical composition of bacterial cells

The techniques of Fourier transformation infra-red spectroscopy, pyrolysis mass spectrometry and UV resonance Raman spectroscopy are used to examine the total chemical composition of bacterial cells (Magee 1993).

Genotypic Methods

DNA base ratio determination (moles percent G+C)

This is one of the classical genotypic methods and is considered part of the standard description of bacterial taxa. The ratio is determined by first heating a DNA sample, e.g. from 60 to 100°C, at a specific rate, e.g. 0.2°C per min. By using a spectrophotometer, the thermal denaturation (T_m) value is determined and the DNA base composition calculated using the equation of De Ley (1970). In general, within a well-defined species, the range observed is 3% (maximum), while a range of 10% is the limit for defining a genus (Stackebrandt and Liesak 1993). The DNA base composition of bacteria vary between 24 and 76 mol% G+C (Vandamme *et al.* 1996).

In general, lactic acid bacteria have an average DNA base composition of less than 50 mol% G+C (Vandamme *et al.* 1996). The genus *Lactobacillus* has a DNA base composition of 32 to 53 mol% G+C (Kandler and Weiss 1986). The obligate heterofermentative species have a G+C content of 34 to 53 mol%, which covers almost the entire span of the genus *Lactobacillus* (Kandler and Weiss 1986). *Lactobacillus pontis*, however, has a DNA-base composition of 53-54 mol% G+C (Vogel *et al.* 1994), but is phylogenetically very closely related to *Lactobacillus reuteri*, which has a G+C content of 40 to 43 mol% (Kandler and Weiss 1986).

The DNA base composition of 74 *Leuconostoc* spp. and one *Pediococcus* sp. have been determined by Dicks *et al.* (1990), the results of which are described in another section. The DNA base composition of all the strains analyzed ranged from 38 mol% G+C (for *Oenococcus oeni* Ea3) to 45 mol% G+C (for *Leuc. lactis* DSM 20202^T, DSM 20198 and DSM 20192).

DNA-DNA hybridization studies

This method delineates species through percentage DNA homology (Wayne *et al.* 1987). The percentage DNA binding (De Ley *et al.* 1970), the DNA-DNA hybridization value, or the relative binding ratio (Brenner *et al.* 1969; Grimont *et al.* 1980; Popoff and Coynault 1980) is an indirect parameter of the sequence similarity between two genomes. The most common methods used are the hydroxyapatite method (Brenner *et al.* 1969), the optical renaturation method (De Ley *et al.* 1970), and the S1 nuclease method (Crosa *et al.* 1973; Grimont *et al.* 1980). Bautz and Bautz (1964), Stackebrandt and Ludwig (1994) and Ullmann and McCarthy (1973) have shown that thermal stabilities decrease from 1 to 2.2% for each 1% of mispairing. Much contention, however, remains as to whether data obtained with short oligonucleotides and experimentally induced mispairing can be extrapolated to entire genomes, making it impossible to convert a percentage DNA-binding or DNA-DNA hybridization value into a percentage of whole genome similarity (Vandamme *et al.* 1996).

DNA-DNA hybridization studies have several disadvantages. Since the technique depends on physicochemical parameters, its various results are not cumulative, it is labour-intensive and requires the use of large quantities of DNA (Stackebrandt and Ludwig, 1994). Grimont *et al.* (1980) have shown that DNA hybridization data obtained from different laboratories do not always correlate. They argue that different methods yield different results, and that the same results are not always obtained under standardized conditions.

Most of these studies were performed before the full establishment of the phylogenetic relationships of lactobacilli, and were performed with emphasis on species of economic interest (Vandamme *et al.* 1996). DNA-DNA hybridization studies have allowed for the proper

description of new species, or have facilitated the reduction of previously heterogeneous taxa, proving to be a useful technique to achieve species determination within the genus *Lactobacillus* (Vandamme *et al.* 1996).

Vandamme *et al.* (1996) describes further, in great detail, how DNA-DNA hybridization studies have delineated two groups within the genus *Lactobacillus*.

Subgroup 1 includes *Lact. delbrueckii*, with the three subspecies *Lact. delbrueckii* subsp. *delbrueckii*, *Lact. delbrueckii* subsp. *bulgaricus* (previously "*Lactobacillus bulgaricus*") and *Lact. delbrueckii* subsp. *lactis* (previously "*Lactobacillus lactis*" and "*Lactobacillus leichmannii*"), which share over 80% DNA homology (Weiss *et al.* 1983a).

Subgroup 2 is represented by *Lact. acidophilus sensu lato*, which consists of at least six species; *Lact. acidophilus sensu stricto* (Hansen and Mocquot 1970), *Lactobacillus gasseri* (Lauer and Kandler 1980), *Lactobacillus crispatus* (Cato *et al.* 1983), *Lactobacillus amylovorus* (Nakamura 1981), *Lactobacillus gallinarum* (Fujisawa *et al.* 1992) and *Lactobacillus johnsonii* (Fujisawa *et al.* 1992).

This method has also been used, together with the numerical analysis of total soluble cell protein patterns, in the classification of *Leuconostoc* spp. This work has resulted in the elucidation of the genotypic relatedness of the strains *Leuconostoc oenos, Leuconostoc lactis, Leuconostoc argentinum, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum* and *Leuconostoc paramesenteroides* (Dicks *et al.* 1995a), recently reclassified as *Weissella paramesenteroides* (Collins *et al.* 1993). DNA-DNA hybridizations have also proved useful in the classification of *Leuconostoc* spp. The results obtained contributed to the clarification of the genotypic relatedness of these organisms, as well as the classification of *Ped. acidilactici* ATCC 12697 as *Leuc. paramesenteroides*, and *Leuc. mesenteroides* NCDO 530 as *Leuc. oenos* (Dicks *et al.* 1990). It was also discovered by DNA-DNA hybridization studies that 13 of the 24 presumed *Lact. brevis* strains actually belonged to either the species *Lactobacillus hilgardii*, *Lactobacillus kefir, Lactobacillus confusus* or *Lactobacillus collinoides* (Vescovo *et al.* 1979). This indicated that identification of *Lact. brevis*-like strains using carbohydrate

fermentation reactions or additional simple phenotypic tests was not conclusive enough (Vescovo *et al.* 1979).

Ribosomal RNA (rRNA) sequence homology

Ribosomal RNA contains highly conserved and more variable domains and is thus a favourable target for studying phylogenetic relatedness (Woese 1987; Schleifer and Ludwig 1989; Stackebrandt and Ludwig 1994). Databases with published and some unpublished partial or complete sequences of 16 and 23S rRNA are available (Olsen *et al.* 1991; De Rijk *et al.* 1992). Hybridization studies (De Ley and De Smedt 1975; Palleroni 1984) or rRNA catalogueing of RNase T_1- resistant oligonucleotides of 16S rRNA (Fox *et al.* 1977; Stackebrandt *et al.* 1985; Fox and Stackebrandt 1987) have been used to elucidate natural relationships with and within several bacterial lineages (Woese 1987; De Ley 1992).

More recently, sequencing of rRNA molecules has resulted in the formation of a rRNA sequence database of 5S rRNA (Wolters and Erdmann 1988). This was the first rRNA molecule to be sequenced for many bacteria due to its less complex primary and secondary structures.

Direct sequencing after cloning of the genes from the bulk of the DNA has yielded a limited number of 16S rRNA gene sequences (Ludwig 1991). An important advance in bacterial phylogeny, resulting in a great increase in 16S rRNA sequences, has been the sequencing of this molecule with conserved primers and reverse transcriptase (Lane *et al.* 1985). Nowadays, these techniques have mostly been replaced by direct sequencing of parts or nearly entire 16S or 23S rDNA molecules by using PCR and appropriate primers. The reliability of the technique depends on the size of the conserved elements, i.e. the larger the conserved elements, the more information they bear, and thus the more reliable the conclusions (Vandamme *et al.* 1996).

Sequencing of universal bacterial fragments in the rRNA molecule has been used to classify and detect unculturable organisms (Amann *et al.* 1995). Initially, rRNA sequence analysis was used to determine relationships between genera, families and other higher ranks. Thereafter, it rapidly

became a tool in species delineation, often used without DNA hybridization studies or equivalent techniques. Many researchers objected to this approach, stating that it gave insufficient information for species classification. Fox *et al.* (1992), for example, stated that results from 16S rRNA sequencing were insufficient to guarantee species identity. This group reported that a 99.5% rRNA sequence similarity existed for three phenotypically similar *Bacillus* strains. DNA hybridization data showed, however, that these strains belonged to two distinct species. Fox *et al.* (1992) thus proposed that taxa with virtually identical rRNA sequences be referred to as rRNA subspecies complexes, or rRNA superspecies.

Alternatively, Stackebrandt and Ludwig (1994) noted that rRNA sequence analysis may replace DNA hybridization studies. Based on this review paper, organisms that generally share more than 97% rRNA sequence similarity may belong to a single species. Strains with a rRNA sequence similarity of less than 97% yielded a DNA reassociation of not more than 60%, irrespective of the DNA-DNA hybridization method used. The authors concluded that rRNA sequence analysis may replace DNA hybridization studies as part of the description of new species, but only if the rRNA similarity level is above 97% and if rRNA sequence data of all relevant taxa are included in the comparison.

Stackebrandt and Ludwig (1994) also stressed that the accuracy of a phylogenetic study depends to a great degree on the selection of the number of organisms and on the selection of reference organisms. Stackebrandt and Ludwig (1994) suggested the inclusion of a wide range of apparently related and apparently unrelated reference strains.

Results of rRNA sequencing or DNA-rRNA hybridizations have in the past several years led to the taxonomic reassignment of many *Lactobacillus* spp. to other taxa. Collins and Wallbanks (1992), for example, have transferred *Lactobacillus minutus, Lactobacillus rimae* and *Lactobacillus uli* to the genus *Atopobium.* These taxonomic reassignments indicate the discrepancies that exist between traditional phenotypic tests and rRNA sequencing. Schleifer and Ludwig (1995a) constructed a phylogenetic tree of the lactic acid bacteria using distance matrix, parsimony and maximum-likelihood analysis (see Fig. 1). These results were largely in agreement with data published by Collins *et al.* (1991).

Ribosomal RNA homology studies have resulted in the creation and refinement of the three main *Lactobacillus* groups, i.e. the *Lact. delbruekii* group (Hammes and Vogel 1995), the *Lact. casei-Pediococcus* group (Collins *et al.* 1991; Schleifer and Ludwig 1995a), and the *Leuconostoc* group (Collins *et al.* 1991). Collins *et al.* (1991) has reported 16S rRNA homologies of 90.8 to 99.3% for the *Lact. delbruekii* group and 90.3 to 99.0% for the *Lact. casei-Pediococcus* group. Within the *Leuconostoc* group, very low homology values of 85.9 to 91.5% and 85.9 to 86.8% were recorded with strains of *Leuc. oenos*. This resulted in the exclusion of *Leuc. oenos* from the genus *Leuconostoc*, and its designation as a new genus *Oenococcus* gen. nov. and species *Oenococcus oeni* (Dicks *et al.* 1995a).

The three main *Lactobacillus* groups, as defined by rRNA homology studies, are:

(i) ***Lact. delbruekii* group**. This group contains the type species of the genus *Lactobacillus*, *Lact. delbrueckii*. *Lact. delbrueckii* contains three subspecies, i.e. *Lact. delbrueckii* subsp. *bulgaricus*, *Lact. delbrueckii* subsp *delbrueckii* and *Lact. delbrueckii* subsp. *lactis*, which cannot be discriminated by rRNA sequence analyses. In addition, the seven species of the *Lact. acidophilus*-group are also included, as well as *Lact. acetotolerans, Lact. hamsteri, Lact. jensenii, Lact. kefiranofaciens, Lact. amylophilus* and *Lact. amylovorus*.

(ii) ***Lact. casei-Pediococcus* group**. This group contains 37 *Lactobacillus* spp. and five *Pediococcus* spp., making it the largest phylogenetic group within the lactobacilli. The four pediococci, *Ped. acidilactici, Pediococcus damnosus, Pediococcus parvulus* and *Pediococcus pentosaceus*, form a separate clade from *Pediococcus dextranicus*, which is closer related to *Lactobacillus coryniformis* and *Lactobacillus bifermentans* than to the other pediococci. *Lact. bifermentans* has, depending on the pH, an aberrant type of fermentation (Pette and Beynum 1943; Kandler *et al.* 1983).

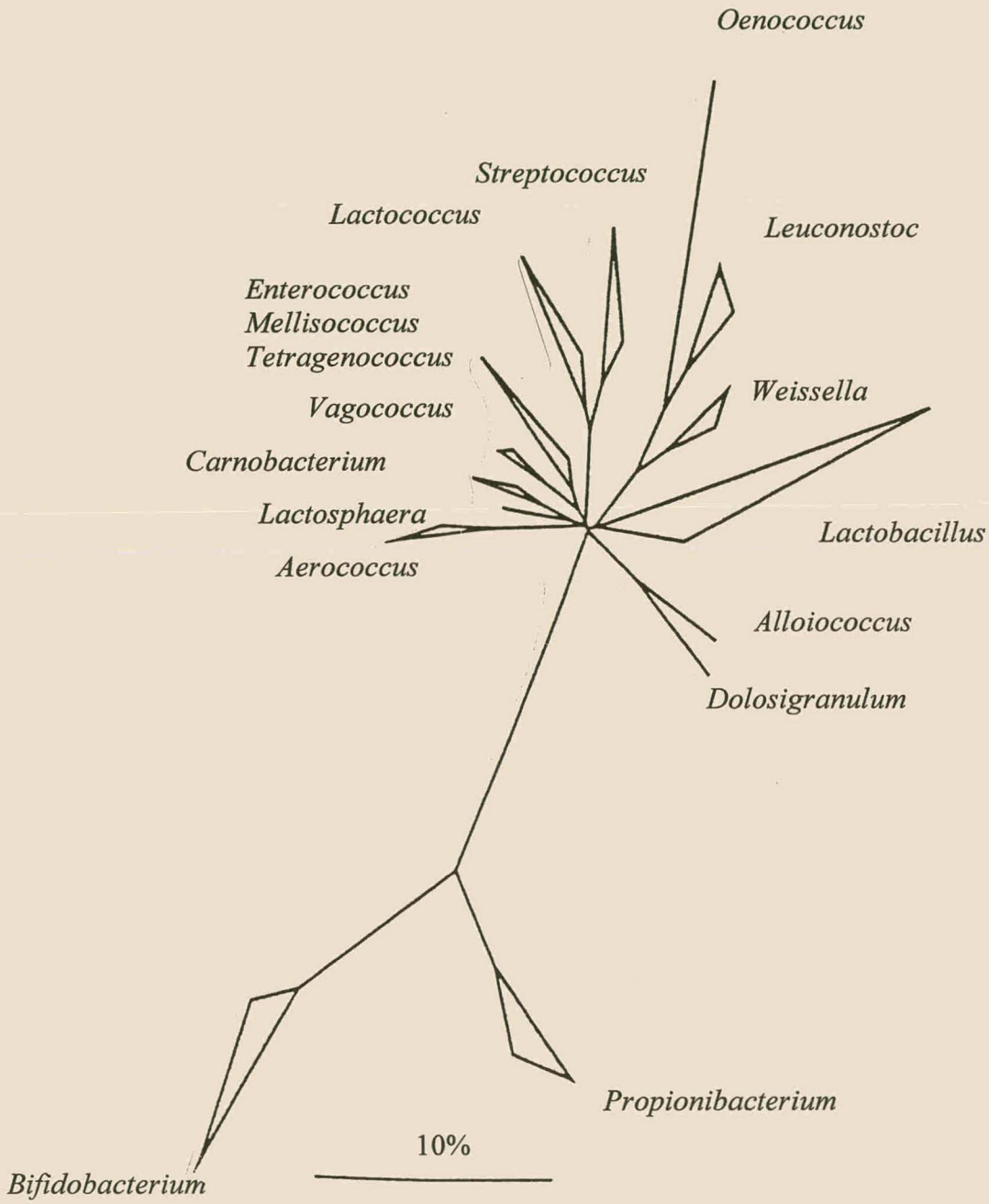

Figure 1 Consensus tree of the major phylogenetic groups of lactic acid bacteria and their relatives based on distance matrix and maximum parsimony analyses of at least 90% complete 16S rRNA sequences. The triangles indicate the phylogenetic groups. The extremes of high and low phylogenetic distances among the members of the particular phylogenetic group are indicated by the length of the edges. The bar indicates 10% estimated sequence divergence (obtained from Schleifer and Ludwig 1995b).

(iii) ***Leuconostoc* group**. Two subgroups are contained within this group. The first subgroup is the *Leuc. paramesenteroides* cluster, which contains the former species *Lactobacillus confusus, Lactobacillus halotolerans, Lactobacillus kandleri, Lactobacillus minor, Lactobacillus viridescens* and *Leuc. paramesenteroides*. The second subgroup comprises the rest of the leuconostocs, as well as *Lactobacillus fructosus* and *Oen. oeni* (Schleifer and Ludwig 1995a). The *Leuc. paramesenteroides* group was recently assigned to the new genus *Weissella*, which consists of the species *Weissella paramesenteroides, Weissella confusus, Weissella halotolerans, Weissella kandleri, Weissella minor, Weissella viridescens*, and a new species, *Weissella hellenica* (Collins *et al.* 1993).

(iv) **Other lactobacilli**. Ribosomal RNA sequence analysis has revealed that many lactobacilli have been misnamed. *Lactobacillus catenaformis* and *Lactobacillus vitulinus* are related to the clostridia and to *Erysipelothrix*. The species which are related to the actinomycete branch of the Gram-positive bacteria are *Lact. minutus, Lact. rimae* and *Lact. uli,* which were all transferred to the genus *Atopobium* (Collins and Wallbanks 1992). *Lactobacillus xylosus* and *Lactobacillus hordniae* were transferred to *Lactococcus lactis* (Kilpper-Bälz *et al.* 1982).

rRNA-targeted oligonucleotide probes

The ever-increasing availability of the number of DNA and rRNA nucleotide sequences has facilitated the comparison of homologous sequences between different taxa of lactic acid bacteria. These homologous oligonucleotide regions may be labelled for use as probes in hybridization experiments to identify unknown isolates. It is also possible to amplify these specific sequences by PCR to enhance detection levels of hybridizations (Vandamme *et al.* 1996). This technique has been applied in the identification of many *Lactobacillus* spp. Ferrero *et al.* (1996) performed strain typing of *Lact. casei* and *Lact. paracasei.* This was achieved by the restriction of genomic DNA of the two species, followed by pulsed-field gel electrophoresis of the DNA fragments. After Southern blotting, the restricted membrane-bound DNA was hybridized with 23S rRNA-targeted probes. Species-specific 23S rRNA targeted oligonucleotide probes have been designed, for example, by Pot and Janssens (1993), to authenticate *Lact.*

acidophilus, Lact. johnsonii and *Lact. gasseri*, by Hertel *et al.* (1993), to identify *Lact. delbruekii, Lact. paracasei* and *Lact. helveticus,* and by Ferrero *et al.* (1996), to identify *Lact. casei* and *Lact. paracasei.*

DNA-based typing methods

These methods refer to techniques used to subdivide species into a number of distinct types (Vandamme *et al.* 1996). Subtyping of species has classically been performed by means of phenotypic analysis, some of which include biochemical or serological tests, antibiotic susceptibility patterning, phage or bacteriocin typing (Maslow *et al.* 1993; Tenover *et al.* 1995) and plasmid profiling (Hill and Hill 1986). A disadvantage of plasmid profiling is that bacterial strains do not always retain their plasmids (Hill and Hill 1986). A variation of this technique, using plasmids as probes for particular strains containing complementary DNA has also been applied (Tannock *et al.* 1992). Due to the instability of many phenotypic characteristics, the use of these strain typing systems is limited (Dykes and von Holy 1994). DNA-directed typing methods have only recently been developed, and have replaced classical typing methods in many laboratories. These methods are reproducible, simple and highly discriminatory (Maslow *et al.* 1993; Tenover *et al.* 1995).

Restriction fragment length analysis

The first DNA-based typing methods included whole-genome restriction fragment length analysis and plasmid DNA analysis. In the former method, whole-genome DNA is digested with restriction enzymes, separated by agarose gel electrophoresis, followed by visualizatiion of the banding patterns. Restriction fragment length polymorphisms are then noted. A disadvantage of this technique is that very complex patterns are often generated which are difficult to compare (Vandamme *et al.* 1996). The technique of restriction fragment length analysis has been applied to plasmid analysis, yielding simpler banding patterns (Vandamme *et al.* 1996). Reduction of the number of DNA fragments produced can be achieved by selecting restriction enzymes which rarely cut DNA by recognising a specific combination of six to eight bases. Since the DNA

fragments yielded from low frequency restriction fragment analysis are very large, they are separated by pulsed-field gel electrophoreses. This method is known to be very discriminatory (Gordillo *et al.* 1993; Maslow *et al.* 1993; Tenover *et al.* 1995) at the species or infraspecific level (Jayaro *et al.* 1991). A modification of this method is the transfer of the DNA fragments to a membrane, which is then hybridised with a labelled probe. Ribotyping is an example of this method, where the probe used is rRNA (Grimont and Grimont 1986). 16S or 23S RNA, or both, may be used, with or without the spacer region, as well as a conserved oligonucleotide part of the RNA (Bingen *et al.* 1994).

Polymerase chain reaction (PCR)

The method of polymerase chain reaction (PCR) has also proved to be very useful in typing bacteria. This method is universally applicable, simple and rapid. Assays in which short arbitrary sequences are used as primers include arbitrarily primed PCR, where 20mer oligonucleotides are used (Welsh and McClelland 1990), as well as randomly amplified polymorphic DNA analysis (RAPD-PCR), where primers may be, for example, 5 bases (Caetano-Anolles *et al.* 1991) or 10 bases in length (Du Plessis and Dicks 1995).

Random amplified polymorphic DNA (RAPD)-PCR has proven to be extremely useful in the classification of many *Lactobacillus* spp. Holzapfel *et al.* (1997) has successfully used this method, together with SDS-PAGE of soluble cell proteins, to distinguish between representative species of the "*Lact. acidophilus*-group", the "*Lact. casei*-group", and between *Lact. fermentum* and *Lact. reuteri*. This technique has also been used to differentiate *Lact. acidophilus, Lact. crispatus, Lact. amylovorus, Lact. gallinarum, Lact. gasseri* and *Lact. johnsonii* (Du Plessis and Dicks 1995). The results corresponded well with results obtained from numerical analysis of soluble cell protein patterns and strains with identical L(+)-nicotinamide adenine dinucleotide-dependant lactic dehydrogenase (nLDH) could also be differentiated (Du Plessis and Dicks 1995). RAPD-PCR has also proved useful for distinguishing among strains of *Lact. plantarum* and *Lact. pentosus* (Van Reenen and Dicks 1996).

Alternatively, the primers used may be consensus motifs complementary to fragments of repetitive elements dispersed throughout the genomes of Gram-positive or Gram-negative bacteria (Versalovic *et al.* 1991; Lupski and Weinstock 1992) or to tRNA gene fragments (McClelland *et al.* 1992). The latter method has been reported to allow for classification at species (Welsh and Weinstock 1992) and infraspecific (Seal *et al.* 1992) levels. Ribosomal DNA genes have also been amplified by using universal rDNA primers. Polymorphisms among different rDNA operons generate simple arrays of DNA fragments with different lengths (Kostman *et al.* 1992).

A combination of PCR-based DNA-typing and restriction enzyme analysis has been developed, called the amplified-rDNA restriction analysis method. The PCR product (16S or 23S rDNA or parts of both genes with or without the spacer region) is amplified using universal primers found in the conserved regions of the rRNA genes. The amplicon is subsequently digested using a combination of restriction enzymes. This method generates mostly species-specific patterns (Gurtler *et al.* 1991; Jayaro *et al.* 1991), due to the conserved character of rRNA genes.

Amplified fragment length polymorphism (AFLP) is another PCR-restriction enzyme-based technique (Zabeau and Vos 1993). This method involves restriction fragment length polymorphism analysis, with PCR-mediated amplification to select specific DNA fragments from the pool of restriction fragments. Screening thus occurs for amplified fragment length polymorphisms by selective amplification of restriction fragments.

CONCLUSION

Although most taxonomic revisions proposed during the last decade were based mostly on rRNA sequence information, traditional phenotypic analyses still play an important role in the identification of strains. The re-evaluation of phenotypic classification systems for lactic acid bacteria has been possible through the use of phylogenetic methods. An example of this is the effective phenotypic description of new genera such as *Oenococcus* and *Weissella*, which form well-defined phylogenetic and phenotypic entities (Vandamme *et al.* 1996).

DNA-DNA hybridization studies play an important role in determining the inter- and intraspecific relationships among strains, which is not revealed by rRNA sequence analyses. SDS-PAGE of whole-cell proteins is reliable in elucidating relationships at species and subspecies levels. Ribosomal RNA hybridization studies reveals the phylogenetic relatedness among strains or species. The disadvantages of this technique are twofold. Firstly, the conserved nature of rRNA molecules may result in cross-reactions between closely related species. The second disadvantage is that variation in hybridization procedures is large, creating problems when large numbers of strains are being identified. A polyphasic approach, i.e. using a combination of all available phenotypic and genetic techniques, is the most effective to identify unknown isolates.

FORTIFIED WINE

Introduction

Sherry, more or less resembling Spanish sherry, has long been produced in other wine-growing regions of the world. In the European Union, the term "sherry" refers to oxidized wines from southern Spain, in particular from the region of Jerez de la Frontera in the Cadiz province of Andalusia (Galletti and Carnacini 1996). Ageing with flor yeasts is, however, practiced in other areas of the world, such as France, the Republic of South Africa, Armenia, California and southern Australia (Galletti and Carnacini 1996). These sherries are often named after the region they are produced in, e.g. "Australian sherry" or "Californian sherry" (Webb and Noble 1976).

In September 1998, a trademark dispute over the use of the names "sherry" and "port" occurred between the European Union (EU) and the South African wine-producing community. The cause of outrage among the wine-producers in South Africa was the EU's refusal to offer its improved free trade agreement for South African agricultural products until South Africa allowed EU fishermen access to South African waters, and until South Africa banned the use of the words "sherry" and "port" on its products. With respect to the use of the words "sherry" and "port", the EU claimed that these two names refer to specific places, i.e. the word port refers to the city Oporto in Portugal, and the word sherry refers to Jerez in Spain. The names "port" and "sherry" should thus be the sole property of Portugese and Spanish producers, respectively. South African wine-producers claimed in their defense that sherry has been made in South Africa for more than a century and port for 300 years. Furthermore, the names "port" and "sherry" have been used to describe these fortified wines for just as long.

South Africa rejected the terms of the agreement, and the talks were suspended until the EU could present South Africa with an improved proposal for the access of SA agricultural products to the EU (Kobokoane and Jordan 1998; Kobokoane 1998). On February 1, 1999, South Africa accepted the EU's new trade agreement, which included allowing South Africa to continue selling its port and sherry under these names in their domestic market indefinitely (Burbridge and Fabricius 1999). South Africa indicated that it would sign the free trade deal, however, five EU

foreign ministers rejected the agreement (Fraser 1999). Spain, supported by France, Portugal, Italy and Greece, claimed that the deal was too generous to South African agricultural exporters and would led to financial losses among European farmers and food processors (Fraser 1999).

The existing agreement allows for the names "sherry" and "port" to be used after a 12 year phase-out period (Fraser, 1999). The Spanish foreign minister, Abel Matutes, argued that they were willing to extend this period to 15 to 20 years, after which these names may no longer be used. Foreign Minister Matutes was also concerned that no guarantees had yet been made for fishing rights in South African waters (Fraser 1999).

Webb and Noble (1976) describe three sherry types; the film (flor) sherry produced in the Jerez de la Frontera region, submerged-culture flor and baked sherries. Goswell (1986) includes oloroso and amontillado sherry as a subgroup to fino sherry. Muscadel/Hanepoot wines belong to a group of sherries which are known for their extreme sweetness (Goswell 1986).

Viticulture and Vintage

Sherry, authentic to the Jerez de la Frontera region of Spain (Manuel and Gonzalez 1972), is traditionally produced by completely fermenting the wine, followed by a fortification step (Goswell 1986). The base wines are therefore always dry, but are also often sweetened before sold (Goswell 1986).

The Palomino grape variety is used in Jerez and in most other regions for sherry production (Manuel and Gonzalez 1972; Goswell 1986). In South Africa, the main grape varieties which are used to produce fino and oloroso sherries are Chenin Blanc, Palomino, Sémillon and Pedro (Hughes *et al* 1992). The most commonly used variety is Palomino Fina (Goswell 1986). The variety of grape used to make sherry must fit certain criteria. The grape has to yield a white wine of clean but neutral character, lacking in acidity and high in readily oxidizable polyphenols– which are essential in the production of good oloroso sherry (Goswell 1986). The grape must

also be low in tannins to avoid the "coarseness" which interferes with the delicate fino sherry flavour (Webb and Noble 1976), and to avoid inhibition of flor growth (Hughes *et al* 1992).

The Palomino grape variety has the ability to give reasonable yields on calcium carbonate-rich but nutrient-poor soils, which are vital in contributing to the specific character of sherry (Goswell 1986). When grafted onto a suitable rootstock, e.g. Chasselas x Berlandieri 41B, Palomino fulfills the above criteria (Goswell 1986). The Palomino variety is known for its modest sugar content (fully ripe at 18° to 20° Balling) and its low level of fixed acid, which does not give much scope to the wine maker (Hughes *et al* 1992). The Palomino vine performs well on higher pH soils under irrigation in warmer climates (Hughes *et al.* 1992). In South Africa, it is one of the oldest known varieties to have been grown in the Cape (Hughes *et al.* 1992).

When the grapes have attained a sugar content of 11.5 to 12.5 Baumé, they are harvested, which is usually during the first and second weeks of September in the Northern hemisphere (Goswell 1986). Ideally, the level of tartaric acid in the must should be approximately 5g l^{-1}. In most musts, however, the concentration of tartaric acid is only half of what it should be. The traditional way to rectify this is to add solid calcium sulphate to the grapes at crushing (approx. 1 kg per 600-liter butt); this reacts with potassium bitartrate in the wine to produce a precipitate, calcium tartrate. This presumably lowers the buffering capacity of the wine and lowers its pH. Additions of tartaric acid are currently used as a means to increase the titratable acidity of the wine (Goswell 1986).

Pedro-Ximinez grapes are used in the production of Pedro-Ximinez sherry. A proportion of this grape variety, together with the Palomino grape is also used to make oloroso sherry (Webb and Noble 1976). The grapes used to produce oloroso sherry have a higher sugar content than those used for fino sherry, due to the dehydration of the grapes for two to three days in the sun (Webb and Noble 1976).

Winemaking Methods

To make fino-style sherry, a must low in solids is desirable (Goswell 1986). Sulphur dioxide is added by most winemakers at crushing, however, not all winemakers perform this practice since they believe that it increases the amount of tannin which is retained in the wine (Goswell 1986). The level of SO_2 during alcoholic fermentation influences the future aroma of the wine, due to its formation of complexes with carbonyls such as acetaldehyde, 2-ketoglutarate and 4-oxobutyrate. These complexes may later dissociate and form aromatic compounds (Webb and Noble 1976).

The presence of naturally occuring yeasts in the must is sufficient to perform a considerably clean fermentation (Goswell, 1986). Inoculation of the must with selected yeast strains produces wine of similar quality, but is only performed when high rainfalls and low temperatures are experienced during the harvest season (Goswell 1986). Some winemakers prefer to inoculate with flor yeast at the beginning of the primary (alcoholic) fermentation and not after the fermentation has been completed (Webb and Noble 1976).

The temperature of the must is approx. 20 °C when it enters the cellar. The must settles for approx. 24 h before the onset of fermentation. The fermentation is traditionally performed at 27 °C in wooden casks (butts) of about 600 l and is completed in approx. one week. More recently, these fermentations have been conducted in large vertical stainless steel tanks which allows for better control of the fermentation temperature (Goswell 1986).

Several factors contribute to the formation of aroma compounds during alcoholic fermentation, i.e. the yeast strain, temperature, SO_2 concentration, juice clarity and aeration (Webb and Noble 1976). The ideal fermentation temperature at which the greatest variety and the highest concentrations of aroma compounds are formed is between 10-15 °C. Film and submerged-culture flor wines are therefore fermented at low to moderate temperatures to optimise the production of aromatic compounds.

Fermentation yeasts

The initially dominant yeasts responsible for performing fermentations in the Jerez area are *Kloeckera apiculata*, *Hanseniaspora valbyensis*, *Metschnikowia pulcherima*, *Saccharomyces cerevisiae*, *Saccharomyces chevalieri*, *Saccharomyces italicus* and *Saccharomyces rosei* (Inigo-Leal *et al.* 1963). As the alcohol concentration increases, the numbers of *Metsch. pulcherima* decrease, while the numbers of *Sacch. chevalieri* increase (Inigo-Leal *et al.* 1963). At the end of the fermentation, the predominant yeasts are *Sacch. cerevisiae*, *Sacch. chevalieri* and *Sacch. italicus* (Inigo-Leal *et al.* 1963). Significant differences exist between the yeasts found in musts from vineyards in different areas of Jerez, and in the early stages of fermentation (Inigo-Leal *et al.* 1963). In all cases, however, the final phase of the fermentation is dominated by *Sacch. cerevisiae*, *Sacch. chevalieri* and *Sacch. italicus*.

In Jerez, malolactic fermentation usually occurs immediately after alcoholic fermentation. The importance of controlling this reaction has only recently been realised in Jerez, which may explain why studies of its occurrence in this area have not yet been published (Goswell 1986).

Post-fermentation treatment

The base wine produced for sherry fermentation has the same characteristics as a dry white wine, but lacks varietal flavours and does not last well in the bottle. However, because of its neutral base, the wine provides a background for the creation of the characteristic sherry flavours (Goswell 1986).

The wine clarifies spontaneously and is racked-off the lees. The alcohol content is increased to 14.5% - 15% (v/v) by the addition of distilled alcohol (Goswell 1986). This fortification prevents the growth of acetic acid bacteria, which are inhibited by alcohol levels of above 13.5% (v/v) (Goswell 1986). Only alcohol distilled from wine is permitted to fortify sherry, but it has been argued that fortification with neutral spirits from other agricultural products would make little difference to the taste of the final product (Goswell 1986).

The sherry blender produces three types of base wine which is used in the production of the final product (Goswell 1986):

- Fino-style wines matured under a layer of flor yeast.
- Amontillado wine, which is matured under flor, followed by a further period of oxidative maturation without flor.
- Oloroso wines, which are matured oxidatively in oak wood without a flor film.

The solera/fractional blending system

The solera system has existed in the Jerez region for centuries (Manuel and Gonzalez 1972). In South Africa, fino-style sherries are produced mainly in the Boberg region (Hughes *et al.* 1992). The main aim of this system is to obtain a wine that does not vary in quality from year to year (Manuel and Gonzalez 1972). The inability of the flor yeast to remain in optimal condition within a single butt of wine for three to six years results in the flavour of sherry not being uniform from cask to cask (Manuel and Gonzalez 1972). This resulted in the development of the solera, or fractional blending system, by the Spanish sherry-makers of the 19th century (Manuel and Gonzalez 1972).

After fortification to 14.5% - 15% (v/v) alcohol, the wine is set aside in casks which are filled to 80% capasity to obtain a large air-to-wine surface (Manuel and Gonzalez 1972). These casks are observed to determine in which a film of yeast (known as flor) would develop spontaneously on the surface, and to determine the quality of the film produced (Manuel and Gonzalez 1972). Fino-style sherries are produced from casks of wine with a thick film of yeast (Manuel and Gonzalez 1972).

The solera

The word solera is used to describe the system of casks used in the production of fino-style sherry. The solera is comprised of several casks stacked in "scales". In a solera of six scales, the first scale (consisting of, for example, 40 or 50 casks) contains the wine ready to be sold. The next scale contains wine approx. a year younger, etc.. The 6th and last scale contains wine of approx. 5 years younger than the wine from the first scale. The same variety of wine is used in all the scales. The name "solera" is usually used to describe the first scale (i.e. the wine ready to be bottled). The remaining scales are named "criaderas" (nurseries), and in some cellars they are called second, third etc. solera scales.

A larger number of scales are used for fino wines than for oloroso and amontillado wines, since the base wine used in fino wines vary much more from year to year. The wine in these casks is treated in the following way. Wine is drawn off from the first scale (the solera), the removed wine from this scale is then replenished with wine from the second scale, which is in turn topped up with wine from the third scale, etc. These drawings are carried out several times a year.

The flor

In Jerez, the base wine undergoes a spontaneous alcoholic fermentation with the flor yeasts naturally present in the cellars (Goswell 1986). In many other sherry producing regions more detailed studies on sherry flor have been conducted (Cruess 1948; Fornachon 1953; Frieberg 1955). According to Fornachon (1953) the ideal conditions for flor film development and maintenance are a dry white base wine without a pronounced varietal flavour, a low tannin content, a pH of 3.1-3.4 and a sulphur dioxide content not more than 100 mg l^{-1}. A wine temperature of 15 °C - 20 °C and a flor film of approx. 15 cm^2 per liter wine is considered to be the ideal. Furthermore, the alcohol content has to be low enough to permit the development of the flor yeast, but high enough to prevent the growth of spoilage organisms, such as *Acetobacter* spp. (Webb and Noble 1976).

According to Goswell (1986), the flor film consists of several yeast species and a few bacteria. Some of the yeasts involved include *Saccharomyces beticus, Saccharomyces cheriensis, Saccharomyces montuliensis, Sacch. cerevisiae* (Goswell 1986) and *Saccharomyces fermentati* (Webb and Noble 1976). Several stages of flor film development have ben described (Fornachon 1953). During the first stage, the yeast grows rapidly to form a complete film over the surface of the wine. Because of this the high redox potential of the wine decreases slowly, ethanol is metabolized to acetaldehyde, glycerol and acetic acid levels decrease and the flor aroma begins to form. The second stage is characterized by a thick complete film covering the wine surface, making the wine anaerobic. In addition, acetaldehyde accumulation slows or stops and the volatile acidity begins to increase. The colour of the wine also lightens to pale gold from its dark amber colour due to the reduction of some of the darker-coloured oxidized phenolic polymers. It is also likely that several of the aroma components are reduced at this stage. During the third stage, the film becomes thinner and turns grey. Aldehydes begin to accumulate and the redox potential starts to increase slowly. It is believed that the reactions of the second stage tend to reverse themselves in the third stage. During the fourth stage the film breaks up and cannot be reestablished. The wine also loses its film odour and taste, and it becomes prone to acetification. In a fino solera, wines in the criadera are in stages 1-2, whilst the older scales contain wines in stages 2-3 (Webb and Noble 1976).

Acetaldehyde, produced by the film stage of the yeast, is one of the main aromatic compounds formed during the production of flor sherry (Egorov and Saakyan 1950). Wines which have been matured on the flor film have acetaldehyde levels of 150 - 350 mg l^{-1}, which is much higher than oxidatively matured wines which have an acetaldehyde level of approximately 80 mg l^{-1} (Goswell 1986).

Amontillado-style sherry

This sherry type is created by drawing off sherry matured under flor, fortifiying it to about 18% (v/v) ethanol to inhibit most of the flor yeasts, and then allowing it to mature in oak casks without the protection of a flor film (Goswell 1986). Self-generated amontillados may also be produced

if the flor dies spontaneously (Goswell 1986). The oak casks containing this wine are usually filled to 95% capacity, and the wine may be subjected to a fractional-blending (using fewer stages than for fino sherry), although the wine would mature satisfactorily even if left in a single cask (Goswell 1986). The oak of the cask has been found to contribute favourably to the flavour of amontillado sherry.

Flavour characteristics of fino and *amontillado sherry*

Fino sherries are characterized as being pungent, very dry, without acidity, pale straw in colour and with a delicate yet pungent aroma (Manuel and Gonzalez 1972). The alcohol content of these sherries ranges between 15.5° and 17° Salleron (Manuel and Gonzalez 1972). Amontillado sherries are also very dry and lean on the nose and palate, and has the same pungent aroma compared to fino sherries, but is usually nuttier and has a fuller body with a deep amber colour which becomes darker with age (Manuel and Gonzalez 1972). The alcohol content of amontillado sherries is 17%-18% (v/v), and may reach 20%-24% (v/v) with age (Manuel Gonzalez 1972).

Oloroso-style sherry

The oloroso sherries of Spain are produced by first allowing the fermentation to proceed to completion, followed by the addition of at least 16% (v/v) alcohol, to prevent the growth of flor yeast and acetic acid bacteria (Goswell 1986). The flor thus has no part in the development of the flavour of oloroso sherry. The wine is placed in wooden casks to mature oxidatively. The oak of the cask and the presence of oxidizable polyphenols are important in contributing to the flavour of oloroso sherries (Goswell 1986). In South Africa, oloroso sherry is produced in the Boberg region and in the Little Karoo (Hughes *et al.* 1992).

Flavour characteristics of oloroso sherry

Oloroso sherries have a less pungent aroma than that of a fino or an amontillado, they have more body or vinosity on the palate, and although dry, they leave a slight sweetness on the palate

(Manuel and Gonzalez 1972). The alcohol content of olorosso sherries is usually 18-20% (v/v), which may reach 24% (v/v) with age. These sherries have a deeper colour than amontillado and may turn to a dark gold colour with age (Manuel and Gonzalez 1972).

Submerged-culture flor sherries

The method used to produce traditional flor sherry is expensive, labour-intensive and uneconomic in terms of cellar space (Goswell 1986). The production of submerged-culture flor sherries is less expensive, less labour-intensive, and a rapid alternative (Goswell 1986).

The base wine used for submerged-culture flor sherries is similar to that used to support flor film growth (Goswell 1986). A suitable yeast is inoculated into the wine, which is aerated by the injection of air or oxygen. The yeast grows rapidly and acetaldehyde is produced at very high concentrations (600 mg l^{-1} or more – twice as high as in traditionally-made fino sherry). Flor yeasts were initially utilized, but many other yeasts were found to give satisfactory results, e.g. *Sacch. beticus* and *Sacch. fermentati* (Ough and Amerine 1960; Webb and Noble 1976). This process is more rapid at alcohol concentrations lower than 14.5% (v/v) alcohol, provided that contamination with acetic acid bacteria is avoided (Goswell 1986).

Two main differences exist between the submerged-culture process and the flor method (Webb and Noble 1976). In submerged cultures a higher surface to volume ratio exists between the yeast at the interface of the wine and the air space, compared to yeast in a flor film (Webb and Noble 1976). Furthermore a higher concentration of acetaldehyde is produced in a much shorter fermentation time, accompanied by various acetals which are not formed in wines fermented with a yeast film (Webb and Noble 1976). Because of the high concentration of acetaldehyde, the sherry is blended with more neutral wines before botteling (Ough *et al.* 1960).

Baked sherries

These sherries are produced by first adding between 18-20% (v/v) alcohol to dry base wine, followed by heating in closed tanks to approximately 50 °C for up to six months, or to 70 °C for six weeks (Webb and Noble 1976; Dinsmoor Webb *et al.* 1964). Sherries with a more favourable taste are produced using a longer incubation time and lower temperatures (Webb and Noble 1976). The basic flavours of these wines arise from Maillard and caramelization reactions (Webb and Noble 1976). After the heating treatment, the wine is matured in wooden containers (Goswell 1986). Baked sherries have been blended with submerged-culture flor sherries to produce a sherry type with a more attenuated aldehydic aroma (Webb and Noble 1976).

Fortification of Sherry After Incomplete Fermentation

These sherries are well known for their extreme sweetness, which is why they are often used to sweeten dry sherries (Goswell 1986). The primary fermentation of these wines is stopped by the addition of distilled alcohol which prevents the yeast from utilizing all of the sugar, hence the sweetness and fruity character of these sherries (Goswell 1986).

Muscatel sherry

Muscatel sherry is a dessert wine which is produced from the Muscatel grape, and is used to sweeten and colour other wines (Manuel and Gonzalez 1972). After harvesting, the grapes are sun-dried for several days (which concentrates the sugar in the grape berry), followed by pressing (Manuel and Gonzalez 1972). This wine is very sweet and has the characteristic Muscatel aroma caused by certain essential oils found near the outer part of the grape berry pip (Manuel and Gonzalez 1972). Moscatel wines have a dark golden colour, which enhances during maturation (Manuel and Gonzalez 1972).

Muscadel/Hanepoot dessert wine

In South Africa, these wines are made from the Muscat d'Alexandrie grape variety (or Muscadel) – known locally as the Hanepoot grape variety (Hughes *et al.* 1992). This grape variety needs considerable heat to reach full maturity, which is why it is usually found in warmer wine regions of the world (Hughes *et al.* 1992). This vine yields sweet, mellow wines of low acidity, with a characteristic 'muscat' flavour (Hughes *et al.* 1992).

The grapes are harvested when they reach a sugar content of approx. 30 °balling, after which they are crushed, and the skins kept with the juice for colour and flavour extraction (Distillers Corporation (Sa) Ltd, pers. comm.). After approx. 24 h, the skins are removed and up to 17% (v/v) alcohol (pure grape spirit) is added to the juice, thus killing the yeasts and therefore preventing any further fermentation (Distillers Corporation (Sa) Ltd, pers. comm.). Since active fermentation is difficult to obtain after 24 h, fermentation does not have a large influence on the character of the wine (Distillers Corporation (Sa) Ltd, pers. comm.). At this point, the juice has a high sugar content and a concentrated grape flavour, which is characteristic of this wine (Distillers Corporation (Sa) Ltd, pers. comm.). After fortification, the wine is matured for several years, after which the wine has a raisin sweetness, described as warm and smooth (Distillers Corporation (Sa) Ltd, pers. comm.). The minimum alcohol requirement for this wine is 16.5% (v/v), but quality Muscadel/Hanepoot wines usually have an alcohol content of approximately 17% (v/v) (Distillers Corporation (Sa) Ltd, pers. comm.). These wines mature well in the bottle for a few years, and an opened bottle (sealed with the original cork) may be kept for a number of months (Distillers Corporation (Sa) Ltd, pers. comm.).

Microbiological Spoilage of Fortified Wines

For many countries, a tax advantage exists in the shipping of wine at less than 18% alcohol (Goswell 1986). As a result, sweet and medium-dry sherries, and similar fortified wines, which were once consumed at 19.5-20.5% (v/v) alcohol, are now produced with an alcohol content of approximately 17.5% (v/v) (Goswell 1986).

Spoilage by yeast

Decreases in the alcohol content of fortified wines raises concern that yeast infections will occur while the wine is in the bottle (Goswell 1986). Many manufacturers practice sterile bottling procedures with microbiological monitoring, but very few cases of yeast contamination have occurred (Goswell 1986). The majority of yeasts present at bottling die within six weeks, but there still remains the concern that virulent organisms will establish themselves in the filling plant (Goswell 1986). Hammond (1975) has also found that several yeasts are alcohol resistant, for example, *Sacch. bisporus* var *bisporus* was isolated from sherries with 22% (v/v) alcohol, and *Sacch. cerevisiae* and *Sacch. bayanus* has been found to grow in 18% ethanol, while most yeasts tolerate 15% (v/v) ethanol. This indicates that even a high alcohol concentration is not always sufficient to inhibit yeast growth and prevent spoilage. Fino sherries are known to be at risk of spoilage if they are slightly sweetened (Goswell 1986).

Spoilage by bacteria

Acetic acid bacteria do not present a problem since they rarely grow in sherries with alcoholic strengths of more than 13.5% (v/v) (Goswell 1986). Some lactic acid bacteria, especially the bacilli, can grow in fortified wines containing up to 21% (v/v) ethanol (Goswell 1986). Most of these strains are slow growing and ferment few substrates other than glucose and fructose, are difficult to isolate in pure culture and are slow growing on test media (Goswell 1986). Only a few positive characteristics allow for characterization (Goswell 1986). *Lact. hilgardii* was first isolated from California wine, and was able to grow in the presence of 18% (v/v) alcohol (Douglas and Cruess 1936). *Lactobacillus trichodes* was first described by Fornachon *et al.* (1949) after it was isolated from spoiled appetizer and dessert wines, and it was found to grow in wine containing up to 21% (v/v) alcohol. *Lact. trichodes* has since been renamed as *Lactobacillus fructivorans* (Weiss *et al.* 1983b). *Lact. fructivorans* often grow as long filaments, or as fragments of cotton wool in contaminated bottled wines, they are very resistant to ethanol, and most strains are sensitive to moderate concentrations of sulphur dioxide (Goswell 1986).

Lact. hilgardii, Lact. fructivorans, Lact. collinoides and *Lact. mali* have been isolated from Douro fortified wines containing 18-20% (v/v) ethanol, and with a pH of 3.5 – 4 (Couto and Hogg 1994).

Gini and Vaughan (1962) have isolated *Bacillus coagulans, Bacillus circulans, Bacillus macerans, Bacillus pumilis, Bacillus sphaericus, Bacillus panothenticus* and *Bacillus subtilis* from spoiled Californian fortified wines and from winery equipment. Some of these bacteria are able to survive conditions normally used to pasteurise these wines, and they are able to grow to such a degree that they create a haze and a sediment.

When levels of mould on the grapes are high, much of the added SO_2 becomes bound to the mould and is thus prevented from inhibiting bacteria (Goswell 1986). The decreased levels of active SO_2 in the wine results in bacterial infections of young, sweet, fortified wines (Goswell 1986). These bacteria can produce high levels of volatile acidity, and once established, they become difficult to control with practical levels of ethanol or sulphur dioxide, the only final control measure being pasteurisation (Goswell 1986).

Mechanisms of Ethanol Tolerance

Ethanol concentrations of 1-10% (v/v) inhibit the growth of most bacteria, but a few organisms are able to grow at ethanol concentrations above 10% (v/v) (Ingram and Buttke 1984). *Lact. fructivorans* is known for its ability to tolerate alcohol levels as high as 21% (v/v) (Fornachon *et al.* 1949). Other lactic acid bacteria able to withstand high alcohol levels include *Lact. brevis, Lact. buchneri* (Farrow *et al.* 1986) and *Lact. hilgardii,* which were isolated from a fortified wine of pH 3-4, and of 18 to 20% ethanol (v/v) (Couto and Hogg 1994). All of the above lactobacilli are members of the *Lact. buchneri* group (Schleifer and Ludwig 1995b).

Alcohol tolerance studies have been performed on *Sacch. cerevisiae* (Thomas *et al.* 1978; Alexandre *et al.* 1994), *Schizosaccharomyces pombe* (Koukou *et al.* 1990), *Kloeckera apiculata* (Alexandre *et al.* 1994), *Zygosaccharomyces bailii* (Couto and Huis in't Veld 1995), *B. subtilis*

(Rigomier *et al.* 1980), *Cl. thermocellum* (Herrero *et al.* 1982), *Lactobacillus heterohiochii,* which has been reclassified as *Lact. fructivorans* (Weiss *et al.* 1983b; Uchida 1975), *Lact. hilgardii* (Uchida 1975; Kandler and Weiss 1986) and *Zymomonas mobilis* (Bringer *et al.* 1985).

Reported mechanisms of ethanol tolerance include the production of heat shock proteins (HSP) by certain bacteria (Völker and Hecker 1992) and changes in the fatty-acid composition and hence the fluidity of the plasma membrane (Couto *et al.* 1996). Ethanol tolerance in *Lact. higardii* is related to changes in the fatty acid composition of the cell, leading to changes in the fluidity of the plasma membrane (Couto *et al.* 1996). Proteins are not involved in the ethanol tolerance mechanism of *Lact. hilgardii* (Couto *et al.* 1997).

Fatty acids of strains of *Lact. hilgardii* encountered in a study on ethanol tolerance include, lactobacillic acid, palmitic acid, oleic acid, vaccenic acid and eicosenoic acid (Couto *et al.* 1996). Organisms showing a high ethanol tolerance do not share a specific pattern of fatty acids (Couto *et al.* 1996). Fatty acids provide a hydrophobic barrier against ethanol, the longer the hydrocarbon chain in the membrane lipids, the greater the surface area for hydrophobic and Van der Waals interactions, and a decrease of the polarity within the hydrophobic core (Carey and Ingram, 1983).

Exposure to ethanol also results in an increase in plasma membrane fluidity of *Lact. hilgardii* (Couto *et al.* 1996). This increase in fluidity is associated with an increase in the level of fatty acids in the plasma membrane (Law *et al.* 1962; McElhaney 1989). Kodicek (1963) proposed that cyclopropane fatty acids perform a role in preventing the close packing of lipids in cell membranes, making these membranes more flexible. Sinensky (1974) explains membrane fluidity as being a compensation for the increase in 'rigidity' due to the action of ethanol. An increase in membrane fluidity under the same conditions has also been reported for *Clostridium* (Herrero *et al.* 1982) and in *Sacch. cerevisiae* (Mishra and Prasad 1989). The opposite was, however, recorded for *Bac. subtilis*, where ethanol induced fatty acid changes resulted in a decrease in membrane fluidity (Rigomier *et al.* 1980).

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3. IDENTIFICATION OF *LACTOBACILLUS* SPP. ISOLATED FROM A SOUTH-AFRICAN FORTIFIED WINE BY NUMERICAL ANALYSIS OF TOTAL SOLUBLE CELL PROTEIN PATTERNS AND 16S rRNA SEQUENCING

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Identification of *Lactobacillus* spp. isolated from a South-African fortified wine by numerical analysis of total soluble cell protein patterns and 16S rRNA sequencing

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A.L. STRATIOTIS, L.M.T. DICKS AND M.D. COLLINS. 1999. Fortified wines contain a high level of unfermented sugars and are prone to spoilage by alcohol-tolerant lactic acid bacteria. A total of 62 strains were isolated from various production stages of one of the more popular fortified wines produced in South Africa. The strains were identified by using numerical analysis of total soluble cell protein patterns and 16S rRNA sequence analyses. The species most predominant were *Lactobacillus vermiforme* (24 strains) and *Lactobacillus casei* subsp. *casei* (32 strains). Twenty-four of the strains of *Lact. vermiforme,* three strains of *Lactobacillus buchneri*, one strain of *Lactobacillus plantarum* and two strains of *Lact. casei* subsp. *casei* were isolated from spoiled fortified wine which contained 22% (v/v) ethanol. The majority of strains of *Lact. casei* subsp. *casei* (27 of the 32) and two strains of *Lactobacillus zeae* were isolated from wine before submerged fermentation. Five strains of *Lact. casei* subsp. *casei* were isolated from wine during the submerged fermentation process. No strains were isolated from unbottled wine which underwent the complete fermentation process and with an alcohol content of 17·20% (v/v). Three distinct phenotypic groups of *Lact. vermiforme* were identified at $r \geq 0{\cdot}70$, separate from *Lactobacillus brevis, Lact. buchneri* and *Lactobacillus hilgardii*. Three phenotypic clusters have been identified for *Lact. casei* subsp. *casei*. This is the first report of the presence of *Lact. vermiforme*, *Lact. zeae, Lact. casei* subsp. *casei* and *Lact. plantarum* in fortified wines.

During the primary fermentation of wine, grape must is fermented by *Saccharomyces cerevisiae* (Goswell 1986). In a secondary fermentation L-malic acid is converted to L-lactic acid and CO_2 by members of the genera *Oenococcus* (previously *Leuconostoc oenos*), *Leuconostoc*, *Lactobacillus* and *Pediococcus* (Davis *et al.* 1985; Wibowo *et al.* 1985; Dicks *et al.* 1995). Wines produced in cold regions, i.e. Germany, France and the Eastern United States, have a high acid content and may benefit from deacidification by MLF. However, wines from warmer viticultural regions, i.e. South Africa, California and Australia, have a lower acidity and a further increase in pH could result in a flat, insipid wine with undesirable sensory characteristics (Davis *et al.* 1985; Wibowo *et al.* 1985) and subsequent growth of spoilage bacteria such as *Pediococcus* and *Lactobacillus* spp. (Rankine and Bridson 1971).

Apart from an increase in pH (Kunkee 1967; Rankine and Bridson 1971; Davis *et al.* 1985), red wines often show a colour reduction of 30% when fermented with *Leuconostoc* spp. (Vetsch and Lüthi 1964). In some cases high concentrations of histamine were reported after MLF, especially in wines of higher pH (Davis *et al.* 1985; Wibowo *et al.* 1985; Radler 1986). These claims are, however, questionable since malolactic bacteria do not produce histamine in wine (Buteau *et al.* 1984).

Little is known about the bacterial population in fortified wines. Malolactic bacteria are generally adapted to alcohol levels of up to 14% (v/v), low pH conditions of 3·2-3·8, and SO_2 levels as high as 30-50 mg l^{-1} (Wibowo *et al.* 1985). The alcohol levels in fortified wines are, however, usually higher than 15% (v/v) and prevents the growth of most malolactic bacteria. However, *Lactobacillus fructivorans, Lactobacillus hilgardii, Lactobacillus brevis* and *Lactobacillus buchneri* can tolerate ethanol levels as high as 20%, v/v (Fornachon *et al.* 1949; Farrow *et al.* 1983; Hecker and Völker 1990) and should thus be able to survive the conditions in most fortified wines, depending on the method of production.

Most fortified wines are produced by adding distilled alcohol after alcoholic fermentation (Goswell 1986). Some of the wines have undergone complete fermentation prior to fortification (flor sherry), whereas others have had their fermentation halted by fortification, i.e. sweet dessert wines (Goswell 1986). The high level of sugars that remain in these wines may become a source of energy for microbial growth and spoilage (Goswell 1986). *Lact. hilgardii*, *Lact. fructivorans* (including previously identified strains of *Lactobacillus trichodes,* Fornachon *et al.* 1949),

Lactobacillus collinoides and *Lactobacillus mali* have been isolated from Douro fortified wines (Couto and Hogg 1994).

To date, microorganisms responsible for causing spoilage in South African fortified wines have not received much attention. The aim of this study was to identify the *Lactobacillus* spp. isolated from a South-African fortified wine. The phenotypic relatedness of the strains was determined by using numerical analysis of total soluble cell protein patterns, and the genetic relatedness by 16S rRNA sequencing.

MATERIALS AND METHODS

Isolation of bacteria and reference strains

Bacteria were isolated from a popular sweetened fortified wine produced in South Africa. Samples were taken from three different stages during production and from a spoiled bottled product. The first sample was taken from dry white wine before the onset of submerged-culture flor sherry fermentation. The second sample was taken from fortified wine during submerged fermentation with an alcohol content of 11·92% (v/v). The third sample was from fortified wine after completion of the fermentation process and with an alcohol content of 17·20% (v/v), before the addition of sweet wine. The fourth sample was taken from a bottle of fortified wine which underwent microbial spoilage. The spoilage was visible as a haze and a sediment in the bottle.

Seven-hundred-and-fifty ml from each of the four samples were centrifuged (8 500 x *g*, 10 min), the pellet resuspended in 1 ml saline solution (80%, w/v, NaCl) and then serially diluted in 10 ml saline. Aliquots from these dilutions were spread-plated onto MRS agar (Biolab). All plates were incubated at 30°C for five days, after which pure cultures were obtained.

The reference strains included in this study were obtained from the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen (DSM) and the National Collection of Food Bacteria (NCFB).

Preliminary identification

All isolates were gram stained, and tested for the production of catalase by using 5% (v/v) hydrogen peroxide. Catalase-negative, Gram-positive rods or cocci were selected and screened for the production of CO_2 from glucose and gluconate, according to the methods described by Dicks and Van Vuuren (1987). All isolates were stored at –80°C in glycerol (40%, v/v).

Numerical analysis of total soluble cell protein patterns

The strains were cultured in 50 ml MRS broth for 18h at 30°C. The methods used for the preparation of whole-cell protein extracts, SDS-PAGE, and preparation of the gels for numerical analysis, were as described by Pot *et al.* (1994b). The software package GEL COMPAR (version 4·0) of Applied Maths (Kortrijk, Belgium) was used to analyze the protein fingerprints (Vauterin and Vauterin 1992). This program recorded the normalized electrophoretic protein patterns of the densitometric traces. Similarity between all pairs of protein patterns was expressed using the Pearson product moment correlation coefficient (*r*), and cluster analysis was performed by the unweighted average pair-group (UPGMA) method.

16S rRNA sequencing

16S rRNA was performed on representative strains selected from the protein profile clusters. The method described by Collins *et al.* (1991) was used. PCR was used to amplify a 16S rRNA gene using conserved primers close to the 3' and 5' ends of this gene. The PCR products were purified with the Prep-A-Gene kit (Biorad), and directly sequenced using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer. The EMBL/Genbank Data Library and the FASTA program were used to locate relatives of the isolates, and the retrieved sequences were then aligned with the newly determined sequences using the program PILEUP.

RESULTS

A total of sixty-two Gram-positive and catalase negative rods were isolated from the wines (Tables 1 and 2). Twenty-seven strains were isolated from wine before the onset of submerged fermentation and five strains from wine which was, at the time, undergoing submerged fermentation. No strains were isolated from wine which underwent the complete fermentation process and with an alcohol content of 17·20% (v/v). Thirty strains were isolated from bottled fortified wine which contained 22% (v/v) ethanol.

Twenty-seven strains produced CO_2 from glucose and were classified as obligately heterofermentative (group III, Kandler and Weiss 1986). All of these strains were isolated from bottled fortified wine which underwent spoilage. The phenotypic relatedness of these strains, as determined by numerical analysis of total soluble cell protein patterns, is shown in Fig. 1. Five clusters were delineated at $r = 0{\cdot}70$, with reference strains of *Lact. brevis, Lact. buchneri* and *Lact. hilgardii* in one cluster at $r \geq 0{\cdot}72$. Three strains formed cluster II at $r \geq 0{\cdot}85$. Cluster III consisted of eight strains which clustered at $r \geq 0{\cdot}80$. The fourth cluster comprised five strains which clustered at $r \geq 0{\cdot}75$. Eleven strains formed cluster V at $r \geq 0{\cdot}79$. Based on 16S rRNA sequence analyses, the strains in cluster II are members of *Lact. buchneri*, whereas the strains in clusters III - V belong to the species *Lactobacillus vermiforme* (Table 1).

Thirty-five strains produced CO_2 from gluconate, but not from glucose and were classified as facultatively heterofermentative. Twenty-seven of these strains were isolated from wine before the onset of submerged fermentation, five strains were isolated from wine which, at that stage, underwent submerged fermentation and three strains from a bottle of spoiled fortified wine (Table 2). The phenotypic relatedness of these strains, based on their protein banding patterns, is shown in Fig. 2. Four clusters were delineated at $r = 0{\cdot}84$. Cluster I contained the type strain of *Lactobacillus plantarum* (ATCC 14917^T), *Lact. plantarum* ATCC 8014 and strain LB100 (2) at $r \geq 0{\cdot}91$. Nineteen wine strains grouped in cluster II at $r \geq 0{\cdot}88$; two strains (A27 and A29) grouped with the type strain of *Lactobacillus casei* subsp. *casei* (ATCC 393^T) at $r \geq 0{\cdot}90$ in subgroup 1, separate from 17 wine strains and *Lact. casei* subsp. *alactosus* ATCC 25180 which grouped at $r \geq 0{\cdot}90$ in subgroup 2. The 12 strains in cluster III grouped at $r \geq 0{\cdot}85$ and linked with the strains in clusters I and II at $r \geq 0{\cdot}80$. The three strains in cluster IV formed a

phenotypic group at r ≥ 0·84, but were less closely related to the strains in clusters I - III. Based on 16S rRNA performed on strains selected from the clusters, strain LB100 (2) in cluster I is a member of *Lact. plantarum*. The strains in clusters II - IV belonged to the same 16S rRNA homology group as *Lact. casei* subsp. *casei* (Table 2).

DISCUSSION

Numerical analysis of total soluble cell protein patterns grouped the five reference strains of *Lact. brevis, Lact. buchneri* and *Lact. hilgardii* into one cluster at $r \geq 0{\cdot}72$ (Fig. 1), suggesting that the three species are phenotypically not that destinct. This is in correlation with our previous findings, i.e. strains of *Lact. buchneri, Lact. brevis* and *Lact. hilgardii* cannot be differentiated by using simple physiological tests (L.M.T. Dicks, M.Sc. thesis, University of Stellenbosch, Stellenbosch, South Africa 1985). Sharpe (1981) proposed the reclassification of *Lact. buchneri* as a subspecies of *Lact. brevis*, based on the many phenotypic similarities between the two species.

Previous results obtained by numerical analysis of total soluble cell protein patterns (Dicks and Van Vuuren 1987) have clearly indicated that *Lact. brevis* is a phenotypically heterogeneous species. This, and the fact that three DNA homology groups have been described for *Lact. brevis* (Vescovo *et al.* 1979), led to an investigation to the merging of *Lact. buchneri* and *Lact. brevis* into one genetic group (personal communication, Dr. A. Kagermeier, Poliklinik für Zahnerhaltung und Parodontologie, Erlangen, Germany). This could explain why strains 85224a, 85759a and 85224b (cluster II) grouped with the type strain of *Lact. buchneri* into the same 16S rRNA cluster (Table 1), despite their low phenotypic relatedness ($r \geq 0{\cdot}65$) with *Lact. buchneri, Lact. brevis* and *Lact. hilgardii* (Fig. 1). The isolation of *Lact. buchneri* from fortified wine is not surprising, since the species is known for its ability to tolerate high alcohol levels (Farrow *et al.* 1986).

The strains in clusters III, IV and V formed tight groups within each cluster, suggesting that they belong to three phenotypic well defined groups. Furthermore, the overall protein patterns of these strains were different from that obtained for the strains in clusters I and II, as evident by the low correlation values recorded (Fig. 1). Results obtained by 16S rRNA sequence analyses have

clearly shown that the strains in clusters III - V are members of *Lactobacillus vermiforme* (Table 1), well separated from *Lact. hilgardii* and any other *Lactobacillus* sp.

DNA hybridization studies performed by Farrow *et al.* (1986) on three strains, designated as *Lact. vermiforme* NCDO 961, NCDO 962 and NCDO 1965, indicated that they shared a high DNA homology (72 - 90%) with the type strain of *Lact. hilgardii* (NCDO 264^T). Based on these results, the species name *Lact. vermiforme* was rejected (Kandler and Weiss, 1986). However, more recent taxonomic studies on two strains (ATCC 11540 and ATCC 13133), which resemled the original description of *Betabacterium vermiforme* (later reclassified as *Lact. vermiforme*), could not be designated into any of the presently known *Lactobacillus* spp. and were classified as an unknown *Lactobacillus* sp. (ATCC Culture Collection Catalogue, 1999). Strain ATCC 11540 was isolated from a ginger beer plant Mayer (1938). The origin of strain ATCC 13133 is not known. Strains ATCC 11540 and ATCC 13133 were not available when we initiated the present study. The strains which we have isolated from bottled fortified wine might represent the authentic strains of *Bet. vermiforme*. Further research is needed to determine the relatedness of these strains to *Lact. vermiforme* ATCC 11540 and ATCC 13133. It might be that the name *Lact. vermiforme* will have to be revived.

Strain LB100 (2), which formed a tight phenotypic cluster with the type strain of *Lact. plantarum* (ATCC 14917^T) and *Lact. plantarum* ATCC 8014 (cluster I, Fig. 2) is also genetically closely related to *Lact. plantarum*, as determined by 16S rRNA sequencing (Table 2). Strain LB100 (2) is thus classified as *Lact. plantarum*.

The remaining strains of the facultatively heterofermentative lactobacilli grouped into three well separated protein profile clusters (Fig. 2), indicating that they belong to at least three phenotypically diverse groups.

Lact. casei subsp. *casei* (ATCC 393) grouped with two wine strains (A27 and A29) in one subgroup, separate from the other strains of *Lact. casei* subsp. *casei* in cluster II (Fig. 2). Similar results were recorded in our previous studies (Dellaglio *et al.* 1991; Dicks *et al.* 1996) which, at the time, led to a proposal to reclassify *Lact. casei* subsp. *casei* ATCC 393 (and *Lactobacillus rhamnosus* ATCC 15820) as *Lactobacillus zeae* nom. rev. (Dicks *et al.* 1996). The proposed reclassification of strain ATCC 393 as *Lact. zeae*, followed by the designation of strain ATCC 334 as the neotype of *Lact. casei* subsp. *casei*, was supported by results obtained from DNA-DNA hybridization studies (Dicks *et al.* 1996). High levels of DNA homology (above 80%)

were recorded between strains ATCC 393 and ATCC 15820, whereas both of these strains shared only a moderate DNA homology (8 - 46%) with strains of *Lact. casei* subsp. *casei* and its subspecies, including *Lact. casei* subsp. *alactosus* (Dellaglio *et al.* 1973). We have also argued that strains originally classified as *Lact. casei* subsp. *alactosus* be reclassified as *Lact. casei* subsp. *casei*, based on total soluble cell protein patterns and DNA-DNA hybridization studies (Dicks *et al.* 1996). Thus, based on the data previously presented (Dellaglio *et al.* 1991 and Dicks *et al.* 1996), and the results obtained in the present study, the strains in subgroup a of cluster II should be classified as *Lact. zeae* and the strains in subgroup b as *Lact. casei* subsp. *casei* (Table 2). This classification is supported by results obtained from 16S rRNA sequencing (Table 2).

Concluded from the 16S rRNA sequencing data, the strains in clusters III and IV belong to the species *Lact. casei* subsp. *casei* (Table 2). The strains from these two clusters were phenotypically not closely related to the *Lact. casei* subsp. *casei* strains in cluster II (Fig. 2). It is also interesting to note that all five strains isolated from wine during submerged fermentation (strains B2, B3, B1, B4 and B5) grouped in cluster III (Fig. 2).

The taxonomic status of *Lact. casei* and its subspecies is uncertain. The species has been subjected to considerable nomenclatural changes (Collins *et al.* 1989; Pot *et al.* 1994a). This is not surprising, since the *Lact. casei* – *Pediococcus* phylogenetic group is the largest and most heterogeneous of all lactic acid bacteria (Collins *et al.* 1991). An indepth taxonomic study is needed on all members of *Lact. casei*, which should also include strains from various niches.

Concluded from the present study, the predominant microorganisms isolated from the wines were *Lact. vermiforme*, *Lact. zeae* and *Lact. casei* subsp. *casei*. The absence of homofermentative or facultatively heterofermentative species from the bottled fortified wine is perhaps not surprising, since members of these two groups are less tolerant to alcohol than species from the obligately heterofermentative group (group III, Kandler and Weiss, 1986). It is furthermore interesting to note that only a few strains (5 out of 62) were isolated from wine during submerged fermentation. The reason for this is unknown. Strains of *Lact. buchneri* and *Lact. plantarum* were less predominant. *Lact. plantarum* has been isolated from table wines (Sharpe, 1981) and grape must (Costello *et al.* 1983). The species seldom proliferate during the grape-must phase of winemaking, and they are usually supressed during alcoholic fermentation, but some strains of *Lact. plantarum* may multiply (Ribéreau-Gayon *et al.* 1975).

No strains of *Lact. brevis, Lact. hilgardii* and *Lact. fructivorans* were isolated, despite their ability to tolerate alcohol levels as high as 20% (Fornachon *et al.* 1949; Farrow *et al.* 1986). Many reports exist regarding the isolation of *Lact. hilgardii* from spoiled fortified wines. *Lact. hilgardii* has, for example, been isolated from Portuguese Douro fortified wine (Couto and Hogg 1994). Strains of *Lact. hilgardii* have also been isolated from fortified wines with an ethanol content of 10 - 20 % (v/v) and a pH of 3 - 4 (Hecker and Völker 1990).

Strains of *Lact. casei* have been isolated from fresh grape must (Costello *et al.* 1983). Prior to the addition of sweet fortified wine, the alcohol concentration of the submerged-culture flor fortified wine is adjusted to approximately 17% (v/v) by the addition of distilled alcohol. The isolated strains of *Lact. casei* probably survived the alcoholic fermentation, but were inhibited during the submerged-culture sherry-production process. The apparent absence of isolates from the final fortified wine sample was probably due to the final alcohol fortification of 17·20%, which seems to be too high for the bacteria to survive.

This is the first report on *Lact. casei, Lact. zeae* and *Lact. plantarum* isolated from South African fortified wine. The few strains isolated for each of the latter species suggests that they do not play a major role in the spoilage of fortified wines.

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Table 1 Classification of obligately heterofermentative strains based on numerical analysis of total soluble cell protein patterns and 16S rRNA sequence analysis

Strain*	PAGE†	Identification based on 16S rRNA sequencing	Classification
ATCC 14869^T	I		*Lact. brevis*
ATCC 8291	I		*Lact. brevis*
ATCC 12935	I		*Lact. buchneri*
ATCC 11305	I		*Lact. buchneri*
ATCC 8290^T	I		*Lact. hilgardii*
85224a	II	*Lact. buchneri*	*Lact. buchneri*
85759a	II		*Lact. buchneri*
85224b	II	*Lact. buchneri*	*Lact. buchneri*
OBS-LEES	III	*Lact. vermiforme*	*Lact. vermiforme*
85752 (1)	III		*Lact. vermiforme*
T392	III		*Lact. vermiforme*
85757 (2)	III	*Lact. vermiforme*	*Lact. vermiforme*
87591	III	*Lact. vermiforme*	*Lact. vermiforme*
85018 (1)	III		*Lact. vermiforme*
A	III		*Lact. vermiforme*
LB100 (5)	III	*Lact. vermiforme*	*Lact. vermiforme*
91476	IV		*Lact. vermiforme*
85760 (1)	IV	*Lact. vermiforme*	*Lact. vermiforme*
85755 (2)	IV		*Lact. vermiforme*
89833	IV		*Lact. vermiforme*
85759b	IV	*Lact. vermiforme*	*Lact. vermiforme*
93337	V		*Lact. vermiforme*
85758	V	*Lact. vermiforme*	*Lact. vermiforme*
8445601	V		*Lact. vermiforme*
93992	V		*Lact. vermiforme*
84456	V		*Lact. vermiforme*
85758	V		*Lact. vermiforme*
85760 (2)	V		*Lact. vermiforme*
93992	V		*Lact. vermiforme*
87602	V		*Lact. vermiforme*
92698	V		*Lact. vermiforme*
92734	V		*Lact. vermiforme*

*All strains were isolated from bottled fortified wine which has been spoiled
†Grouping of strains based on numerical analysis of total soluble cell protein patterns (Fig. 1).

Table 2 Classification of facultatively heterofermentative strains based on numerical analysis of total soluble cell protein patterns and 16S rRNA sequence analysis

Strain*	PAGE†	Identification based on 16S rRNA sequencing	Classification
ATCC 8014	I		*Lact. plantarum*
LB100 (2)	I	*Lact. plantarum*	*Lact. plantarum*
ATCC 393	IIa		*Lact. zeae*
A27	IIa		*Lact. zeae*
A29	IIa		*Lact. zeae*
ATCC 25180	IIb		*Lact. casei* subsp. *alactosus*
A15	IIb		*Lact. casei* subsp. *casei*
A17	IIb		*Lact. casei* subsp. *casei*
A1	IIb		*Lact. casei* subsp. *casei*
A3	IIb		*Lact. casei* subsp. *casei*
A2	IIb		*Lact. casei* subsp. *casei*
A9	IIb	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A14	IIb		*Lact. casei* subsp. *casei*
A16	IIb		*Lact. casei* subsp. *casei*
A4	IIb	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A6	IIb		*Lact. casei* subsp. *casei*
T394	IIb		*Lact. casei* subsp. *casei*
A21	IIb	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A22	IIb		*Lact. casei* subsp. *casei*
A23	IIb		*Lact. casei* subsp. *casei*
A24	IIb		*Lact. casei* subsp. *casei*
A18	IIb	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A25	IIb		*Lact. casei* subsp. *casei*
B2	III		*Lact. casei* subsp. *casei*
B3	III		*Lact. casei* subsp. *casei*
B1	III		*Lact. casei* subsp. *casei*
B4	III		*Lact. casei* subsp. *casei*
A28	III		*Lact. casei* subsp. *casei*
B5	III		*Lact. casei* subsp. *casei*
A11	III		*Lact. casei* subsp. *casei*
A12	III		*Lact. casei* subsp. *casei*
T395 (1)	III		*Lact. casei* subsp. *casei*
A31	III	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A32	III	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A26	III	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A19	IV		*Lact. casei* subsp. *casei*
A20	IV	*Lact. casei* subsp. *casei*	*Lact. casei* subsp. *casei*
A5	IV		*Lact. casei* subsp. *casei*

*Numbers starting with an "A" refer to strains isolated from wine before the onset of submerged fermentation, a "B" refers to strains isolated from wine undergoing submerged fermentation. Strains LB100 (2), T394 and T395 (1) were isolated from bottled fortified wine which underwent spoilage.

†Grouping of strains based on numerical analysis of total soluble cell protein patterns (Fig. 2).

Fig. 1 Dendrogram showing the clustering of obligately heterofermentative stains of lactobacilli isolated from fortified wine. All strains were isolated from bottled fortified wine which has been spoiled, except strain A which was isolated from wine before the onset of submerged fermentation. Grouping was by the unweighted average pair-group method. Strains indicated in bold numbers were selected for 16S rRNA sequencing.

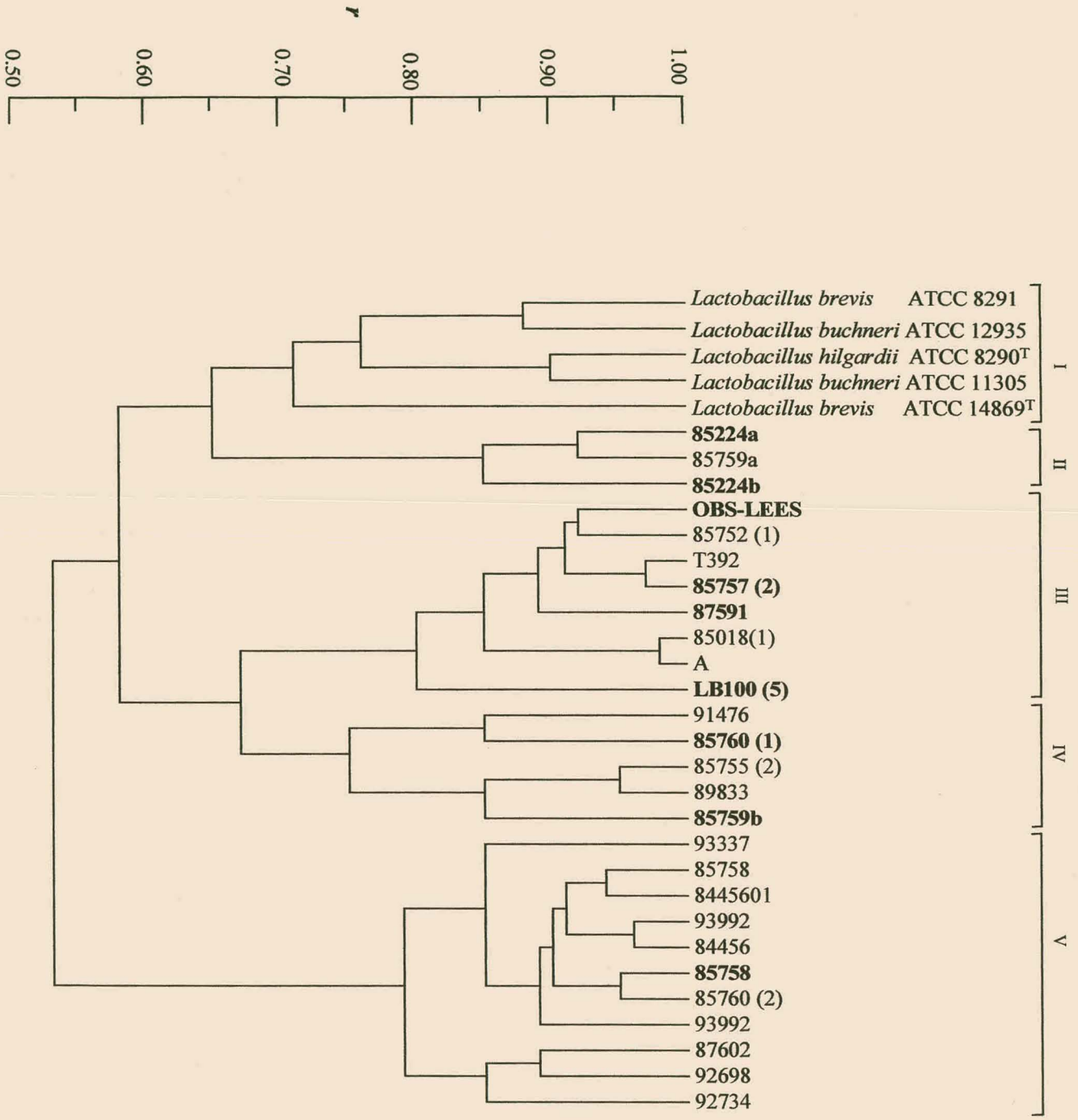
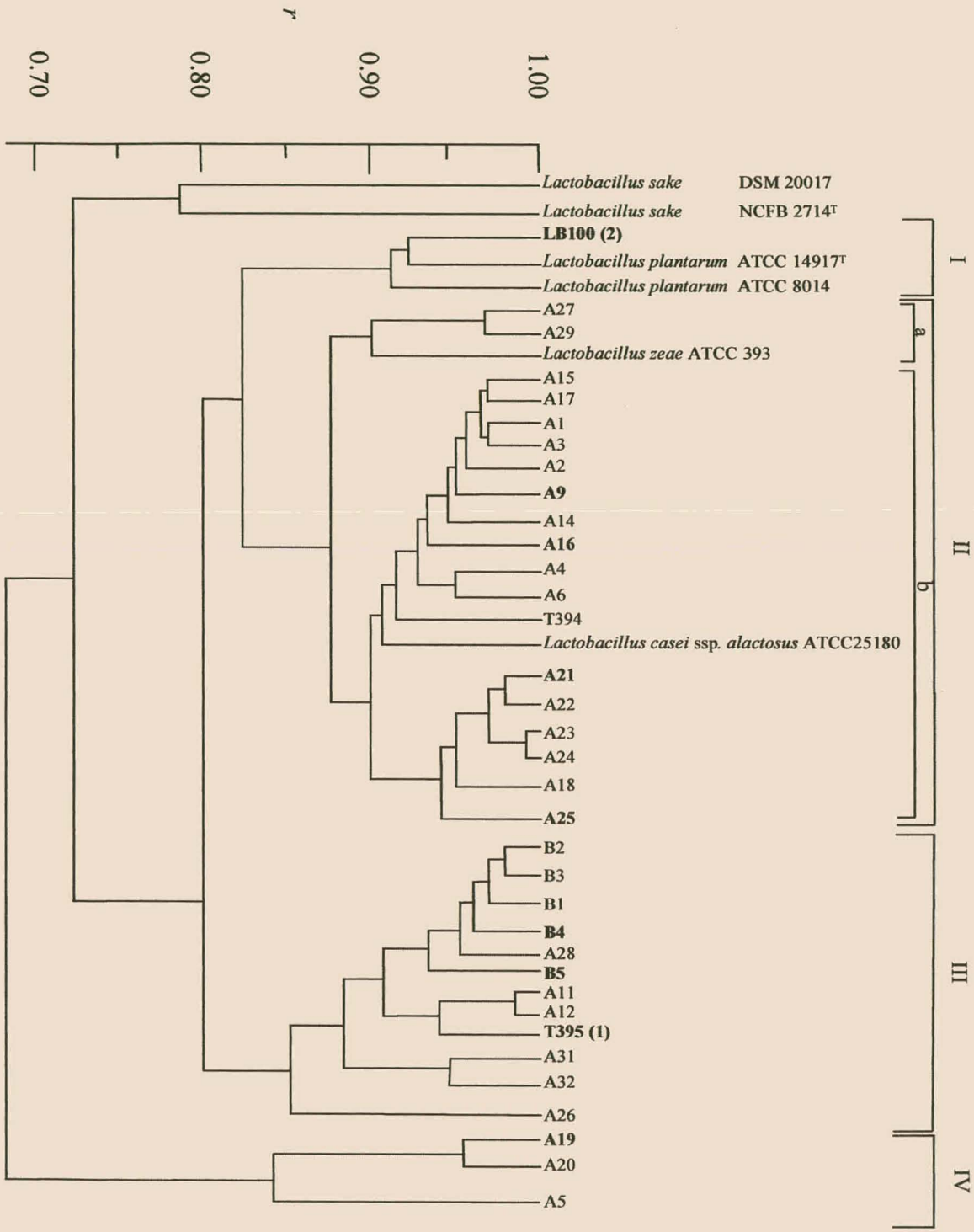


Fig. 2 Dendrogram showing the clustering of facultatively heterofermentative stains of lactobacilli isolated from fortified wine. Numbers starting with an "A" refer to strains isolated from wine before the onset of submerged fermentation, a "B" refers to strains isolated from wine which was at he time undergoing submerged fermentation. Strains LB100 (2), T394 and T395 (1) were isolated from bottled fortified wine which underwent spoilage. Grouping was by the unweighted average pair-group method. Strains indicated in bold numbers were selected for 16S rRNA sequencing.



4. GENERAL DISCUSSION AND CONCLUSIONS

Lactic acid bacteria are Gram-positive, catalase-negative cocci or rods and include the genera *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Kandler and Weiss, 1986; Holzapfel and Wood 1995; Vandamme *et al.* 1996). These bacteria are found in many habitats, e.g. bread, buttermilk, cheese, fermented vegetables, fermented meats, yoghurt, sour dough, silage, wine and various fermented beverages, sewage and the genital, intestinal and respiratory tracts of man and animals (Nissen *et al.* 1991; Hammes *et al.* 1991; Vandenbergh 1993). Homofermentative lactic acid bacteria ferment hexoses to produce mainly lactic acid (more than 85%), whereas heterofermentative lactic acid bacteria produce lactate, acetate, ethanol, CO_2, and formate or succinate in equimolar amounts (Kandler and Weiss, 1986). The low pH resulting from acid production prevents the growth of a number of food spoilage and pathogenic bacteria. Various other metabolic compounds are also produced, such as diacetyl, H_2O_2, CO_2, bacteriocins and acetic acid, which may work together or individually to extend the shelf life and/or organoleptic qualities of foods and beverages (De Vuyst and Vandamme 1994).

The presence of malolactic bacteria in wines and the role they play during the secondary fermentation have been well documented (Davis *et al.* 1985; Wibowo *et al.* 1985; Dicks *et al.* 1995). In wines of high pH malolactic bacteria may proliferate and deacidify the wine to a flat and insipid product, often accompanied by undesirable sensory characteristics (Davis *et al.* 1985; Wibowo *et al.* 1985). In some red wines a colour reduction of 30% has been recorded when fermented by *Leuconostoc* spp. (Vetsch and Lüthi 1964). Trace amounts of histamine were also reported after MLF, especially in high pH wines (Davis *et al.* 1985; Wibowo *et al.* 1985; Radler 1986).

Little is known about the bacterial population in fortified wines, especially in South African wines. The alcohol levels in fortified wines are usually higher than 15% (v/v) and prevents the growth of most malolactic bacteria (Fornachon *et al.* 1949; Farrow *et al.* 1983; Hecker and Völker 1990). However, *Lactobacillus fructivorans, Lactobacillus hilgardii, Lactobacillus brevis* and *Lactobacillus buchneri* can tolerate ethanol levels as high as 20%, v/v (Fornachon *et al.*

1949; Farrow *et al.* 1983; Hecker and Völker 1990) and should thus be able to survive the conditions in most fortified wines, depending on the method of production. The high level of sugars that remain in these wines may become a source of energy for microbial growth and spoilage (Goswell 1986).

The development of sophisticated genotypic classification methods resulted in the identification of many new species and the reclassification of a number of species which were previously classified based on results obtained by phenotypic methods (De Vuyst and Vandamme 1994). In more recent papers sequencing of 16S rRNA is often used without DNA hybridization studies or equivalent genotypic data. Many researchers have objected to this approach. In one study, for example, results from 16S rRNA sequencing reported a 99.5% rRNA sequence similarity for three phenotypically similar strains of a *Bacillus* sp. (Fox *et al.* 1992). DNA-DNA hybridization data showed, however, that these strains belonged to two distinct species.

The *Lact. vermiforme* strains isolated from South African fortified wines have shown a close genetic relatedness based on results obtained by 16S rRNA sequencing. However, the fact that three distinct phenotypic groups (protein clusters) of *Lact. vermiforme* were identified suggests the species consists of a phenotypically heterogeneous collection of strains.

Lact. hilgardii, *Lact. fructivorans*, *Lact. collinoides* and *Lact. mali* have been isolated from Douro fortified wines (Farrow *et al.* 1986). The results obtained in the present study reflect the results predicted for spoilage in fortified wines in the literature. *Lact. vermiforme* was the dominant spoilage organism in this particular type of fortified wine, followed by *Lact. buchneri, Lact. casei* and *Lact. plantarum*. It is possible that if *Lact. vermiforme* and *Lact. buchneri* had been present during the production-phase of the fortified wine. They would have survived the alcohol fortification step to cause spoilage.

Betabacterium vermiforme was originally isolated from Californian table wines (Farrow *et al.* 1986). DNA hybridization studies performed on three strains of *Lactobacillus vermiforme* (NCDO 961, NCDO 962 and NCDO 1965) showed that these three strains shared a DNA homology of 72 to 90% with the type strain *Lact. hilgardii* NCDO 264^T (Farrow *et al.* 1986).

The species name *Lact. vermiforme* has since lost nomenclatural standing over the name *Lact. hilgardii*. Later work with 16S rRNA sequencing showed that these two species are phylogenetically distinct. This study suggests that these two species are also phenotypically distinct (with respect to the method used). The method of DNA-DNA hybridization therefore gave insufficient evidence for the reclassification of *Lact. vermiforme* in the past. Further comparisons, using phenotypic and genotypic taxonomic methods, between different strains of *Lact. vermiforme* and *Lact. hilgardii*, may elucidate the true taxonomic position of *Lact. vermiforme*.

The decrease in the prevalence of strains of *Lact. casei* from the second phase of submerged-culture sherry production may suggest inhibition by specific compounds produced by the yeast during the submerged-culture sherry production process. Lactic acid bacteria were not isolated from the final phase of production of this wine, suggesting that the bacteria did not survive the final alcohol fortification step, or their numbers were too low for isolation. The apparent absence of *Lact. casei* strains from the final phase of production indicates that it is not a potentially serious spoilage organism of the fortified wine studied. *Lact. casei* has been isolated from diverse habitats, e.g. dairy products, silage, human intestinal tracts and mouths, and sewage (Kandler and Weiss, 1986). It is possible that this organism originated from irrigation water contaminated with sewage, and slowly adapted to the gradually increasing ethanol level and the decreasing pH during the primary fermentation. They may have also become the natural inhabitants of the winery equipment.

The two subgroups obtained for *Lact. casei* supports previous findings, i.e. the designation of a neotype for *Lact. casei* and the description of a new species *Lact. zeae* (Dicks *et al.* 1996). This is the first report of the presence of *Lact. casei, Lact. zeae* and *Lact. plantarum* from a spoiled fortified wine, their low numbers suggesting a minor role in spoilage. This is perhaps due to the high alcohol level of the wine, or perhaps due to inhibitory compounds produced by the yeast or by the other lactic acid bacteria.

Oenococcus oeni is one of the major microorganisms responsible for performing malolactic fermentation in wines. In a previous study on South African fortified wines, strains of *Oen. oeni*

have been isolated (Dicks *et al.* 1995). However, no strains of *Oen. oeni* have been isolated from the wines included in this study.

Although the results of this study reflect the bacterial flora of one particular sweetened fortified wine, the data obtained may be used to predict the bacterial flora of other fortified wines. Dry (virtually no sugar present), medium cream (medium sweet) and full cream (very sweet) fortified wines differ from each other only in the level of sugar they contain. The alcohol levels of these fortified wines are between 16-20%, the lower limit of 16% being the limiting factor for bacterial and yeast growth. This limiting factor determines which bacteria (and yeasts) will survive, the sugar level indicating the rate at which spoilage will occur.

Bacterial spoilage of fortified wines in the pre- and post-production phases causes financial losses in the South African wine industry every year. More work on the identification of spoilage microorganisms from other South African fortified wines is necessary, in addition to studies on the conditions responsible for the contamination and spoilage of South African fortified wines.

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