The role of lactic acid bacteria in brandy production

by

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DECLARATION

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

Heinrich du Plessis

SUMMARY

The presence and growth of lactic acid bacteria (LAB) in wine and their influence on wine quality has received much attention in recent years. Lactic acid bacteria are responsible for conducting malolactic fermentation (MLF) in wine. The benefits associated with malolactic fermentation in terms of deacidification of wine and the contribution to wine flavour and complexity have also recently been the topic of research. It is impossible to describe malolactic fermentation as distinctly desirable or undesirable in terms of its influence on the final quality of wine. The benefits and disadvantages are dependent upon viticultural region, grape variety, wine composition, winemaking techniques and the style and objectives of the winemaker.

Brandy production is a multi-stage process in which base wine production, distillation technique and wood maturation all have a large influence on the final chemical profile and organoleptic quality of the brandy. The volatile composition of the base wine, which basically undergoes a concentration process during the subsequent double distillation phase, is critical in determining the aroma and flavour quality of the final brandy product. Thus, the brandy is only as good as the base wine it is distilled from.

The aims of this study were to determine the effect of lactic acid bacteria and spontaneous malolactic fermentation on the quality of brandy base wine and the resulting distillate, and to determine which LAB species had been responsible for the occurrence of spontaneous MLF.

This study showed that LAB are present at high numbers and are able to conduct spontaneous MLF of brandy base wines. It was shown that the incidence of spontaneous MLF varied from year to year. In 1998, 50% of the commercially produced base wines had undergone partial MLF prior to distillation. In 1999 and 2000 respectively, 34% and 45% of the commercial base wines had undergone partial MLF prior to distillation. The occurrence of spontaneous MLF had an influence on the chemical composition and the sensory quality of the base wine and distillate. There was an increase in the concentrations of ethyl lactate, acetic acid and diethyl succinate in samples that had undergone MLF. There was also a decrease in the concentrations of esters, such as iso-amyl acetate, ethyl acetate, ethyl caproate, hexyl acetate and 2-phenethyl acetate in these same samples. Sensory evaluation of the base wines and distillates demonstrated that samples that had undergone MLF differed significantly from samples that had not undergone MLF. It was also shown that distillates that had not undergone MLF had a slightly better aroma profile than those that had. Sweet aromas, like chocolate and caramel, as well as negative aromas, like chemical or solvent, were more prominent in brandy distillates that had undergone MLF. Herbaceous and fruity aromas were more intense in distillates not having undergone MLF.

Fifty-four strains, all Gram-positive and catalase negative, were isolated at different stages of brandy production. Seven strains were isolated from the grape juice, 15 strains

were isolated from the base wine, 20 strains were isolated during MLF and 12 strains were isolated from the base wine after MLF had been completed. Based on CO_2 production from glucose and gluconate, 17 strains were classified as facultatively heterofermentative and 37 strains as obligately heterofermentative. Fifteen of the 37 obligately heterofermentative strains were rod-shaped and were regarded as lactobacilli. The remaining 22 strains were oval or cocci-bacilli shaped. The isolates were identified to species level by using numerical analysis of the total soluble cell protein patterns, 16S rRNA sequencing and polymerase chain reaction (PCR) with species-specific primers.

The facultative heterofermentative lactobacilli were identified as *Lactobacillus paracasei* and *Lactobacillus plantarum*. The fifteen obligately heterofermentative lactobacilli were identified as members of the species *Lactobacillus brevis*, *Lactobacillus vermiforme*, *Lactobacillus buchneri* and *Lactobacillus hilgardii*. The 22 obligate heterofermentative isolates, with a coccoid morphology, could be grouped into two clusters and were identified as *Oenococcus oeni*. *O. oeni* was the species responsible for the occurrence of spontaneous MLF in most of the commercial base wines. *Lb. brevis*, *Lb. hilgardii* and *Lb. paracasei* were also isolated from commercial base wines that had undergone spontaneous MLF. In nine out of 14 experimental base wine samples that had undergone spontaneous MLF, *O. oeni* was again the predominant species. *Lb. brevis*, *Lb. hilgardii* and *Lb. paracasei* were identified in the remaining experimental base wine samples. This is the first report of the presence of *Lb. paracasei* and *Lb. vermiforme* in brandy base wine. It was shown that the occurrence of spontaneous MLF had a negative effect on the quality of brandy base wine, but that was shown to be due to the different species and strains performing MLF. In the non-preferred distillate samples, *Lactobacillus* spp. had performed MLF or had developed after or during MLF.

OPSOMMING

Die teenwoordigheid en die vermoë van melksuurbakterieë (MSB) om in wyn te groei, is 'n onderwerp wat al heelwat nagevors is. Melksuurbakterieë is verantwoordelik vir die uitvoering van appelmelksuurgisting (AMG) in wyn. Die voordele verbonde aan appelmelksuurgisting, ten opsigte van die verlaging van die totale suurinhoud en die bydrae tot die verbeterde geur en kompleksiteit van die wyn, is ook al goed bestudeer. Wat die invloed op die finale wynkwaliteit betref, is dit byna onmoontlik om AMG as uitsluitlik gewens óf ongewens te beskou. Die voordele en nadele van AMG is afhanklik van verskeie faktore, nl. wingerdkundige streek, druifkultivar, wynsamestelling, wynmaakpraktyke, asook die styl en doelwitte van die wynmaker.

Die produksie van brandewyn is 'n multistapproses waarin die bereidingsmetode van die basiswyn, die distillasietegniek en houtveroudering 'n groot invloed op die finale kwaliteit en chemiese samestelling van die brandewyn het. Die vlugtige verbindings van die basiswyn, wat tydens die dubbele distillasieproses gekonsentreer word, is van wesenlike belang in die bepaling van die aroma en geur van die finale brandewynproduk. Brandewyn is dus inderdaad net so goed soos die basiswyn waarvan dit gestook is.

Die doelwitte van hierdie studie was om te bepaal wat die invloed van MSB en die voorkoms van spontane AMG op die kwaliteit van die basiswyn en die distillaat is, asook om die MSB wat vir die voorkoms van spontane AMG verantwoordelik was, te identifiseer.

Hierdie studie het bewys dat MSB in hoë getalle teenwoordig was en dat dit in staat is om die spontane AMG van basiswyne uit te voer. Daar is bewys dat die voorkoms van spontane AMG moontlik van jaar tot jaar kan verskil. In 1998 het 50%, in 1999 het 34% en in 2000 45% van die kommersieel-geproduseerde basiswyn gedeeltelike AMG spontaan voor distillasie ondergaan. Daar is ook gevind dat spontane AMG 'n invloed op die chemiese samestelling en sensoriese kwaliteit van die basiswyn en die distillaat gehad het. Daar was 'n toename in die konsentrasies van etiellaktaat, asynsuur en diëtielsuksinaat in monsters wat spontane AMG ondergaan het. In dieselfde monsters was daar ook 'n afname in die konsentrasies van iso-amielasetaat, etielasetaat, etielkaproat, heksielasetaat en 2-fenielasetaat. Sensoriese evaluering van die basiswyne en distillate het getoon dat daar betekenisvolle verskille was tussen die monsters wat AMG ondergaan het en dié wat nie AMG ondergaan het nie. Daar is bewys dat die distillate wat nie AMG ondergaan het nie, 'n beter aromaprofiel gehad het as dié wat AMG ondergaan het. Soet geure, soos sjokolade en karamel, en negatiewe geure, soos "chemies" en "oplosmiddel", was prominent in distillate wat AMG ondergaan het. Kruidagtige en vrugtige geure was meer intensief in distillate wat nie AMG ondergaan het nie.

Vier-en-vyftig bakteriese rasse, almal Gram-positief en katalase-negatief, is gedurende die verskillende stadia van brandewynproduksie geïsoleer. Sewe rasse is uit druiwesap, 15 rasse gedurende die alkoholiese fermentasie, 20 rasse gedurende AMG en 12 rasse na voltooiing van AMG geïsoleer. Op die basis van koolstofdioksied (CO_2)-produksie vanaf

glukose en glukonaat is 17 rasse as fakultatief heterofermentatief en 37 rasse as obligaat heterofermentatief geklassifiseer. Vyftien van die 37 obligaat-heterofermentatiewe rasse was staafvormig en is as lactobacilli geïdentifiseer. Die oorblywende 22 het ovaal of kokkus-bacillusvormige selmorfologie getoon. Identifikasie tot op spesievlak is gedoen deur van numeriese analise van die totale oplosbare selproteïenprofiele, 16S-rRNA-volgordebepalings en spesie-spesifieke inleiers vir die polimerasekettingreaksie (PKR) gebruik te maak.

Die fakultatief-heterofermentatiewe rasse is as *Lactobacillus paracasei* en *Lactobacillus plantarum* geklassifiseer. Die 15 obligaat heterofermentatiewe stafies is as *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus hilgardii* en *Lactobacillus vermiforme* geïdentifiseer. Die 22 ovaal, obligaat heterofermentatiewe isolate kon in twee groepe ingedeel word en is as *Oenococcus oeni* geïdentifiseer.

Daar is bevind dat *O. oeni*-isolate vir die voorkoms van spontane AMG in die meeste van die kommersiële basiswyne verantwoordelik was. *Lb. brevis*, *Lb. hilgardii* en *Lb. paracasei* is ook uit kommersiële basiswyne wat spontane AMG ondergaan het, geïsoleer. In nege uit 14 van die eksperimentele basiswyne wat spontane AMG ondergaan het, was *O. oeni* die dominante spesie. In die oorblywende eksperimentele wyne is *Lb. brevis*, *Lb. hilgardii* en *Lb. paracasei* aangetref. Hierdie is die eerste vermelding van die teenwoordigheid van *Lb. paracasei* and *Lb. vermiforme* in brandewynbasiswyn. Daar is gevind dat die voorkoms van spontane AMG 'n negatiewe invloed op brandewynkwaliteit het, maar dit is as gevolg van die verskeidenheid van MSB-spesies en rasse wat voorkom. In die distillate wat deur die proepaneel afgekeur is, het *Lactobacillus* spesies die AMG deurgevoer, of het dit tydens of na AMG ontwikkel.

This thesis is dedicated to my family for their continuous love and support. Hierdie tesis is aan my familie opgedra vir hulle volgehoue liefde en ondersteuning.

BIOGRAPHICAL SKETCH

Heinrich du Plessis was born in Paarl, South Africa on 3 November 1975. He attended Paarlzicht Primary School and matriculated at Noorder Paarl Secondary School in 1993. Heinrich enrolled at Stellenbosch University in 1994 and obtained his BSc degree in 1997, majoring in Microbiology and Genetics. He completed his HonsBSc degree in Wine Biotechnology in 1998. Heinrich is currently employed by the Agricultural Research Council (ARC) at ARC Infruitec-Nietvoorbij.

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PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the *Journal of Applied Microbiology*, in which Chapter 3 was published. Chapter 4 is written according to the style of the *International Journal of Food Microbiology*, to which it is to be submitted for publication.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **LITERATURE REVIEW**
The occurrence of lactic acid bacteria in wine

Chapter 3 **Research Results**
The occurrence of malolactic fermentation in brandy base wine and its influence on brandy quality

Chapter 4 **Research Results**
Identification of lactic acid bacteria isolated from South African brandy base wines

Chapter 5 **General Discussion and Conclusions**

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The winemaking process is a complex ecological niche where the biochemistry and interaction of microorganisms play a pivotal role in the final product (Du Toit and Pretorius 2000). Yeast, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and fungi occur naturally on the grape berry at harvesting. Yeasts are well adapted to growth in grape must and the onset of alcoholic fermentation starts quickly after crushing. During the alcoholic fermentation, sugars are fermented to ethanol, while minor end products are also produced. *Saccharomyces cerevisiae* is the dominant yeast during alcoholic fermentation. Acetic acid bacteria survive at low concentrations and the fungi and other bacteria are suppressed by the increase in ethanol concentrations. At the end of the alcoholic fermentation, yeast numbers decline and LAB growth occurs (Lonvaud-Funel 1999; Ribéreau-Gayon *et al.* 2000). Malolactic fermentation (MLF) follows the alcoholic fermentation and is a so-called secondary fermentation that results from the metabolic activity of certain LAB in wine and consists of the conversion of L-malic acid to L-lactic acid and CO_2 (Kunkee 1991; Lonvaud-Funel 1995; Versari *et al.* 1999).

Base wine used for brandy production has the following characteristics: high acid and low pH, low alcohol content (10-11%), and contains no or little sulphur dioxide (SO_2) (Leaute 1990). Sulphur dioxide is the wine maker's most powerful antimicrobial agent. During the harvest season, distilleries struggle to cope with the large influx of base wine and some base wines are forced to spend time on the yeast lees. Yeast autolysis can occur, resulting in the leaching of nutrients into the wine. Conditions in the base wine therefore are quite suitable for the development of LAB.

Wine aroma and flavour has a profound influence on the quality of an alcoholic beverage. Wine aroma and flavour are determined primarily by the secondary metabolites of the grape and, to a lesser extent, by the yeast that conducts the primary fermentation. Bacteria can also influence the flavour (Bartowsky and Henschke 1995). During their growth, LAB ferment residual sugars, mostly hexoses and pentoses, and transform numerous wine components (Lonvaud-Funel 1999). Lactic acid bacteria may improve the quality and stability of the wine; however, they may also spoil it. Lactic acid bacteria can ferment carbohydrates, producing acetic acid and lactic acid as the major end products of this fermentation. This results in an increase in the volatile acidity of the wine. Faults in wine, such as a "mousy taint" and "ropiness", can also result from the growth of LAB (Lonvaud-Funel 1999). The influence of LAB on the flavour and therefore on the commercial value of wine can be considerable. Whether or not the changes in flavour are regarded as desirable or not, depends on the concentration of the compounds produced and the style and type of wine (Davis *et al.* 1985). Malolactic fermentation has been shown

to have a considerable influence on the aroma and flavour properties of wine (Laurent *et al*. 1994; Bartowsky and Henschke 1995; De Revel *et al*. 1999).

Brandy is a distillate of wine and subsequently its initial composition depends on the compounds present in the base wine. Although ethanol and water are the two major compounds in any distilled beverage, the aroma and flavour character depend on a multitude of minor compounds, which often are referred to as brandy congeners. The most abundant congeners of brandy are minor products of the alcoholic fermentation. The relative quantities of these minor compounds recovered in the distillate are affected by distillation conditions (Guymon 1974). It is apparent that a detailed knowledge of the relative concentrations of aroma-forming compounds, and the exact origin of these compounds, could be extremely useful in maintaining or improving the quality of base wines and brandies.

South African vineyards provide raw material for one of the world's largest brandy producers. The quantity of base wine going through South African distilleries is about 250 million litres annually. A lot of brandy is produced, yet very little is exported (Hughes *et al*. 1994). In 2000, about 174 429 litres of brandy was exported and 488 954 litres was imported. Brandy has been a favourite with South Africans for a long time, with annual sales of over 45 million litres. It accounts for about 50% of all local spirit sales and revenue of about 546·6 million rand [South African Wine Industry Information & Systems (SAWIS)]. Due to the versatility of this beverage, South Africans from all social spheres enjoy brandy and it is not necessary to drink it to be a brandy lover. Brandy has become one of the most important features in the South African kitchen and its hearty zest livens many traditional dishes. This popularity has allowed the brandy industry to become a stalwart in the local economy and South Africa currently is the fifth largest brandy producer in the world (The South African Brandy Foundation). Legislation changed in 1990 to allow for the production of pot-still brandy and for fine-aged brandy to be sold at 38% (v/v) rather than the regular strength of 43% percent alcohol. This results in a more natural and more finely flavoured brandy product (Hughes *et al*. 1994).

Any information that may lead to an improvement in brandy quality would be of great value. It is clear that a greater understanding of the influence of MLF on the organoleptic quality of brandy will be of great economic benefit to the South African brandy industry. There are always requests for brandy with varying styles or flavour characteristics. This research might provide some options for new or varying brandy characteristics.

1.2 PROJECT AIMS

This study forms an integral part of an extensive research programme at the Institute for Wine Biotechnology aimed at the improvement of the quality of South African wines and brandies. It has been shown that the presence and growth of lactic acid bacteria may be beneficial or detrimental to the quality of wine. The objectives of this project were to determine if spontaneous MLF occurred in South African brandy base wines, the effect of

lactic acid bacteria and the occurrence of spontaneous MLF on the quality of brandy base wine and the resulting distillate, and to determine which LAB species were responsible for spontaneous MLF.

The specific aims and approaches of this study were as follows:
  i.   isolation of lactic bacteria from grape juice and commercially and experimentally produced brandy base wines;
  ii. identification of each isolate, using total soluble cell protein patterns, 16S rRNA sequencing, and PCR by using species-specific primers;
  iii. to determine the extent to which spontaneous malolactic fermentation occurs in South African brandy base wines;
  iv. to focus on the effect of spontaneous MLF on the volatile and non-volatile compounds present in brandy base wine;
  v.   to determine the effect of spontaneous MLF on the volatile compounds of brandy distillate; and
  vi. to determine the effect of spontaneous MLF on the organoleptic quality of the brandy base wines and the resulting brandy spirits.

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CHAPTER 2

LITERATURE REVIEW

The occurrence of lactic acid bacteria in wine
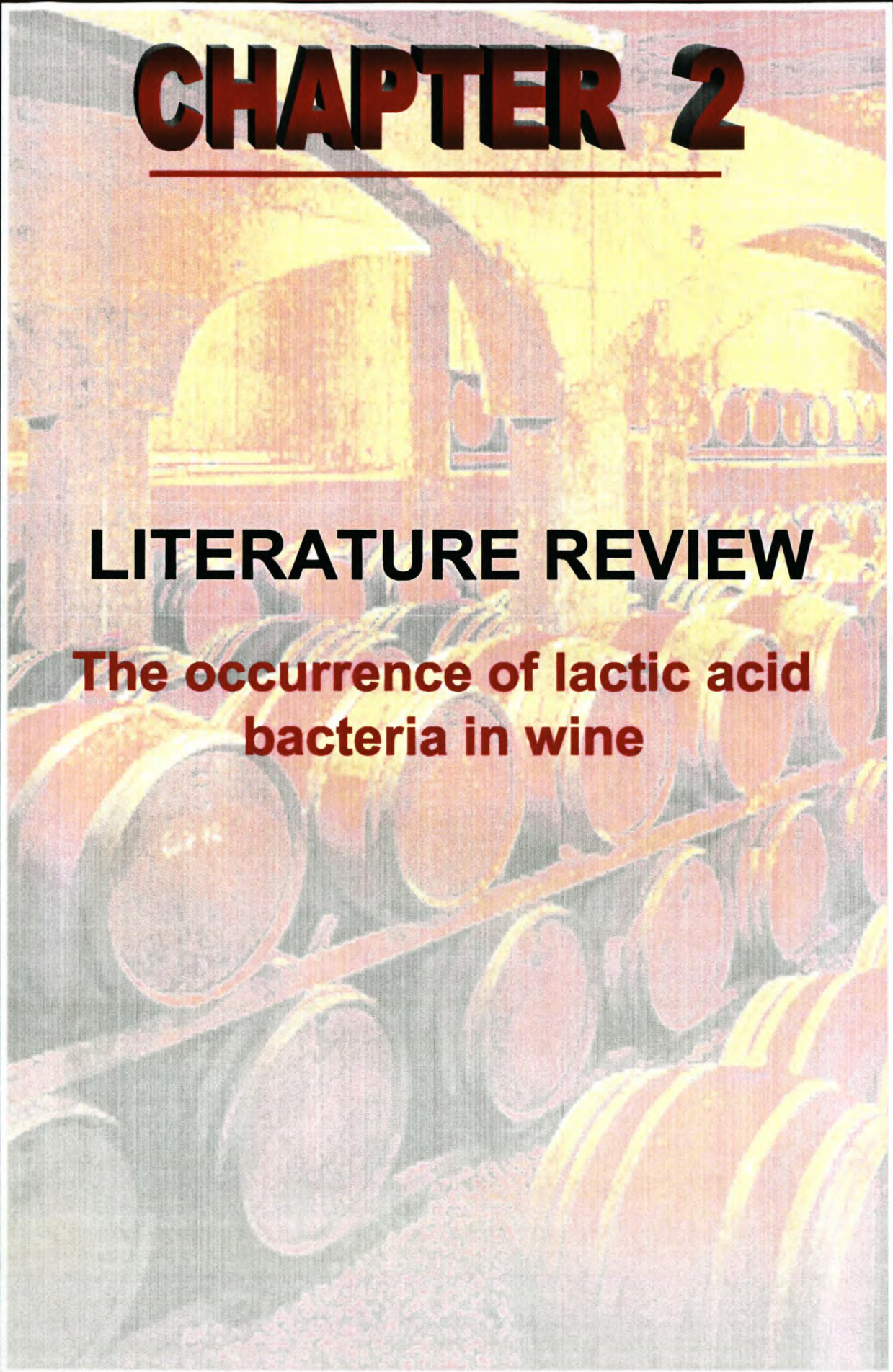
2. LITERATURE REVIEW

2.1 INTRODUCTION

Lactic acid bacteria (LAB) are involved in a large number of food fermentations and are closely associated with the human environment. Lactic acid bacteria are Gram-positive, catalase-negative, non-motile, non-spore forming rods, cocci or coccobacilli and produce mainly lactic acid from the fermentation of carbohydrates (Stiles and Holzapfel 1997). Lactic acid bacteria isolated from wines are classified in two families and four genera. The *Lactobacillaceae*, represented by the genera *Lactobacillus* and *Pediococcus*, include rods and cocci-shaped species and their respective strains. The *Leuconostocaceae*, represented by the genera *Oenococcus* and *Leuconostoc,* include cocci or coccobacilloid-shaped isolates (Kandler and Weiss 1986; Stiles and Holzapfel 1997; Lonvaud-Funel 1999). These may include lenticular or sausage-shaped cells, as in the case of *Oenococcus oeni*, or filamentous chains, such as *Lactobacillus fructivorans,* and tetrads that result from division in two planes, such as *Pediococcus damnosus* (Fugelsang 1997).

2.2 TAXONOMY OF WINE LACTIC ACID BACTERIA

2.2.1 TAXONOMY AND PHYLOGENY

The objective of taxonomy is to identify, describe and classify microorganisms. Classification is made according to several hierarchical levels. The highest level corresponds with the classification among prokaryotes and the lowest level is at the species level (Kandler and Weiss 1986; Ribéreau-Gayon *et al.* 2000).

Lactic acid bacteria belong to the *Clostridium* branch of the phylogenetic tree, representing Gram-positive bacteria with a G + C content of less than 55%. The LAB of importance in foods comprise the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Vandamme *et al.* 1996). The lactic acid bacteria from the genera *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus* (Kandler and Weiss 1986; Stiles and Holzapfel 1997; Lonvaud-Funel 1999) are usually associated with grape juice and wine and are included in the first group of the *Clostridium* branch (Ribéreau-Gayon *et al.* 2000).

Sequencing of the 16S rRNA divides LAB into three phylogenetic groups:

1) The *Lactobacillus delbrueckii* group, which contains *Lb. delbrueckii, Lactobacillus acidophilus* and other obligately homofermentative lactobacilli. However, at least three facultatively heterofermentative species, *Lactobacillus acetotolerans,*

Lactobacillus hamsteri and *Lactobacillus intestinalis,* also belong to this group (Collins *et al.* 1991; Schleifer and Ludwig 1995a, b);

2) The *Lactobacillus casei–Pediococcus* group. This group is a more heterogenous group since it contains obligate and facultatively heterofermentative species, as well as obligate homofermentative species. This group contains 37 *Lactobacillus* spp. and five *Pediococcus* spp. (Collins *et al.* 1991; Schleifer and Ludwig 1995a, b); and

3) The *Leuconostoc* group, which can be divided into three subgroups. The first subgroup contains *Leuconostoc paramesenteroides* and some heterofermentative lactobacilli. The *Lc. paramesenteroides* group has been assigned to the new genus *Weisella,* which consists of seven species (Collins *et al.* 1993). The second subgroup comprises the remaining leuconostocs, as well as *Lactobacillus fructosus,* and the third subgroup contains *O. oeni* (Schleifer and Ludwig 1995a, b).

Grouping by means of 16S rRNA sequences is based on phylogenetic relationships between bacteria. It does not support the grouping achieved by using phenotypic characteristics, such as morphology and physiology. Physiological and biochemical criteria remain useful, but molecular taxonomy is more reliable since it is related directly to the genetic heritage of a strain (Ribéreau-Gayon *et al.* 2000).

2.2.2 CLASSIFICATION OF WINE LACTIC ACID BACTERIA

The LAB can be divided into three groups according to their metabolic activity. They can be homofermentative, facultatively heterofermentative or obligately heterofermentative. Homofermentative bacteria produce more than 85% lactic acid from glucose. Heterofermentative bacteria produce CO_2, ethanol and acetic acid, in addition to lactic acid (Ribéreau-Gayon *et al.* 2000).

The bacteria from the genera *Leuconostoc* and *Oenococcus* are obligately heterofermentative and those from the genus *Pediococcus* obligately homofermentative. The genus *Lactobacillus* contains both homo- and heterofermentative species. Lactobacilli can be divided into the three groups mentioned above:

a) Group I: Obligately homofermentative,

b) Group II: Facultatively heterofermentative, and

c) Group III: Obligately heterofermentative.

The obligately homofermentative LAB ferment glucose to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway (**Fig. 2.1**) and do not ferment pentoses. Homofermentative LAB produce two molecules of lactic acid and two molecules of ATP from one molecule of glucose (hexose) via the EMP pathway. Depending on the species, either the L- or D-lactic acid isomer is formed. *O. oeni* produces only D (−)-lactate, whereas *Pediococcus* spp. produce either D or L- (+)-lactate. *Lactobacillus* spp. produce both D- (−) and L- (+)-lactate (Fugelsang 1997).
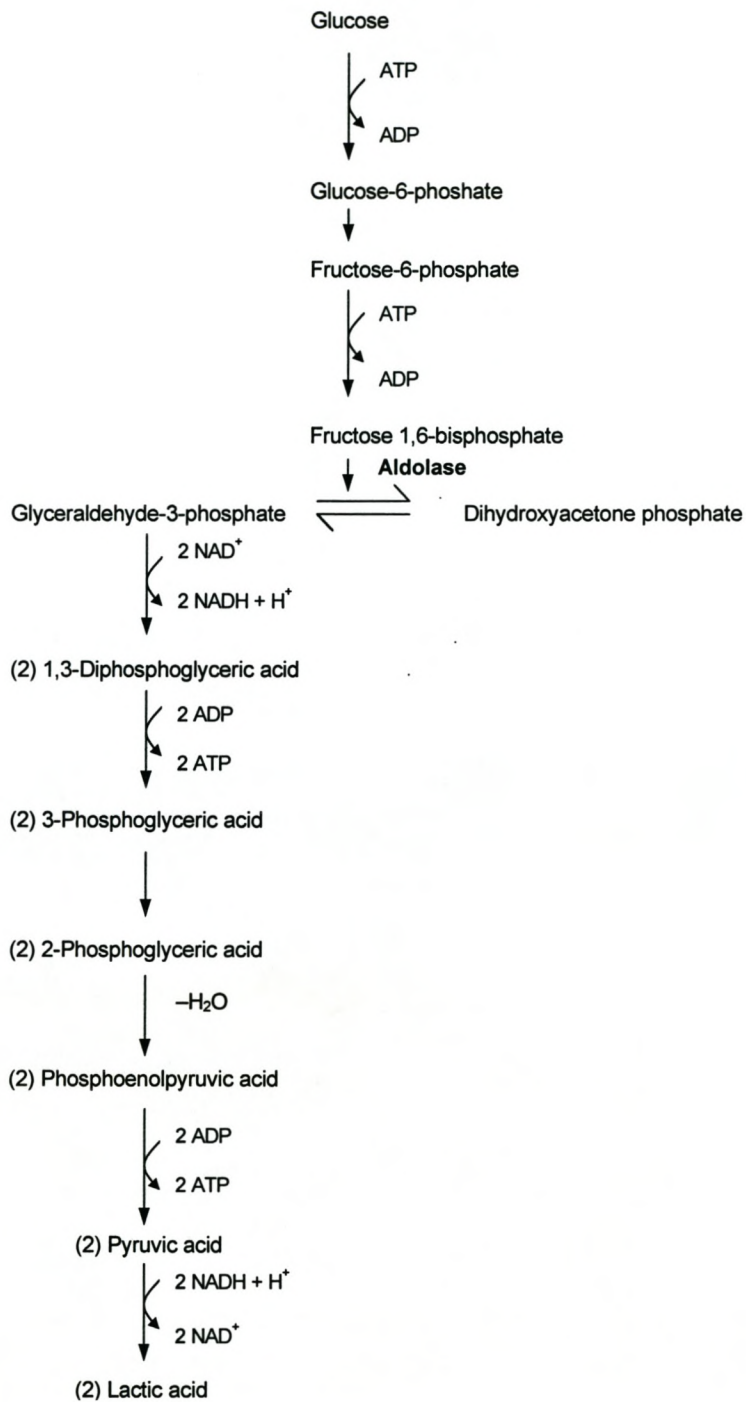
Fig. 2.1 Embden-Meyerhof-Parnas (EMP) pathway for the metabolism of glucose by obligately homofermentative LAB (Adapted from Fugelsang 1997).

In facultatively heterofermentative lactobacilli (Group II), glucose is converted to lactic acid, as in the case of Group I, but pentoses are fermented into lactic acid and acetic acid via the pentose phosphate pathway (**Fig. 2.2**). The obligately heterofermentative LAB of Group III lack the fructose diphosphate aldolase enzyme of the EMP pathway. They ferment glucose to CO_2, lactic acid, acetic acid and ethanol via the pentose phosphate pathway. Pentoses are fermented into lactic acid and acetic acid in the same manner as the lactobacilli from Group II (Ribéreau-Gayon *et al.* 2000).

Several species and strains of lactobacilli have been isolated from grape must and wine. These include *Lactobacillus brevis* (Vaughn 1955; Du Plessis and Van Zyl 1963; Pilone *et al.* 1966; Chalfan *et al.* 1977; Sharpe 1981; Dicks and Van Vuuren 1988), *Lactobacillus buchneri* (Vaughn 1955; Du Plessis and Van Zyl 1963; Pilone *et al.* 1966; Sharpe 1981; Dicks and Van Vuuren 1988), *Lb. casei, Lactobacillus curvatus, Lb. delbrueckii, Lactobacillus fermentum* (Vaughn 1955; Dicks and Van Vuuren 1988), *Lb. fructivorans* (Amerine and Kunkee 1968), *Lactobacillus hilgardii* (Douglas and Cruess 1936; Vaughn 1955; Du Plessis and Van Zyl 1963; Dicks and Van Vuuren 1988), *Lactobacillus jensenii, Lactobacillus kunkeei* (Edwards *et al.* 1998), *Lactobacillus nagelii* (Edwards *et al.* 2000), *Lactobacillus plantarum* and *Lactobacillus sakei* (Costello *et al.* 1983; Fugelsang 1997). Three species from the genus *Pediococcus* have been isolated from wine, namely *Pediococcus parvulus, Ped. damnosus* and *Pediococcus pentosaceus* (Wibowo *et al.* 1985; Edwards and Jensen 1992; Fugelsang 1997; Gindreau *et al.* 2001). The only species from the genus *Leuconostoc* that has been found in wine is *Leuconostoc mesenteroides* (Lafon-Lafourcade *et al.* 1983; Lonvaud-Funel 1999). *Leuconostoc oenos* has been renamed *O. oeni* (Dicks *et al.* 1995). *Oenococcus oeni* is the only species in the genus *Oenococcus* and is usually associated with malolactic fermentation (MLF) (Wibowo *et al.* 1985).

2.3 IDENTIFICATION OF LACTIC ACID BACTERIA

In the past, the identification of LAB has been based primarily on phenotypic characteristics, such as morphology, physiology and biochemical features. More discriminating methods, such as the chemical composition of microorganisms (i.e. fatty acids and proteins), were then used. More recently, the use of molecular techniques have made the identification of LAB at genus, species and even strain level more accurate. Molecular techniques have revolutionised the phylogeny and taxonomy of living organisms. It has also led to the development of polyphasic taxonomy. The aim of polyphasic taxonomy is to use genotypic, phenotypic and phylogenetic information to identify and classify microorganisms (Vandamme *et al.* 1996).
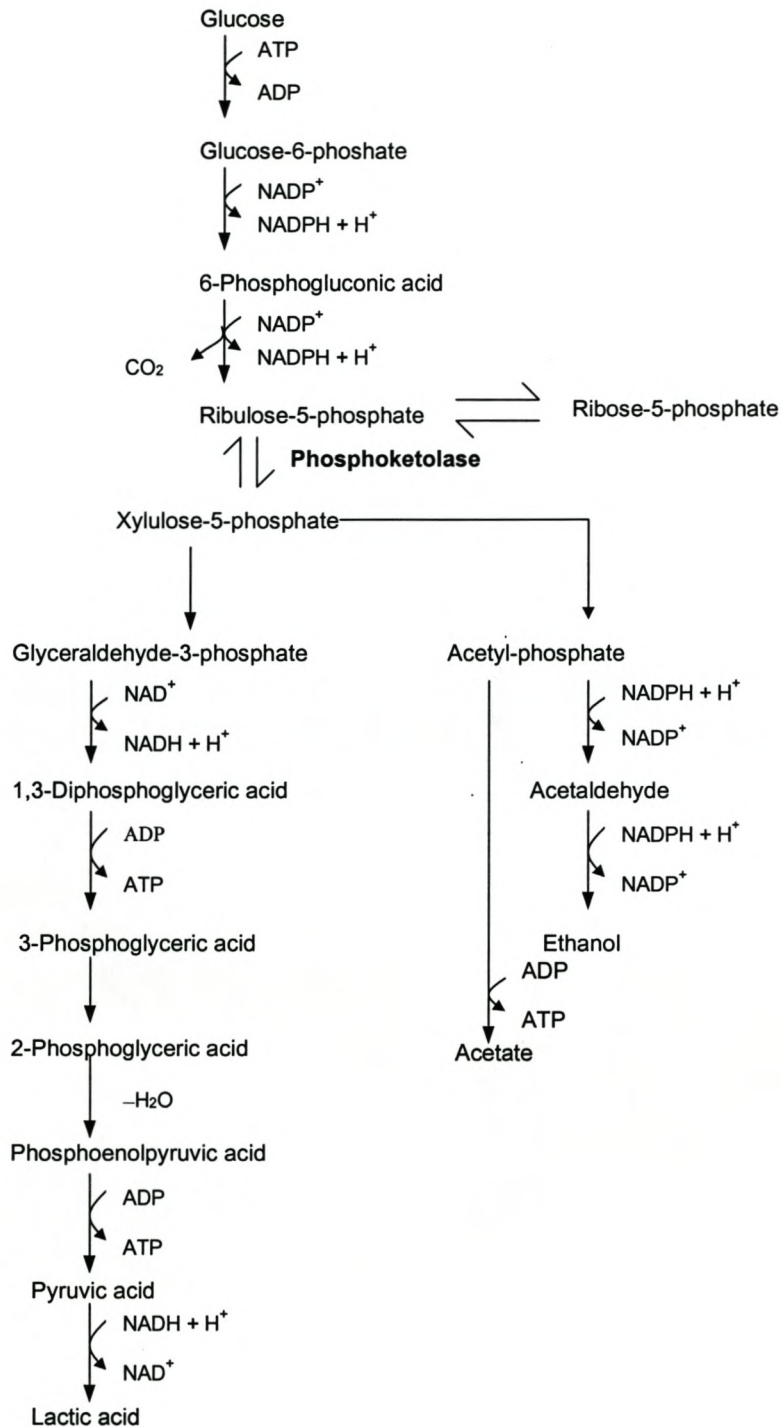
Fig. 2.2 Pentose phosphate (6-phosphogluconate) pathway for the metabolism of glucose by heterofermentative LAB (Adapted from Fugelsang 1997).

2.3.1 PHENOTYPIC METHODS

Phenotypic analysis includes the morphology, physiology and biochemical assimilation of diverse substrates and the nature of the metabolic products. As a whole, all these characteristics provide useful information. Morphological features include both cellular (shape, endospore, Gram staining, inclusion bodies and flagella) and colony (form, size, colour and dimension) characteristics. Physiological features include the ability to grow at different temperatures, pH values, salt concentrations and atmospheric conditions, and growth in the presence of different chemicals (e.g. antimicrobial agents). Examples of biochemical features are the presence and activity of different enzymes and the metabolism of different compounds (Vandamme *et al.* 1996).

2.3.1.1 Carbohydrate fermentation

The API system (Biomerieux, France) is commonly used for the identification of bacteria. This system entails the utilisation of different carbohydrates as energy sources. The fermentation profile for the examined strain can therefore be determined. The API system is widely used to identify LAB isolated from wine (Costello *et al.* 1983; Davis *et al.* 1986; Jensen and Edwards 1991). Dicks and Van Vuuren (1988) reported the API 50 CHL system to be reliable and rapid for the identification of wine lactobacilli. Tracey and Britz (1987) grouped 54 *O. oeni* strains into six clusters using the API 50 CHL system.

However, Pardo *et al.* (1988) found differing fermentation patterns when *O. oeni* were inoculated in the API 50 CHL system and in conventional test tubes. Jensen and Edwards (1991) proved that the API 50 CHL system was not adequate for the identification of *O. oeni*. However, when the pH of the API 50 CHL medium was adjusted from 7·0 to 5·2 and the incubation period was extended, the results corresponded well with results obtained with the test tube method (Jensen and Edwards 1991). A disadvantage of this technique is that a good LAB database is needed. The current API databases contain a limited number of LAB isolated from wine and the identification of unknown isolates is not always possible.

2.3.1.2 Enzyme fingerprinting

Commercially available enzyme fingerprinting systems consist of a series of dehydrated reagents to which a standardised inoculum is added. The enzyme activity is recorded after a short incubation. The API ZYM system (API Laboratories and Analytab Products) has been used successfully to identify the enzymatic pattern of strains of *Lb. casei*. The system allows for the detection of enzymes such as esterase, lipase, alkaline phoshatase and α-galactosidase (Bascomb 1987). For these tests to be accurate, it is important that they are performed under standardised conditions (Vandamme *et al.* 1996).

2.3.1.3 Cell wall composition

The cell walls of Gram-positive bacteria are composed mainly of peptidoglycan (30-70% of the total cell wall), polysaccharides and/or teichoic acids and often teichuronic acid (Schleifer and Kandler 1972). Teichoic acid may be analysed by using gas-liquid chromatography (Fischer *et al*. 1980, 1981).

The different murein (peptidoglycan) types of the cell wall have been used to classify LAB (Hammes and Vogel 1995). The murein types present in the cell walls of *Leuconostoc* spp. are characteristic for each species (Garvie 1986). The interpeptide bridges in the murein of *O. oeni* are different from those found in non-acidophilic leuconostocs (Garvie 1986; Schillinger *et al*. 1989). The Lys-D-Asp type of murein is specific for the homofermentative, as well as the facultatively heterofermentative, lactobacilli from the *Lb. delbrueckii* group. The Lys-D-Asp type or the diaminopimelic-direct type is also the type found in *Lb. brevis, Lb. buchneri, Lb. hilgardii* (Sharpe 1981) and *Lb. fructivorans* (Weiss *et al*. 1983). *Lb. fermentum* contains the L-Orn-D-Asp type (Sharpe 1981). The obligately heterofermentative organisms from the *Leuconostoc* group all have the Lys amino type [Lys-L-Ser-L-Ala$_2$ and Lys-L-Ala$_2$] (Dellaglio *et al*. 1995). The *Pediococcus* spp. associated with wine contain the L-Lys-L-Ala-D-Asp type of peptidoglycan (Garvie 1986). The chemical composition of the cell wall can change in response to culture conditions and age of the culture.

2.3.1.4 Electrophoretic mobility of enzymes

The electrophoretic mobility of lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH), glucose-6-phoshate (G6PDH) and 6-phosphogluconate dehydrogenase (6-PGD) are useful to differentiate between LAB (Gasser 1970; Garvie 1975; Garvie and Farrow 1980; Irwin *et al*. 1983). This technique has been used in the classification of leuconostocs (Garvie 1969, 1975; Garvie and Farrow 1980; Irwin *et al*. 1983) and lactobacilli (London 1976; Sharpe 1981). The use of LDH in starch gels (Gasser 1970) or in polyacrylamide gels (Hensel *et al*. 1977) has proved useful for the classification of LAB. Fujisawa *et al*. (1992) showed that this method was especially reliable for the discrimination of phenotypically similar strains.

2.3.1.5 Cellular fatty acids

The variety of lipids present in the bacterial cells can also be used for taxonomic purposes. Polar lipids and sphingolipids, which are present in a restricted number of taxa (Jones and Krieg 1984), are examples of these. Fatty acids have also been used for classification purposes (Suzuki *et al*. 1993). The variability of fatty acid chain length, double bond position and substituent groups has proved useful in the characterisation of bacterial taxa (Suzuki *et al*. 1993). This technique has been used for the identification and classification of *O. oeni* (Tracey and Britz 1989), *Ped. acidilactici, Ped. damnosus, Lb. fermentum* and *Lb. plantarum* (O'Leary and Wilkinson 1988).

It is essential to standardise the growth conditions and experimental procedures to obtain reproducible results. This method is rapid, simple and has become highly automated for the analysis of large numbers of strains (Vandamme *et al.* 1996). A disadvantage of the technique is that it is not always reliable. For some genera, the results may allow for the differentiation and identification of individual species or even subspecies, while, for other genera, different species have identical fatty acid profiles (Welch 1991).

2.3.1.6 Analysis of total soluble cell protein patterns

This identification method involves subjecting the total cell protein contents of bacteria to electrophoresis. The comparison of whole-cell protein patterns obtained under standardised sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) conditions provides the taxonomist with a phenotypic tool to distinguish isolates on a species and/or subspecies level (Pot *et al.* 1994). Previously, a certain degree of pre-identification was required to know which reference strains must be selected. This was overcome with the development of computer-assisted software that led to the compilation of databases.

Databases containing digitised and normalised protein patterns of known LAB species have been used with great success (Vauterin and Vauterin 1992; Vauterin *et al.* 1993; Pot *et al.* 1994). A good correlation exists between the results of SDS-PAGE, DNA-DNA hybridisation and primers for randomly amplified polymorphic DNA (RAPD-PCR) (Dellaglio *et al.* 1991; Dicks *et al.* 1996). Dicks *et al.* (1990) used protein profiles to determine the genotypic relatedness between *O. oeni* and the *Leuconostoc* spp. The use of protein profiles to differentiate among species of the *Lb. acidophilus* group (Pot *et al.* 1993; Du Plessis and Dicks 1995; Boot *et al.* 1996), *Lb. casei*, *Lactobacillus paracasei*, *Lactobacillus zeae* and *Lactobacillus rhamnosus* (Dellaglio *et al.* 1991; Klein *et al.* 1995; Dicks *et al.* 1996), *Lb. plantarum* and *Lactobacillus pentosus* (Van Reenen and Dicks, 1996) has been very successful.

Protein fingerprints produce complex but stable patterns under standardised conditions and, when analysed with computer-assisted software, will yield valuable information in the typing of large numbers of isolates. A disadvantage of this technique is that expensive computer-assisted analysis is needed for the protein fingerprints.

2.3.2 GENOTYPIC METHODS

2.3.2.1 Restriction fragment length polymorphism (RFLP)

The restriction fragment length polymorphism (RFLP) method consists of digesting DNA with restriction enzymes. These enzymes produce fragments of different sizes, which are then separated by electrophoresis. The electrophoretic profile differs depending on the species and strain. Different fragments, varying in size and number, can be obtained from

the same DNA by using a variety of restriction enzymes. The restriction enzymes that are often used for lactobacilli are *Sma*I, *Bgl*I, *Apa*I, *Xho*I and *Sfi*I (Roussel *et al*. 1993; Ferrero *et al*. 1996; Charteris *et al*. 1997). Sato *et al*. (2000) used RFLPs to identify *O. oeni* strains isolated from wine and the method has also been used in the characterisation of *Lb. plantarum* (Johansson *et al*. 1995b) and *Lb. casei* (Ferrero *et al*. 1996). The technique is reliable and well adapted for the identification of yeast and LAB.

Enzymes that cut the DNA too often will produce complicated profiles that are difficult to study. Enzymes that cut only a few times produce simpler profiles, but the diffrence in the size of the fragments produced might require pulse field electrophoresis to separate them.

2.3.2.2 Specific probe DNA/DNA hybridisation

The DNA/DNA hybridisation technique is based on the ability of double strand DNA to separate, reversibly, into two single strands under conditions that breaks their hydrogen bonds. Along with the ionic force of the medium, temperature is the determining parameter of DNA denaturation. Strains are considered to belong to the same species if they have 70% homology of their genomic DNA sequence (Ribèreau-Gayon *et al*. 2000).

Most of the wine LAB can be identified to species level in this manner. The first successful probe was developed for *O. oeni* (Lonvaud-Funel *et al*. 1989). The DNA probes of *O. oeni* do not hybridise with the genomic DNA of other species, and the inverse is also true. The presence of *O. oeni* can be identified even in a mixture containing other bacteria. This method proved well suited for other LAB species found in grape must and wine (Lonvaud-Funel *et al*. 1991). Strains of *Lb. hilgardii*, *Lb brevis* and *Lb. fructivorans* were identified in spoiled wine through *in situ* hybridisation (Sohier and Lonvaud-Funel 1998).

DNA-DNA hybridisation studies have a few disadvantages. They can be labour intensive and require the use of large quantities of DNA (Stackebrandt and Ludwig 1994). Cross hybridisation can occur between closely related species, such as *Lb. brevis* and *Lb. hilgardii* (Lonvaud-Funel *et al*. 1991; Sohier and Lonvaud-Funel 1998).

2.3.2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction consists of amplifying one or several DNA fragments by nucleotide polymerisation. The obtained product can be viewed by electrophoresis. In recent years, species-specific primers have been developed for the identification of LAB. Specific probes have been developed for several dairy and wine LAB species (**Table 2.1**). Species-specific primers have been used for the identification and detection of *O. oeni* (Zapparoli *et al*. 1998) and for the differentiation of *Lb. plantarum, Lb. pentosus* and *Lactobacillus paraplantarum* (Torriani *et al*. 2001; Berthier and Ehrlich 1998). This is a rapid and reliable PCR technique.

The primers for randomly amplified polymorphic DNA (RAPD)–PCR are used under low stringency annealing conditions, which allows them to bind DNA at places to which they have full or partial homology. The primers can be derived either from specific DNA or RNA sequences or from randomly selected DNA primers. The target DNA is usually purified DNA, crude cell extracts or colonies. RAPD analysis has been a valuable tool for the rapid typing of LAB strains in wine (Zapparoli *et al.* 2000). This technique has also been used in the typing of *Lactobacillus* strains (Cocconcelli *et al.* 1995; Du Plessis and Dicks 1995; Johansson *et al.* 1995a; Drake *et al.* 1996; Van Reenen and Dicks 1996). The disadvantages of this method are that computer-assisted analysis is needed for the interpretation of complicated fingerprints and that reproducibility can also be a problem. Small changes in annealing conditions can affect banding patterns (Cusick and O'Sullivan 2000).

Another PCR technique that has also been used for the identification of LAB is nested PCR. Nested PCR consists of two rounds of PCR with two distinct primer sets. The first round amplifies a fragment of DNA that contains the primer sites for the second round of PCR within its sequence (Stewart and Dowhanick 1996). This technique has been used by Zapparoli *et al.* (1998) for the identification of *O. oeni* in wine and for the screening of *Lactobacillus* and *Pediococcus* spp. in beer (Stewart and Dowhanick 1996).

2.3.2.4 Ribosomal RNA (rRNA) sequence homology

Ribosomal RNA contains highly conserved and more variable domains and is a good target for studying phylogenetic relatedness (Woese 1987; Schleifer and Ludwig 1989; Stackebrandt and Ludwig 1994). Ribosomal RNA sequencing allows us to trace the ancient origins of the prokaryotes and also reveals the phylogenetic relationships between bacteria.

The sequencing of 5S rRNA molecules resulted in the formation of a sequence database (Wolters and Erdmann 1988). The sequencing of 16S rRNA with conserved primers and reverse transcriptase (Lane *et al.* 1985) was a very important advance in bacterial phylogeny. Nowadays, these techniques have mostly been replaced by direct sequencing of parts of, or virtually entire 16S or 23S rDNA molecules using the PCR technique. Databases are available that contain published and some unpublished partial or complete sequences of 16S and 23S rRNA (Olsen *et al.* 1991; De Rijk *et al.* 1992). The analysis of 16S and 23S rRNA sequences is now being used increasingly as an alternative to DNA-rRNA hybridisations and oligonucleotide cataloguing (Lane *et al.* 1985). 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).

Table 2.1   Specific primer sequences for the identification of wine lactic acid bacteria

Primer	Specificity	Sequence 5' - 3'	Target	Reference
forw	*Lb. brevis*	CTTGCACTGATTTTAACA	16S rRNA	Guarneri *et al.* 2001
rev	*Lb. brevis*	GGGCGGTGTGTACAAGGC	16S rRNA	Guarneri *et al.* 2001
Y2	*Lb. casei*	CCCACTGCTGCCTCCCGTAGGAGT	16S rRNA	Young *et al.* 1991
casei	*Lb. casei*	TGCACTGAGATTCGACTTAA	V1 region of 16S rRNA	Ward and Timmins 1999
16	*Lb.curvatus*	GCTGGATCACCTCCTTTC	16S rRNA	Berthier and Ehrlich 1998
Lc	*Lb.curvatus*	TTGGTACTATTTAATTCTTAG	16S rRNA	Berthier and Ehrlich 1998
Del I	*Lb. delbrueckii*	ACGGATGGATGGAGAGCAG	16' end of spacer region	Tilsala-Timisjärvi and Alatossava 1997
Del II	*Lb. delbrueckii*	GCAAGTTTGTTCTTTCGAACTC	23' end of spacer region	Tilsala-Timisjärvi and Alatossava 1997
FERM1	*Lb. fermentum*	GTTGTTCGCATGAACAACGCTTAA	V1 region of 16S rRNA	Chagnaud *et al.* 2001
LOWLAC	*Lb. fermentum*	CGACGACCATGAACCACCTGT	Conserved 16S rRNA	Chagnaud *et al.* 2001
H2	*Lb. hilgardii*	ACTNATTTGACATTAAGA	Ribosomal operon	Sohier *et al.* 1999
8623	*Lb. hilgardii*	CTGGTTCACTATCGGTCTC	Ribosomal operon	Sohier *et al.* 1999
Pr I	*Lb. paracasei*	CAGACTGAAAGTCTGACGG	16' end of spacer region	Tilsala-Timisjärvi and Alatossava 1997
Pcas II	*Lb. paracasei*	GCGATGCGAATTTCTTTTTC	23' end of spacer region	Tilsala-Timisjärvi and Alatossava 1997
16	*Lb. paraplantarum*	GCTGGATCACCTCCTTTC	16S rRNA	Berthier and Ehrlich 1998
Lpapl	*Lb. paraplantarum*	ATGAGGTATTCAACTTATT	16S rRNA	Berthier and Ehrlich 1998
paraF	*Lb. paraplantarum*	GTCACAGGCATTACGAAAAC	*recA* gene	Torriani *et al.* 2001
pREV	*Lb. paraplantarum*	TCGGGATTACCAAACATCAC	*recA* gene	Torriani *et al.* 2001

Primer	Specificity	Sequence 5' – 3'	Target	Reference
16	*Lb. pentosus*	GCTGGATCACCTCCTTTC	16S rRNA	Berthier and Ehrlich 1998
Lpe	*Lb. pentosus*	GTATTCAACTTATTAGAACG	16S rRNA	Berthier and Ehrlich 1998
pentF	*Lb. pentosus*	CAGTGGCGCGGTTGATATC	*rec*A gene	Torriani *et al*. 2001
pREV	*Lb. pentosus*	TCGGGATTACCAAACATCAC	*rec*A gene	Torriani *et al*. 2001
LbP11	*Lb. plantarum*	AATTGAGGCAGCTGGCCA	16S rRNA	Quere *et al*. 1997
LbP12	*Lb. plantarum*	GATTACGGGAGTCCAAGC	16S rRNA	Quere *et al*. 1997
16	*Lb. plantarum*	GCTGGATCACCTCCTTTC	16S rRNA	Berthier and Ehrlich 1998
Lpl	*Lb. plantarum*	ATGAGGTATTCAACTTATG	16S rRNA	Berthier and Ehrlich 1998
planF	*Lb. plantarum*	CCGTTTATGCGGAACACCTA	*rec*A gene	Torriani *et al*. 2001
pREV	*Lb. plantarum*	TCGGGATTACCAAACATCAC	*rec*A gene	Torriani *et al*. 2001
16	*Lb. sakei*	GCTGGATCACCTCCTTTC	16S rRNA	Berthier and Ehrlich 1998
Ls	*Lb. sakei*	ATGAAACTATTAAATTGGTAC	16S rRNA	Berthier and Ehrlich 1998
16	*Lb. zeae*	GCTGGATCACCTCCTTTC	16S rRNA	Berthier and Ehrlich 1998
*zeae*ITS	*Lb. zeae*	CGATGCGAATTTCTAAATT	16S-23S intergenic spacer	Berthier *et al*. 2001
16reverse	*Lb. zeae*	GAAAGCAGGTGATCCAGC	16S rRNA	Berthier *et al*. 2001
*zeae*16S	*Lb. zeae*	GCATCGTGATTCAACTTAA	16S rRNA	Berthier *et al*. 2001
Lmes-f	*Lc. mesenteroides*	AACTTAGTGTCGCATGAC	16S rDNA	Lee *et al*. 2000
Lmes-r	*Lc. mesenteroides*	AGTCGAGTTACAGACTACAA	16S rDNA	Lee *et al*. 2000
On 1	*O. oeni*	TAATGTGGTTCTTGAGGAGAAAAT	MLE gene	Zapparoli *et al*. 1998
On 2	*O. oeni*	ATCATCGTCAAACAAGAGGCCTT	MLE gene	Zapparoli *et al*. 1998
Forward	*O. oeni*	GTGAAGTGAGGCAATGAC	16S rRNA gene	Bartowsky and Henschke 1999
Reverse	*O. oeni*	GCACCACCTGTATCCAATG	16S rRNA gene	Bartowsky and Henschke 1999

Ribosomal RNA homology studies have resulted in the creation and refinement of the three main *Lactobacillus* groups, i.e. the *Lb. delbrueckii* group (Hammes and Vogel 1995), the *Lb. casei-Pediococcus* group (Collins *et al.* 1991; Schleifer and Ludwig 1995a), and the *Leuconostoc* group (Collins *et al.* 1991). This technique was also used to determine the phylogenetic relatedness of the leuconostocs (Martinez-Murcia *et al.* 1993). 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).

2.3.2.5 RNA-targeted oligonucleotide probes

The availability of the number of DNA and rRNA nucleotide sequences has facilitated the comparison of homologous sequences between different taxa of LAB. These homologous oligonucleotide regions may be labelled for use as probes in hybridisation experiments to identify unknown isolates. It is also possible to amplify these specific sequences by PCR to enhance detection levels of the hybridisations (Vandamme *et al.* 1996). This technique has been used in the identification of many *Lactobacillus* spp. A number of species-specific 23S-rRNA targeted oligonucleotide probes have been designed. Hertel *et al.* (1993) identified *Lb. delbrueckii*, *Lb. paracasei* and *Lactobacillus helveticus*, while Ferrero *et al.* (1996) identified *Lb. casei* and *Lb. paracasei* using this technique.

2.3.2.6 Ribotyping

Ribotyping or RFLP of rRNA-encoding genes generates fewer bands than REA (restriction endonuclease analysis) or RAPD-PCR and the results are interpreted more easily. This method was used by Roussel *et al.* (1993) to differentiate between strains within the *Lb. acidophilus* group. This method provides useful information about polymorphisms among strains with a low DNA homology. Ribotyping was also used successfully to study the genetic heterogeneity among strains of *Lb. plantarum* (Duffner and O'Connell 1995) and at species level by Johansson *et al.* (1995b). This technique was also used for the typing of *O. oeni* and *Leuconostoc* strains (Viti *et al.* 1996).

2.4 ECOLOGY OF LACTIC ACID BACTERIA IN WINE

Many studies have indicated that the LAB in wine originate from the grapes and the winery equipment. Generally, the organisms occur on the surface of grapes and vine leaves, mainly in low numbers (<10^3 CFU g^{-1}), depending on the maturity and condition of the berries and vine (Wibowo *et al.* 1985; Sponholz 1993; Lonvaud-Funel 1995). Grapes spoiled by acetic acid bacteria and fungi have been reported to stimulate the growth of LAB (Fugelsang 1997). Winery equipment, such as storage tanks, pumps, valves and transfer lines (Gini and Vaughn 1962; Webb and Ingraham 1960; Fugelsang 1997), wood

barrels and bottling machines (Wibowo *et al.* 1985) have also been implicated as sources of LAB.

2.4.1 EVOLUTION OF LACTIC ACID BACTERIA

Studies in several countries have shown that *O. oeni* occurs mainly during MLF. *Pediococcus* spp. is found mostly after MLF and predominantly in wines with a higher pH. Together with several *Lactobacillus* spp., which occur mainly in must and after MLF, these are the main species in wine (Lafon-Lafourcade *et al.* 1983; Davis *et al.* 1985b; Wibowo *et al.* 1985; Ribéreau-Gayon *et al.* 2000).

Fig. 2.3 shows several stages of vinification, during which different species of LAB may occur and grow. Musts, soon after crushing, contain LAB at populations of 10^3 to 10^4 cells ml^{-1}. The yeast and bacteria multiply during the first days of alcoholic fermentation (Ribéreau-Gayon *et al.* 2000). The yeasts are better adapted and rapidly reach elevated populations. Usually, the production of ethanol, the amount of sulphur dioxide (SO_2) added and the pH limit the growth of the LAB. In fermentations to which LAB starter cultures are not added, native populations decline rapidly and may not redevelop. Typically, populations fall by 1-2 log units and remain at low numbers (generally <100 CFU ml^{-1}).
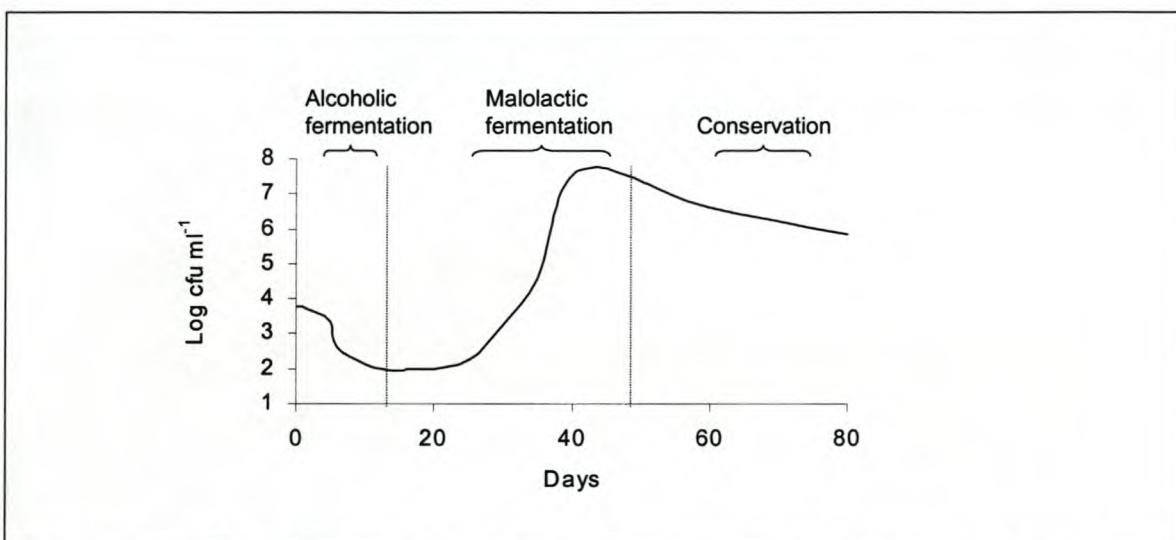
Fig. 2.3 The development of lactic acid bacteria during alcoholic and malolactic fermentation (Adapted from Wibowo *et al.* 1985).

After a lag phase, the length of which depends on wine properties, the surviving cells commence multiplication to conduct MLF. This phase is characterised by vigorous bacterial growth and populations of as high as 10^6 to 10^8 cells ml^{-1} can be attained (Wibowo *et al.* 1985; Fugelsang 1997; Ribéreau-Gayon *et al.* 2000). The fate of the LAB during the subsequent maturation and cellar storage depends on how the wine is handled. The addition of SO_2 and storage of the wine at high temperatures leads to a progressive

loss of the viability of these LAB (Lafon-Lafourcade 1975; Lafon-Lafourcade *et al*. 1983). The pH of the wine also plays an important role (Costello *et al*. 1983).

2.4.2 EVOLUTION OF VARIOUS SPECIES OF LACTIC ACID BACTERIA

During fermentation, the LAB microflora evolve not only in numbers, but also in the variety of species. Just after its arrival in the tank, grape must contains very diverse microbial populations. The major species present at this stage include *Lb. plantarum*, *Lb. casei*, *Lb. hilgardii*, *Lb. brevis*, *Lc. mesenteroides*, *O. oeni*, *Ped. damnosus* and *Ped. pentosaceus* (Costello *et al*. 1983; Lafon-Lafourcade *et al*. 1983; Fleet *et al*. 1984; Ribéreau-Gayon *et al*. 2000). All the species are not always present, but a natural selection takes place during alcoholic fermentation. As with many other foods fermented by LAB, there is a successional growth of several LAB species during vinification (Wibowo *et al*. 1985; Ribéreau-Gayon *et al*. 2000). *Lactobacillus* species, *Pediococcus* and *Lc. mesenteroides* progressively disappear during the alcoholic fermentation, or are present at a concentration that is too low to be detected. *Oenococcus oeni* is the main species identified at the end of fermentation (Lonvaud-Funel 1999). Even more interesting is the observation that several different strains of *O. oeni* may evolve during MLF (Fleet *et al*. 1984). However, some of the other species are not completely eliminated. The natural selection of *O. oeni* during the fermentation is due mainly to the increase in ethanol and other products of yeast metabolism (Lonvaud-Funel 1999). *Oenococcus oeni* has a high alcohol (up to 14% v/v) and low pH tolerance. However, in wines with a high pH (e.g. pH 3·5 to 4·0), *Pediococcus* and *Lactobacillus* spp. may grow and conduct MLF (Costello *et al*. 1983).

The same natural selection process discussed above also applies to brandy base wine. Lafon-Lafourcade *et al*. (1983) studied the development of LAB in cognac base wine. They isolated *Lc. mesenteroides* and *O. oeni* from the freshly produced must. In the pressings they found *Lb. brevis* and *Lb. casei*, along with *O. oeni*. *Leuconostoc mesenteroides* was shown to be the dominant species on the first day of alcoholic fermentation, but *O. oeni* and *Lb. plantarum* were also present. After day three, *O. oeni* was the only species found in the base wine, continued to grow and was responsible for MLF.

2.5 FACTORS AFFECTING THE GROWTH OF LACTIC ACID BACTERIA

2.5.1 PHYSICOCHEMICAL FACTORS

2.5.1.1 pH

Wine pH is one of the most important parameters that affects the proliferation of LAB in wine.

The pH influences:
1) the species that grow in wine;
2) the metabolic behaviour of these species;
3) the survival of the LAB;
4) the growth rate of the LAB; and
5) the lag phase leading to MLF.

Generally, wines with a pH below 3·5 do not support the growth of *Pediococcus* and *Lactobacillus* spp. and, invariably, *O. oeni* dominates in these wines. *Oenococcus oeni* exhibits the greatest tolerance to pH, with strains being able to degrade L-malic acid at a pH below 3·0 (Davis *et al.* 1988; Henick-Kling *et al.* 1989). Lactic acid bacteria can grow at a pH of 2·9-3·0, but their growth is slow (Ribèreau-Gayon *et al.* 2000). Wines in the pH range of 3·5-4·0 generally show a more rapid onset and completion of MLF than wines in the pH range of 3·0-3·5 (Costello *et al.* 1983; Wibowo *et al.* 1985). The sensitivity of LAB to ethanol and free molecular SO_2 increases at a low pH. At low pH, sugars are not metabolised and the catabolism of L-malic acid is enhanced to reach a pH level more favourable to the cell (Firme *et al.* 1994). At higher pH values, when sugar metabolism is easier and lower amounts of ATP are formed from malate (Cox and Henick-Kling 1989), the energy required for growth and maintenance is supplied by sugar catabolism, and malic acid degradation follows biomass formation (Firme *et al.* 1994).

Base wines used for the production of brandy usually have a lower pH than normal white wines. The grapes used for base wines have a higher acid and lower sugar content. The low pH of base wine also helps prevent the growth of spoilage organisms.

2.5.1.2 Sulphur dioxide

Sulphur dioxide (SO_2) is used widely in winemaking as an antioxidant and to control the growth of wild yeast and bacteria. Upon addition to wine, SO_2 enters a pH-dependent equilibrium consisting of bound SO_2, molecular or free SO_2, and bisulphate and sulphite ions. Together, these different forms represent the total level of SO_2 (Wibowo *et al.* 1985). Wine pH affects the dissociation of SO_2, with lower pH values giving a higher proportion of free, molecular SO_2. Free SO_2 is the most inhibitory form (Carr *et al.* 1976; Macris and Markakis 1974). The mechanism of action of SO_2 was studied particularly in yeast, but it is most likely very similar in bacteria. According to Romano and Suzzi (1993), SO_2 penetrates the cell in molecular form by diffusion. In the cytoplasm, where the pH is highest, it dissociates and reacts with essential biological molecules (enzymes, coenzymes and vitamins). The result is cessation of growth and, finally, cell death. Carbonyl compounds, such as acetaldehyde, are responsible for binding some of the SO_2 (Ribèreau-Gayon *et al.* 2000).

A total SO_2 concentration of >100 mg l^{-1}, bound SO_2 of 50 to 100 mg l^{-1} or free SO_2 of 1 to 10 mg l^{-1}, is sufficient to inhibit the growth of LAB (Rankine and Bridson 1971; Somers and Wescombe 1982; Wibowo *et al.* 1985). These values vary depending on the species

(Fornachon 1963), wine pH (Liu and Gallander 1983; Van der Westhuizen and Loos 1981) and the amount of insoluble solids present in wine (Liu and Gallander 1982). In general, strains of *O. oeni* appear to be the most sensitive. Moreover, it is important to distinguish between those concentrations that merely retard growth and those that destroy cell viability (Macris and Markakis 1974; Lafon-Lafourcade 1975; Lafon-Lafourcade *et al.* 1983). It is recommended that base wines contain no SO_2 or less than 20 mg l^{-1} (Léauté 1990).

2.5.1.3 Temperature

In wine, a temperature of 18 to 20ºC is optimal for the growth of LAB and the induction of MLF. The optimal decarboxylation of malic acid occurs between 20 and 25ºC (Ribèreau-Gayon *et al.* 2000). Growth and MLF are strongly inhibited by a low temperature and only a few strains of *O. oeni* can conduct MLF below 15ºC. Malolactic fermentation is rarely observed at temperatures below 10ºC (Wibowo *et al.* 1985). Excessive temperatures of 25ºC and above increases the risk of bacterial spoilage and an increase in the production of volatile acidity. A fermentation temperature below 24ºC is recommended for base wines to minimise higher alcohol formation (Guymon 1974).

2.5.1.4 Ethanol

Like most organisms, LAB are sensitive to ethanol. In general, *Lactobacillus* species are more alcohol tolerant than either *Pediococcus* or *Oenococcus* species. *Lactobacillus fructivorans* is the most alcohol-tolerant wine lactic acid bacteria, surviving in dessert wines with 20% (v/v) alcohol (Fornachon 1943; Vaughn 1955; Wibowo *et al.* 1985). *Lactobacillus buchneri, Lb. casei, Lb. plantarum* and *Lb. vermiforme* strains were isolated from spoiled fortified wine (Stratiotis and Dicks 2002). Other lactobacilli are more sensitive (<15% v/v, alcohol). The alcohol tolerance of *Pediococcus* and *Oenococcus* spp. is 12-14% (v/v) (Fugelsang 1997). Strains of *Lb. fructivorans, Lb. brevis* and *Lb. hilgardii* are frequently isolated from fortified wines with alcoholic strengths of 16 to 20% (v/v). They seem to be naturally adapted to ethanol, but lose this adaptation after isolation (Ribèreau-Gayon *et al.* 2000).

Alcohol tolerance decreases with an increase in temperature and low pH values (Lafon-Lafourcade *et al.* 1983). Growth of *O. oeni* was inhibited at 4% (v/v) ethanol. At pH 3.0, the optimal pH for whole-cell malolactic enzyme activity, the effect of ethanol on the malolactic enzyme activity of non-growing cells was negligible until a concentration of 12% was reached. An increasing inhibition was observed at higher ethanol concentrations. At pH 5.0, malolactic enzyme activity was higher for all ethanol concentrations tested up to 20% (v/v) (Capucho and San Romão 1994). The alcohol concentration of brandy base wines varies considerably, ranging between 8 and 11·5% (v/v) or even more in Armagnac wines (Bertrand 1995), and between 7 and 9% (v/v) in Cognac wines (Léauté 1990). The inhibiting effect of alcohol might be accentuated by the lower pH values of base wine.

2.5.1.5 Oxygen and carbon dioxide

Anaerobic to microaerophilic conditions stimulate the growth of LAB in wines and thus the rate of MLF, although some dissolved oxygen (O_2) appears necessary (Gilliland and Speck 1975). Current observations indicate that limited aeration, after the running off or racking of the wine, strongly favours the initiation of MLF (Ribèreau-Gayon *et al.* 2000). Kelly *et al.* (1989) observed more rapid growth of *O. oeni* under anaerobic conditions. The growth of *O. oeni* was not stimulated by carbon dioxide (CO_2), but rather inhibited by the presence of oxygen.

2.5.1.6 Nutritional status

Compared to growth in supplemented expansion cultures and during alcoholic fermentation, wine represents a relatively limited environment in terms of critical nutrients. Lactic acid bacteria are nutritionally demanding, requiring a variety of compounds, which generally include B vitamins and a variety of amino acids (Fugelsang 1997). Amino acids, and often peptides, supply LAB with their assimilable nitrogen. Amino acid requirements vary with respect to the species and even the strain. These amino acids may be strictly indispensable or simply growth activators. The following amino acids are necessary as a whole or in part, depending on the strain: Ala, Arg, Cys, Glu, His, Leu, Phe, Ser, Trp, Tyr and Val. Cocci are more demanding than bacilli with strains of *O. oeni* being the most fastidious of all LAB. Among nitrogen compounds, puric and pyrimidic bases play an important role in stimulating growth (Ribéreau-Gayon *et al.* 2000).

Minerals such as Mg^{2+}, Mn^{2+}, K^+ and Na^+ are necessary. The first two are often used as key enzyme co-factors of the metabolism. Vitamins are co-enzymes or co-enzyme precursors. Lactic acid bacteria are incapable of synthesising B-group vitamins, in particular nicotinic acid, thiamin, biotin and pantothenic acid (Ribéreau-Gayon *et al.* 2000). A glycoside derivative of pantothenic acid, 4-*O*-(β-D-glucopyranosyl)-D-pantothenic acid, was identified in grape juice; it initially had been purified from tomato juice (Amachi 1975). This factor was termed the tomato juice factor and is crucial for the growth of *O. oeni*. Among the important chemical elements, phosphorus plays a primordial role in LAB, as in all cells, in the composition of nucleic acids and phospholipids and in the stocking of energy in the form of ATP (Ribéreau-Gayon *et al.* 2000). Supplementation is recommended if the nutritional status of the must is known or thought to be problematic. Unlike yeast, which grows well on formulations that are rich in diammonium phosphate, LAB does not find this source of nitrogen very stimulatory, rather needing preformed amino acids. Proprietary formulations are available that are specific for LAB (Fugelsang, 1997).

2.5.2   FACTORS ASSOCIATED WITH VINIFICATION

2.5.2.1 Cellar practices

Winery practices may affect the presence, survival and growth of LAB. These include operations such as clarification, contact with grape skins, time of racking, thermovinification and the addition of preservatives (Wibowo et al. 1985).

Clarification of the must or freshly fermented wine by sedimentation, filtration or centrifugation may remove a substantial proportion of the LAB and so reduce the possibility of spontaneous MLF by the indigenous flora. If excessive, these operations can remove nutrients important for the growth of LAB (Fornachon 1957; Van Wyk 1976). Wine fermented in contact with grape skins undergoes more consistent and rapid MLF than wine fermented in the absence of skins (Beelman and Gallander 1970; Kunkee 1967). This correlates with the leaching of growth stimulating nutrients from the skins (Amachi and Yoshizumi 1969). Delayed racking or leaving the wine in contact with the lees after alcoholic fermentation also stimulates the growth of LAB (Fornachon 1968; Van Wyk 1976). It is generally accepted that yeasts in the lees undergo autolysis to release nutrients that favour the growth of LAB (Kunkee and Amerine 1970). Thermovinification is often used for better colour extraction in red wine fermentations. LAB growth and MLF are rarely observed in wines produced by this technique (Beelman and Gallander 1970; Beelman et al. 1980). The reasons for this difference are not clear, but may be related to a greater retention of SO$_2$ by thermovinified wines (Beelman and Gallander 1979).

The addition of preservatives, such as SO$_2$, to control the growth of LAB in wine is well established. However, other preservatives used/considered include fumaric acid (Cofran and Meyer 1970; Pilone et al. 1974), sorbic acid (Sofos and Busta 1981), chloramphenicol, tyrothricine, monobromoacetic acid, polyphenol substances, diethyl pyrocarbonic acid (Wibowo et al. 1985) and the heptyl ester of parabenzoic acid (Chan et al. 1975; Wibowo et al. 1985). More recently, bacteriocins, such as nisin, have also been used in winemaking (Radler 1990a, b). The use of a bacteriolytic enzyme, like lysozyme, has also been effective in preventing the growth of LAB and the occurrence of MLF in wine (Gerbaux et al. 1997, 1999).

Other vinification factors that could influence the survival and growth of LAB include cold stabilisation, ion exchange and heat pasteurisation. Ion exchange and cold stabilisation could remove the bacteria or the nutrients important to their growth. The heat sensitivity of wine LAB varies with species and wine properties (Splittstoesser et al. 1975; Wibowo et al. 1985).

2.5.2.2 Other compounds

The literature contains very little information about the role of pesticide residues or wine processing aids in the suppression of LAB growth. Copper sulphate is used to remove sulphides from wine, but the sensitivity of LAB to Cu^{2+} appears to be low (Haag et al.

1988). From a laboratory survey of 22 pesticides, 13 were inhibitory to different degrees against the 11 strains (*O. oeni*, *Lb. brevis*, *Lb. hilgardii* and *Lb. plantarum*) tested. Cymoxanil and dichlofluanid have been reported to inhibit MLF (Haag *et al.* 1988). Vidal *et al.* (2001) reported that copper and dichlofluanid had an ihibitory effect on *O. oeni* and MLF. The minimum inhibitory concentrations that affected MLF were just under 5 mg L^{-1} and were enhanced by ethanol.

The action of phenolic compounds on LAB growth remains relatively unknown. Past results have shown that polyphenols tested alone or in a mixture had an inhibitory effect. The inhibitory effect of phenolic acids (coumaric acid, protocatechuic acid, etc.) and tannins on the growth of LAB and the activating role of anthocyanins have been reported (Ribéreau-Gayon *et al.* 2000). Vivas *et al.* (1997) reported that protocatechuic acid had no influence and that vanillic acid inhibited cell viability. They also reported that gallic acid and free anthocyanins stimulated the LAB and enhanced MLF.

2.5.3 BIOLOGICAL FACTORS

Must contains an extensive variety of microorganisms, viz. fungi, yeast, acetic acid bacteria (AAB), LAB and bacteriophages. Initially, these microorganisms come from the grapes and harvesting equipment and, later, from the equipment used to transport the whole or crushed grapes to the tanks. The winemaking microorganisms are naturally selected from this mixture. This selection takes place due to changes in environmental conditions (composition, oxidation-reduction potential) and specific antagonistic and synergistic interactions between the different microorganisms (Ribéreau-Gayon *et al.* 2000).

Yeast and bacteria interact not only with the different types of microorganisms, but also at species and strain level. Due to the diversity of the microorganisms and their adaptation ability in the wine, numerous interactions take place. Only a few are well known. Some are very difficult to identify and to study. The yeast/bacteria interactions during fermentation seem to be the most important (Wibowo *et al.* 1985; Ribéreau-Gayon *et al.* 2000).

2.5.3.1 Yeast/lactic acid bacteria interactions

Historically, the source of yeast/LAB antagonism has been ascribed to either competition for available nutrients (Beelman *et al.* 1982) or inhibition resulting from the formation of biological SO_2 by yeasts (Rankine and Pocock 1969). The growth of native yeast species, such as *Saccharomycodes ludwigii*, *Candida pulcherrima* and *Pichia* spp., has also been reported to be antagonistic to the growth of LAB (Fornachon 1968). During the rapid growth period at the beginning of fermentation, yeast depletes amino acids from the medium. These deficiencies, combined with toxic metabolites, can hinder bacterial multiplication (Ribéreau-Gayon *et al.* 2000). Lipophilic fatty acids liberated by the yeast, such as hexanoic, octanoic, decanoic and especially dodecanoic acid, are inhibitory to

LAB (Lonvaud-Funel *et al.* 1988a). These acids target and alter the bacterial membrane. The incubation of cells in the presence of these fatty acids results in an ATP leak and a loss of malolactic activity.

The formation of high concentrations of ethanol by yeast also influences the growth of LAB. The effect of ethanol on the malolactic activity depends on the pH of the medium.

In contrast to inhibition, yeast may also promote the growth of LAB in wines and stimulate MLF. Vitamins, nitrogen bases, peptides and amino acids are released during yeast autolysis (Kunkee and Amerine 1970). Furthermore, bacteria accelerate the yeast death phase at the end of alcoholic fermentation due to the secretion of glucosidase and protease enzymes (Lonvaud-Funel *et al.* 1988b), which in turn hydrolyse the yeast cell wall.

Bacterial growth may contribute to slow or stuck fermentations (Edwards *et al.* 1998, 1999a, b). Acetic acid and associated products of the LAB metabolism represent potent inhibitors to the fermentation of *Saccharomyces* yeasts, delaying the onset of fermentation with the potential of causing stuck fermentations (Jones *et al.* 1994; Edwards *et al.* 1999b). Lactic acid bacteria may also produce yeast inhibitors. Grape must fermented with certain LAB does not support yeast growth as well as unfermented (control) must (Ribéreau-Gayon *et al.* 2000).

2.5.3.2 Interactions between lactic acid bacteria

The succession of bacterial species during alcoholic fermentation can be explained by a difference in the sensitivity of bacteria to interactions with yeast. Interactions between different species of LAB also exist simultaneously. Like other microorganisms, LAB can synthesise and release substances with antimicrobial activities (Ribéreau-Gayon *et al.* 2000). Bacteriocins belong to a class of proteins whose bactericidal activity generally has a narrow range of action. It is sometimes even limited to the same species as the producing strain. Rammelsberg and Radler (1990) described bacteriocins from *Lb. brevis* and *Lb. casei*, which particularly inhibited LAB that are important in wine. Brevicin, the bacteriocin from *Lb. brevis,* has a broad spectrum of action and inhibits strains of *O. oeni* and *Ped. damnosus*, in addition to strains of *Lb. brevis*. Caseicin from *Lb. casei* is active only against *Lb. casei*.

A strain of *Lb. plantarum* had antibacterial activity against many bacterial species in wine, including lactobacilli and cocci (Rammelsberg and Radler 1990; Lonvaud-Funel and Joyeux 1993). The active protein synthesised has not yet been isolated. A *Ped. pentosaceus* strain produces a bactericidal protein that is active against strains of *Lb. hilgardii*, *Ped. pentosaceus* and *O. oeni* (Ribéreau-Gayon *et al.* 2000). This bacteriocin, which is produced in large quantities in grape juice, is stable in the acidic conditions and ethanol concentrations of wine. Lonvaud-Funel and Joyeux (1993) identified small peptides from *Ped. pentosaceus* and *Lb. plantarum* that strongly inhibit the growth of *O. oeni*. These small peptides are thermostable and degraded by protease. Their toxic effect is only temporary. They do not kill the bacteria, but merely lower the growth rate and

the final population. Lactic acid and other organic acids have also been known for their inhibitory effect (Gibbs 1987). The production of hydrogen peroxide inhibits bacteria (Dahiya and Speck 1968).

2.5.3.3 Bacteriophages

Bacteriophages are viruses capable of extensively destroying cultures of sensitive bacterial strains. Two groups of bacteriophages can be identified, the lytic or virulent phage, whose immediate activity kills the bacterial host, and the lysogenic or temperate phage. The lytic phage enters the bacterial cell and utilises the host-replication system to reproduce many copies of viral nucleic acid. Upon completion of the process, the phage escapes, killing the host cell through cell lysis. The lysogenic phage may remain dormant within the host cell for a number of generations. With the lysogenic phage, the virus genome remains integrated in the bacterial chromosome in the form of a prophage and is replicated and transmitted to the daughter cells. Eventually, some host-related physiological or environmental change might bring about latent activity. This is referred to as induction, and the now virulent phage may start another lytic cycle (Fugelsang 1997). Both lytic and prophages have been described for wine LAB (Gnaegi and Sozzi 1983; Davis *et al*. 1985a; Henick-Kling *et al*. 1986a, b; Cavin *et al*. 1991). Bacteriophages have a specific host range and mixed starter cultures are therefore sometimes used to limit the failure of MLF. Changing the LAB strain regularly is another means of limiting the accumulation of bacteriophages in a winery. It appears that bacteriophages are of a much greater concern to starter culture producers when conditions are more conducive to the destructive lytic cycle (Henschke 1993).

2.6 METABOLISM OF LACTIC ACID BACTERIA

2.6.1 SUGARS

The composition of grape juice or wine has a profound influence on the outcome of MLF. Sugars are the most significant fermentable compounds present (Davis *et al*. 1986). Most dry wines contain between 1 and 3 g l^{-1} residual hexoses and pentoses. Glucose and fructose are the main sugars present in grape juice and wine. Other sugars present are mannose and galactose (hexoses), several pentoses (arabinose, xylose, ribose and rhamnose) and saccharides (maltose, melibiose, raffinose, melezitose and stachyose) (Bartowsky and Henschke 1995). These sugars are utilised by LAB for cell maintenance and growth during MLF. There is also considerable heterogeneity in the ability of species and strains of LAB to metabolise these sugars. Ethanol and pH also influences the ability to ferment sugars (Wibowo *et al*. 1985).

2.6.2 ORGANIC ACIDS

Organic acids are important to wine flavour, the maintenance of colour and wine pH. The low wine pH serves to restrict the growth of bacteria. Consequently, any changes to the concentration of the individual acids by LAB are likely to affect wine quality (Wibowo *et al.* 1985). The two main organic acids usually degraded by LAB are malic acid and citric acid. The majority of the LAB present after alcoholic fermentation metabolise these two acids. Winemakers may consider the transformation of L-malic acid as the most important change as a result of LAB growth, but other changes should also be taken into account.

2.6.2.1 Malic acid

The conversion of L-malic acid, a dicarboxylic acid, by LAB to the monocarboxylic acid, L-lactic acid, is the most important chemical change that occurs during MLF (**Fig. 2.4**). This reaction results in an overall reduction in wine acidity. There are three different pathways that can be followed by malic acid degradation (Radler 1986; Henick-Kling 1993):

I. Decarboxylation of malic acid by the malolactic enzyme, which is conducted by most LAB. The malolactic enzyme catalyses the direct decarboxylation of L-malic acid to L-lactic acid.

II. Conversion of L-malate to pyruvate by *Lb. casei* and *Enterococcus faecalis*. The malic enzyme catalyses the decarboxylation of L-malate to L-pyruvate, using malate as a carbon source.

III. The reduction of malate to oxaloacetate. This reaction is catalysed by the malate dehydrogenase (MDH) enzyme.

The last two metabolic routes are rarely associated with MLF. When D- or D/L-malic acid are added to wine, the D-malic acid is normally not degraded (Bartowsky and Henscke 1995).
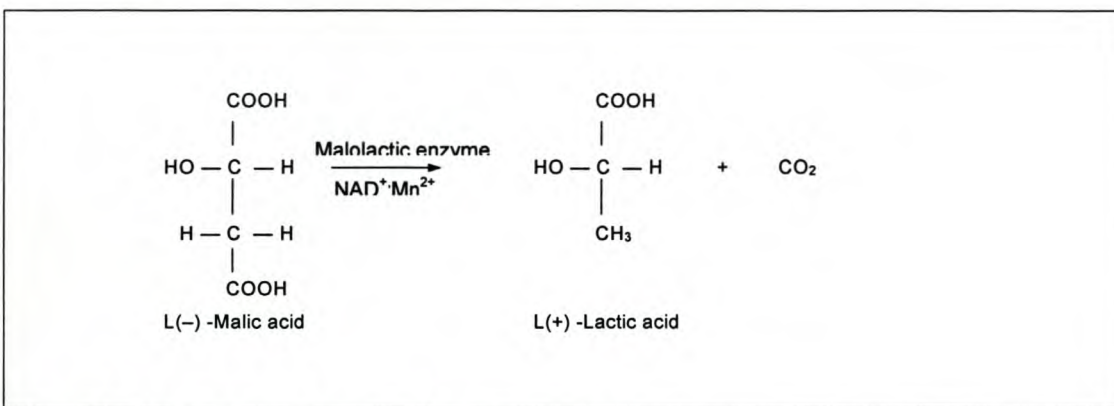
Fig. 2.4   Equation of the malolactic reaction.

2.6.2.2 Citric acid

Certain heterofermentative cocci and homofermentative bacilli are able to degrade citric acid. Among the species found in wine, *Lb. plantarum*, *Lb. casei*, *O. oeni* and

Lc. mesenteroides use citric acid rapidly. *Pediococcus* strains, *Lb. hilgardii* and *Lb. brevis* cannot degrade citric acid (Ribéreau-Gayon *et al.* 2000).

Lactic acid bacteria isolated from wine may contain a citrate permease (essential for the uptake of citric acid), but its role is inconsequential, since at the pH of wine, the non-dissociated substrate diffuses across the membrane without the assistance of permease. Inside the bacteria, citric acid is split into an oxaloacetate molecule and an acetate molecule by the enzyme, citrate lyase (**Fig. 2.5**). Oxaloacetate is then decarboxylated to pyruvate by *Leuconostoc* spp. In certain lactobacilli, it can also lead to the partial formation of succinate and formate (Ribéreau-Gayon *et al.* 2000).

Pyruvate is the source of the carbonyl compounds diacetyl, acetoin and 2,3-butanediol (Ribéreau-Gayon et *al.* 2000). Diacetyl is considered to be an important flavour compound and is associated with the "buttery" character. Acetoin and butanediol, which are derived from the reduction of diacetyl, are almost odourless, with thresholds of 150 and 600 mg l^{-1}, respectively. In addition to carbonyl compounds, the pyruvate molecules arising from citrate can also result in other products. Lactate can be formed if the co-enzyme, NADH, is available. Pyruvate can also be decarboxylated, followed by a reduction reaction to produce ethanol. Pyruvate also participates in the synthesis of fatty acids and lipids via acetyl-CoA (Ribéreau-Gayon *et al.* 2000).
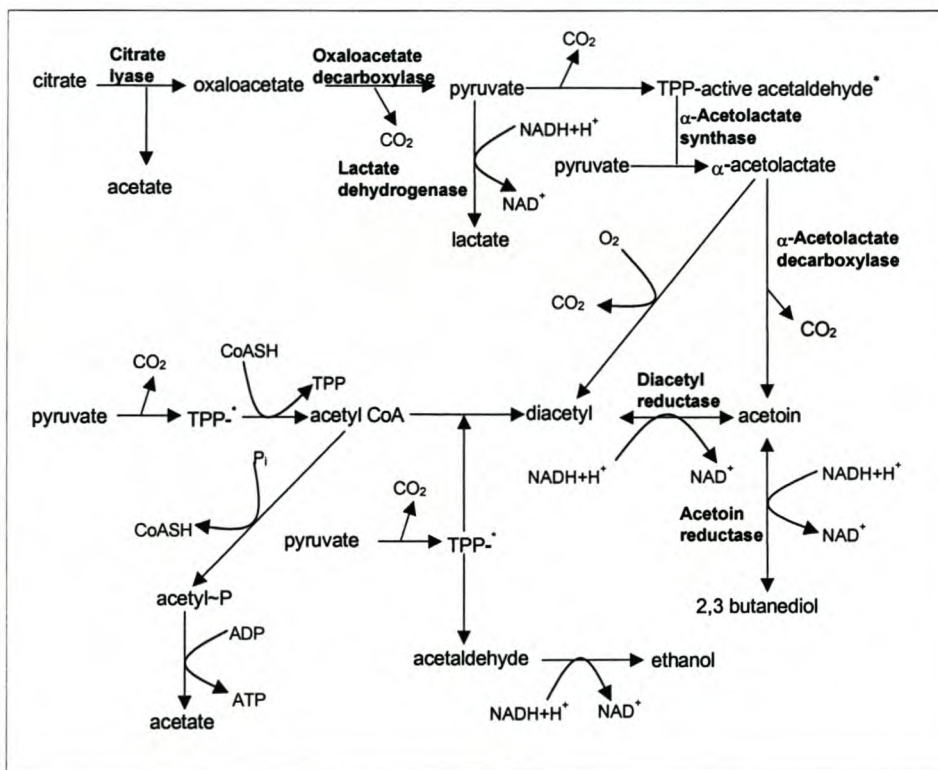
Fig. 2.5 Citric acid metabolism by lactic acid bacteria (Du Toit and Pretorius 2000).

In limited glucose conditions, at a low pH and the presence of growth inhibitors, citric acid degradation results in the formation of carbonyl compounds. To maintain its intracellular pH, the cell must eliminate pyruvate. The pathway for the formation of carbonyl compounds is considered to be a detoxification process. As a result of the conditions, pyruvate is not orientated towards the synthesis of cellular material, since growth is difficult, nor towards lactate and ethanol, because of the lack of reduced co-enzymes. Conversely, when growth is easy, fatty acid synthesis pathways utilise pyruvate and acetic acid is produced in larger quantities (Ribéreau-Gayon *et al.* 2000).

2.6.2.3 Tartaric acid

Lactic acid bacteria that are able to degrade tartaric acid are not widespread (Krumperman and Vaughn 1966; Radler 1975; Sponholz 1993), but MLF can be accompanied by a decrease of 3 to 30% in the concentration of tartaric acid (Pilone *et al.* 1966; Bousbouras and Kunkee 1971; Wibowo *et al.* 1985). Degradation of tartaric acid can be considered as wine spoilage. Pasteur described this process and named it *tourne* disease. The disappearance of tartaric acid lowers the fixed acidity and is accompanied by an increase in volatile acidity. The degradation can be total or partial, depending on the species and the level of development, but it always lowers the wine quality (Ribéreau-Gayon *et al.* 2000). This spoilage is rare and causative strains belonging to different species have been isolated, but certain strains of *Lb. plantarum* and *Lb. brevis* with these characteristics have been described (Krumperman and Vaughn 1966). Some homofermentative lactobacilli can metabolise tartaric acid to oxalacetic acid, pyruvic acid, lactic acid and acetic acid. The metabolites of heterofermentative lactobacilli are oxalacetic acid, pyruvic acid, malic acid, fumaric acid and acetic acid. The main enzyme in tartaric acid metabolism is tartrate dehydratase (Bartowsky and Henschke 1995).

2.7 MALOLACTIC FERMENTATION

2.7.1 BIOCHEMISTRY AND PHYSIOLOGY

With a few exceptions, the LAB found in wine are unique among microorganisms in that they are capable of conducting MLF, a secondary fermentation in wines. Malolactic fermentation is an enzyme-mediated decarboxylation of L-malic acid to L-lactic acid and CO_2 (Kunkee 1967). The reaction is catalysed by malate carboxylyase, the so-called malolactic enzyme, and requires the coenzyme NAD^+ and manganese (Mn^{2+}) (Lonvaud-Funel and Strasser de Saad 1982). Unlike the formation of lactic acid from glucose, only the L-isomer is produced during MLF.

The fact that there is no immediately apparent benefit to the cell, in terms of energy (ATP) production, has led to a great deal of study and speculation. At least part of the answer has been known since 1961, when Mitchell proposed the chemiosmotic hypothesis

for ATP production (Fugelsang 1997). The system first requires a strong transmembrane pH gradient between the intracellular environment (cytoplasm) and that of the surrounding medium and, secondly, a membrane-associated enzyme complex (ATPase) that can take advantage of this gradient for the production of ATP from ADP and P_i (Gottschalk 1985). Cox and Henick-Kling (1989, 1990) reported activity similar to that of a proton pump and ATPase in the formation of ATP during MLF. L-Malate enters the cell through the action of a specific transport enzyme and is decarboxylated as described previously. To prevent proton accumulation and, eventually, cell death, it is necessary to export the protons continually. This is accomplished by the transport of L-lactate along with a single proton (symport). Repeated proton translocation creates a protonmotive force across the membrane. The entry of protons through membrane-associated ATPase generates ATP. Theoretically, one ATP is produced for every three protons that enters the ATPase complex (Olsen *et al*. 1991).

2.7.2 BENEFITS OF MALOLACTIC FERMENTATION

Malolactic fermentation has several roles in wine production. The most important is the deacidification of high acid wines. Wine flavour can be influenced by the formation of organoleptically active compounds arising from bacterial metabolism. The microbial stability of wine can also increase through the removal of growth-stimulating nutrients.

2.7.2.1 Deacidification

Most wines contain 0·55 to 0·85% titratable acid and MLF is the major means by which the acidity can be reduced (Davis *et al*. 1985b). Reduction in wine acidity by MLF may vary from 0·1 to 0·3% and the pH may increase by 0·1 to 0·3 of a unit. Reduction in acidity increases the smoothness and drinkability of red wines (Jackson 2000). Wine deacidification is important for wines produced in cooler regions. Wines from these regions are usually produced from grapes of high acidity. Wines from warmer regions have a low acidity and the occurrence of MLF could reduce the acid level too much, resulting in a flat tasting wine and the growth of spoilage bacteria due to an even higher pH (Davis *et al*. 1985b).

2.7.2.2 Microbial stability

Increased microbial stability is considered one of the prime benefits of MLF. Microbial stability is believed to result from the metabolism of residual nutrients left after alcoholic fermentation. Malic and citric acid is consumed and the more microbially stable tartaric and lactic acid remains. In addition, the complex nutrient demands of LAB also reduce the concentrations of amino acids, nitrogen bases and vitamins (Jackson 2000). Wines that have completed MLF still contain nutrients and may support bacterial growth (Davis *et al*. 1985b; Costello *et al*. 1983). Malolactic fermentation occasionally may decrease the

microbial stability. This is a problem in wines with a high pH. A further increase in the pH places the wines in a pH range that favours the growth of spoilage LAB such as *Lactobacillus* and *Pediococcus* spp. (Davis *et al*. 1985b; Jackson 2000).

2.7.2.3 Flavour modification

In terms of quantity, the transformation of malic acid to lactic acid is the main event during MLF. The total acidity is lowered and the strong green taste of malic acid is replaced by the less aggressive taste of lactic acid (Lonvaud-Funel 1999). Flavour comprises taste and aroma. The flavour modifications that occur during MLF have been the topic of many discussions. Significant differences can be observed when different strains of LAB initiate MLF (Krieger *et al*. 1990; Laurent *et al*. 1994). There is considerable variability among strains and species of LAB, as well as the factors that influence their metabolism (Jackson 2000). Most commercial strains have been selected because they are the easiest to cultivate, best resistant to the industrial process and best to survive in wine. Wine type and especially flavour intensity of the wine, will further affect the perception of flavour generated by MLF. Malolactic fermentation has been shown to influence both the aroma and the flavour of wine (Bartowsky and Henschke 1995). Only certain wine types benefit from MLF, and this is essentially a wine style decision. Many white wines, such as Chenin Blanc, Sauvignon Blanc, Semillon and Riesling, do not benefit from MLF. Chardonnay, however, does benefit from MLF (Shaw 1993). Current winemaking practice encourages MLF in most styles of red wine. Malolactic fermentation in sparkling base wine and some non-floral, wood-matured white wines is also gaining acceptance. However, MLF is commonly prevented in both floral and sweet white wines (Gockowiak *et al*. 1991).

Several attributes, such as an increased buttery and a reduced vegetative character and improved mouth feel and flavour persistence, are associated with MLF (Laurent *et al*. 1994; Bartowsky and Henschke 1995). Sauvageot and Vivier (1997) reported that MLF increased the hazelnut, fresh bread and dried fruit aromas of Chardonnay wines, whereas Pinot Noir wines partially lost their berry notes in favour of animal and vegetative aromas. Other attributes associated with MLF are caramel, fruity and sweaty flavour (Bartowsky and Henschke 1995). Malolactic fermentation can result in an increase or decrease of sensory attributes. Increases in aroma/flavour descriptors, such as buttery and wine finish, were commonly observed, while wine body, burnt sweet aroma, citrus, fruity, maple syrup and sweaty were less common. Attributes that decreased during MLF were banana, burnt sweet, buttery, citrus, fruity and floral. Sufficient data is available to clearly demonstrate that different strains of LAB may increase or decrease the intensity of wine aroma/flavour attributes (Bartowsky and Henschke 1995). Some of the cultivar aroma revealed during the alcoholic fermentation disappears or changes after MLF. In red wines, MLF significantly decreases the free anthocyanins and astringency by increasing the reaction between tannins and anthocyanins (Lonvaud-Funel 1999). Some flavour compounds

associated with MLF are diacetyl, acetoin, 2,3-butanediol, acetaldehyde, acetic acid and its esters (Davis *et al.* 1985b; Henschke 1993; Zoecklein *et al.* 1995).

Diacetyl (2,3-butanedione) is probably the most important of the flavour compounds produced during MLF. Diacetyl is perceived as having a buttery, nutty or toasty aroma (Jackson 2000). The desirability of this compound depends on its concentration in wine and on the type and style of the wine. The odour threshold in wine is 3 mg l^{-1} (Etiévant 1991). Diacetyl has a taste threshold of 9·5 mg l^{-1} in white wine and 14 mg l^{-1} in red wine (Bertrand *et al.* 1984). At a concentration between 1 and 4 mg l^{-1}, diacetyl is regarded as adding complexity to wine flavour. However, at concentrations higher than 5 mg l^{-1}, the buttery character becomes undesirable (Davis *et al.* 1985b). Acetoin (3-hydroxy-2-butanone) and 2,3-buntanediol (butylene glycol) have an aroma threshold of 150 mg l^{-1} and 600 mg l^{-1} respectively (Bertrand *et al.* 1984). Neither of these compounds has a significant impact on the sensory characteristics of wine (Boulton *et al.* 1995).

The desirability of acetic acid depends on its concentration, the wine type and the style of the wine. In delicately flavoured wines, acetic acid can be perceived when its concentration exceeds 0·4 g l^{-1} (Henick-Kling *et al.* 1993; Bartowsky and Henschke 1995). Acetaldehyde is an important wine component. It contributes a bruised apple, nutty character to wine when near its threshold value (approx. 100 mg l^{-1}). A sharper green leaf, oxidative or sherry-like aroma can be imparted at higher concentrations (Bartowsky and Henschke 1995).

Esters are some of the most volatile compounds present in wine and distillates and they contribute to the character and quality of distillates. Ethyl lactate, which has been described as giving a broader, fuller taste to wine, is one of the main esters produced during MLF (Bartowsky and Henschke 1995). It may be formed at concentrations exceeding its flavour threshold of 60 to 110 mg l^{-1}. Ethyl lactate is considered to be a tails fraction component (Von Adam *et al.* 1996). Depending on the distillation technique used and the point at which the tails fraction is drawn, only 7-16% of the total ethyl lactate concentration is found in the heart fraction of the distillate. Concentrations above 250 mg l^{-1} usually point to microbial activity in the base wine. A wine distillate that contained 60 mg l^{-1} ethyl lactate was found to be sensorially poor and unfavourable, whereas a wine distillate containing 22 mg l^{-1} was judged to be pleasant and of good quality (Cantagrel 1989). This cannot be ascribed purely to the ethyl lactate content.

Lactic and acetic acid bacteria (AAB) can also produce ethyl acetate, which is the major ester produced by yeast during the alcoholic fermentation. Ethyl acetate has a low aroma threshold of approximately 10 mg l^{-1}. At concentrations lower than 50 mg l^{-1}, it imparts complexity and a fruity aroma (Bartowsky and Henschke 1995; Jackson 2000). At higher concentrations, it has an undesirable solvent/nail polish remover odour (Bartowsky and Henschke 1995). In the charentais method of potstill distillation, ethyl acetate is found mainly in the heads fraction of the distillate (Von Adam *et al.* 1996). By increasing the

volume of the heads fraction collected, the ethyl acetate concentration can be reduced in the final product. Postel and Von Adam (1988) recommended that there be a minimum of 250 mg l^{-1} and a maximum of 850 mg l^{-1} of ethyl acetate in wine distillates.

Ethyl propionate, diethyl succinate and ethyl butyrate can all be influenced by MLF and can be used as spoilage indicators. The concentration of ethyl propionate should not exceed 15 mg l^{-1}. Higher values are generally found in distillates produced from microbially spoiled wines (Von Adam et al. 1996). According to Cantagrel (1989), diethyl succinate increases during storage. Diethyl succinate can be found at levels between 0·5 and 32 mg l^{-1} in wine distillates. It possesses similar behaviour to ethyl lactate during distillation and can be reduced by carefully controlling the point at which the tails fraction is cut. Generally, when ethyl lactate concentrations are high, elevated levels of diethyl succinate can also be expected (Von Adam et al. 1996). Ethyl butyrate levels in distillates made from wines of good quality are in the order of 1 mg l^{-1} (Cantagrel 1989). In distillates produced from spoiled wines, this value may be as high as 40 mg l^{-1}. A noticeable organoleptic defect becomes apparent when ethyl butyrate is present at concentrations of 7 mg l^{-1}.

Other compounds that increase during MLF include iso-amyl acetate (Laurent et al. 1994); n-propanol, 2-butanol, n-hexanol (Davis et al. 1985b); ethyl hexanoate (fruity, rum-like) and isobutanol (Avedovech et al. 1992). Small decreases in 3-methyl-n-butyl acetate, n-hexyl acetate (fruity), 2-phenylethylacetate and ethyl hexanoate (Zeeman et al. 1982), isobutyraldehyde and isobutyl acetate (Avedovech et al. 1992) have also been observed in wines that have undergone MLF.

2.8 CONTROL OF MALOLACTIC FERMENTATION

2.8.1 INDUCTION OF MALOLACTIC FERMENTATION

To overcome problems with delayed MLF, the winemaker uses various methods to induce MLF in wine.

2.8.1.1 Addition of prepared starter cultures

The main advantages of inoculation for MLF include better control over the timing and progress of MLF and the effect of MLF on the wine aroma and flavour, as well as the LAB strain conducting the process. The general principle should be that the physiological condition of the LAB must be good, to minimise loss of viability upon inoculation and to enhance rapid growth and malic acid degradation (Henschke 1993). Starter cultures can be in the form of liquid cultures; either prepared by the winemaker or purchased from commercial wine laboratories, or in the form of frozen or freeze dried preparations supplied by starter culture manufacturers (Pilone 1995).

2.8.1.2 Addition of wine undergoing malolactic fermentation

In some wines, an unsuccessful MLF may be stimulated by inoculation with wine already undergoing MLF or having just completed MLF. This technique is similar to that used by winemakers in promoting alcoholic fermentation, when an active fermenting culture is used as the inoculum. This method assures that the MLF bacteria are viable and active under the harsh wine conditions (Pilone 1995). There are, however, some disadvantages to this method. The wine to be inoculated needs to be of the same or similar type, otherwise the identity of the wine will be lost. For example, it may not be wise to use a Chardonnay wine to inoculate a Cabernet Sauvignon wine (Pilone 1995). A large volume of inoculum is usually needed, since the bacterial population in wine is not that high (Firme *et al.* 1994). Castino *et al.* (1975) suggested a 5% inoculum level, while Kunkee (1967) recommended 15 to 50%. Thirdly, there is a possibility that contaminating microflora in the inoculum may develop (Costello 1993). Careful monitoring is necessary to avoid potential spoilage problems (Pilone 1995).

2.8.1.3 Addition of wine lees

The addition of wine lees from wine having undergone MLF to stimulate the onset of MLF has been used with some success. Advantages of using lees are that it contains concentrated levels of LAB ($\pm 1 \times 10^8$ cfu ml^{-1}) and that the lees can be used immediately after racking (decanting) or centrifugation of the wine, or can be stored cold and used later (Pilone 1995). A major disadvantage of this method is that the stored lees increases in pH because of yeast cell autolysis. As a result of the higher pH, there is a greater danger of increasing unwanted microflora, such as yeast, acetic acid bacteria (AAB) and spoilage LAB (Granchi *et al.* 1990). These contaminants may compete with the MLF bacteria and develop in the inoculated wine, causing unwanted flavours and aromas. The use of MLF lees requires strict microbiological monitoring (Pilone 1995).

2.8.1.4 The use of immobilised cells or enzymes

It has been established that resting or non-proliferating cells of LAB (*O. oeni*) at high concentrations (10^6 to 10^7 cfu ml^{-1}) can carry out rapid degradation of malic acid (Davis *et al.* 1985b). Conducting MLF in wine normally involves two stages, namely the growth of bacteria and deacidification. By using immobilised cells or a bioreactor process, the cells are pregrown under optimum conditions using inexpensive substrates, and the wine is then passed through the system containing a large density of cells. Thus, the rate of deacidification and the throughput is high, since the growth phase is not a prelude to the deacidification phase (Henschke 1993).

Immobilised cells and reactors are still experimental. There are several advantages for the deacidification of wine, e.g. continuous operation, greater tolerance of wines with high alcohol and SO_2 concentrations and low pH, better control over the timing and extent of deacidification and an absence of the effects of bacterial growth in wine. Disadvantages of

the technique include the possibility of microbial contamination of the reactors, the transfer of taints from the reactor to the wine, a loss of activity on prolonged operation, and leakage of cells or immobilisation substrate into the wine, as well as infection of the cells by phages (Davis *et al.* 1985a). Attempts to deacidify wine using the malolactic enzyme immobilised on gels have not been successful, probably because the enzyme loses activity at wine pH and the required cofactor, NAD, is unstable in wine (Gestrelius 1982).

2.8.1.5 Carbonic maceration

Carbonic maceration as a winemaking technique is practised widely in some parts of France and is being used successfully in other countries, such as Australia. Intact grapes are held in tanks filled with CO_2 before crushing and alcoholic fermentation. The intracellular enzymes of the grape degrade the malic acid in the intact berries, producing ethanol as the main end product. Up to 50% of the malic acid can be degraded by this process. Yeast and LAB usually metabolise the remaining malic acid in the alcoholic and malolactic fermentation. Apart from the rapid degradation of malic acid in wines made by carbonic maceration, the sensory quality is also significantly different from wines made by traditional vinification techniques (Davis *et al.* 1985b).

2.8.2 PREVENTION OF MALOLACTIC FERMENTATION

If MLF is not desired for a specific wine style, the growth of LAB and the occurrence of MLF can be avoided. Inhibition can be achieved through the use of chemical preservatives or selection of conditions that will restrict or prevent bacterial growth. These conditions include: wine pH below 3·2, total SO_2 concentrations above 50 mg l^{-1}, ethanol concentration in excess of 10%, low fermentation and storage temperature (< 15°C), early racking and clarification (Kunkee 1967), reduction in skin contact (Beelman and Gallander 1970) and sterile filtration (Kunkee 1974). The influence of SO_2, ethanol, temperature and pH has already been discussed, therefore this section will focus on the use of chemical and biopreservatives.

2.8.2.1 Chemical preservatives

Sorbic acid is a short chain unsaturated fatty acid that is generally used in the food and beverage industries. In some countries, it is used in sweetened wines to prevent re-fermentation by *Saccharomyces cerevisiae* (Zoecklein *et al.* 1995). Sorbic acid is not an effective inhibitor of LAB, AAB and certain yeasts, such as *Brettanomyces*, *Saccharomycodes* and *Zygosaccharomyces* (Du Toit and Pretorius 2000). Certain LAB can metabolise sorbic acid to produce geranium-like off-flavours. Wine pH, alcohol concentration, SO_2 concentration and the level of spoilage yeasts (Zoecklein *et al.* 1995) influence the effectiveness of sorbic acid. Fumaric acid has been shown to inhibit certain LAB (Cofran and Meyer 1970; Tchelistcheff *et al.* 1971; Ough and Kunkee 1974; Pilone

et al. 1974), but this acidulant is metabolised by yeast (Wagener *et al.* 1971), lactobacilli, pediococci and oenococci (Ough and Kunkee 1974; Pilone *et al.* 1974). The use of fumaric acid is restricted in some countries. Dimethyldicarbonate (DMDC) is more commonly known as Velcorin. Velcorin is lethal against yeast and bacteria and can be used as a sterilant in wine to ensure a controllable fermentation. Velcorin is hydrolysed to CO_2 and methanol (Du Toit and Pretorius 2000).

2.8.2.2 Biopreservatives

The traditional preservatives are still used widely, but there is a growing demand from consumers for products containing fewer preservatives, or preserved with alternative and natural methods.

Unspecific inhibitors or antibiotics will prevent the growth of LAB, but such compounds cannot be added to wine. Contrary to antibiotics, the macromolecular bacteriocins are very specific and only affect a small group of microorganisms. Nisin is a polypeptide that is produced by *Lactococcus lactis* subspecies *lactis* and has antimicrobial activity against LAB and other Gram-positive bacteria (Hurst 1981). Nisin is the only LAB bacteriocin with GRAS (Generally Regarded As Safe) status and its use is approved in 47 countries (Delves-Broughton 1990). Daeschel *et al.* (1991) have reported that 100 IU ml^{-1} of nisin can prevent MLF in wine. The use of nisin in winemaking has been evaluated (Radler 1990a, b; Strasser de Saad *et al.* 1995). The results indicated that most of the LAB of importance in winemaking were inhibited by low concentrations of nisin. The wine yeast was not affected and the sensorial quality of the wine was not influenced. However, *O. oeni* was affected and nisin would not be applicable in wines that have to undergo MLF. Daeschel *et al.* (1991) addressed this problem by developing nisin-resistant mutants of *O. oeni*.

Lysozyme is an enzyme with bactericidal properties that is used as a preservative in the food industry. It is non-toxic and has GRAS status. The commercial source is hen egg white. Lysozyme cleaves the β-1,4-glycosidic bonds present in the cell wall of Gram-positive bacteria (Cunningham *et al.* 1991). In contrast to SO_2, the activity of lysozyme increases with an increase in the pH. Lysozyme can be used for the inhibition or control of MLF and for stabilisation after MLF (Amati *et al.* 1996; Gerbaux *et al.* 1997, 1999). As in the case of nisin, lysozyme does not have anti-oxidative properties and cannot replace SO_2 completely, but enables the use of reduced SO_2 levels.

2.9 SPOILAGE BY LACTIC ACID BACTERIA

The growth of LAB and the occurrence of MLF are not always beneficial and can be responsible for undesirable changes in wine.

2.9.1 ACID FORMATION

Acetic acid, in addition to D-lactic acid, is an obligate product of the fermentation of hexose sugars by heterofermentative LAB (*O. oeni* and *Lactobacillus* spp.). The D-lactic acid is associated with spoilage (Sponholz 1993). Acetic acid is significant because of its contribution to the volatile acidity (VA) of wines. Acetic acid is also one of the end products when citric acid is metabolised. Acetic acid produced by LAB is often sensorially different from that resulting from the growth of acetic acid bacteria (AAB). In the latter case, VA is often perceived as a mixture of acetic acid and ethyl acetate. In the case of LAB, the ethyl acetate component is either absent or present at very low levels (Henick-Kling *et al.* 1993). A 0·1-0·2 g l^{-1} increase in the concentration of acetic acid invariably accompanies MLF performed by *O. oeni* (Davis *et al.* 1985b; Henschke 1993; Henick-Kling *et al.* 1993).

2.9.2 ACROLEIN PRODUCTION

Some species of LAB are able to ferment glycerol, with a major metabolite being acrolein (Sponholz 1993). Acrolein is formed by the dehydration of 3-hydroxypropionaldehyde, either through heating or as a result of long-term storage in acid solutions (Sponholz 1993). A concentration of 10 mg l^{-1} is sufficient to indicate spoilage (Margalith 1981). Acrolein can also react with the phenolic groups of anthocyanins in wine to give a bitter taste (Rentschler and Tanner 1951). Red wines are more commonly associated with this cause of bitterness because of their higher phenolic concentrations. Davis *et al.* (1988) reported that *Ped. parvulus*, *O. oeni* and certain *Lactobacillus* spp. have the ability to degrade glycerol, but that this is strain dependent.

2.9.3 FORMATION OF BIOGENIC AMINES

Biogenic amines are organic bases and are derived from the decarboxylation of amino acids. The main biogenic amines in wine are histamine, tyramine, putrescine (diaminobutane), cadaverine and phenylethylamine. While ethylamine seems to be produced during alcoholic fermentation, the other amines are more abundant after MLF. As with other foodstuffs, such as cheese, sauerkraut and beer, LAB are involved in the production of these substances (Coton *et al.* 1999). The various authors differ on the total biogenic amine contents that are considered to be dangerous to health. At the concentrations usually found in wine, biogenic amines are not considered to be dangerous to consumers (Cabanis 1985), although ethanol may enhance their toxicity (Aerny 1982). Histamine has been studied the most from a toxicological point of view and for its biosynthesis by microorganisms. *Pediococcus* spp. are regarded as the most important producers of histamine. Lonvaud-Funel and Joyeux (1994) also isolated *O. oeni* strains that produce histamine from histidine. The histamine concentration rarely exceeds 10 mg l^{-1} (Radler and Fäth 1991). As far as wine is concerned, some authors have recommended

a limit of 10 mg l⁻¹. The biogenic amine content also creates new difficulties for the marketing of the wines (Coton *et al.* 1999).

2.9.4 ARGININE METABOLISM

Ethyl carbamate, or urethane, is a known human and animal carcinogen found in wine (Ough 1976, 1993). Precursors in wine are urea, produced by yeast, citrulline and carbamyl phosphate, produced by LAB via the arginine deiminase (ADI) pathway (Lonvaud-Funel 1999).

The ADI pathway involves the activity of three enzymes:

$$\text{L-arginine} + H_2O \xrightarrow{\text{Arginine deiminase (ADI)}} \text{L-citrulline} + NH_3$$

$$\text{L-citrulline} + PO_4 \xleftrightarrow{\text{Ornithine transcarbamylase (OTC)}} \text{L-ornithine} + \text{carbamyl-}PO_4$$

$$\text{carbamyl-}PO_4 + ADP \xleftrightarrow{\text{Carbamate kinase (CK)}} ATP + NH_3 + CO_2$$
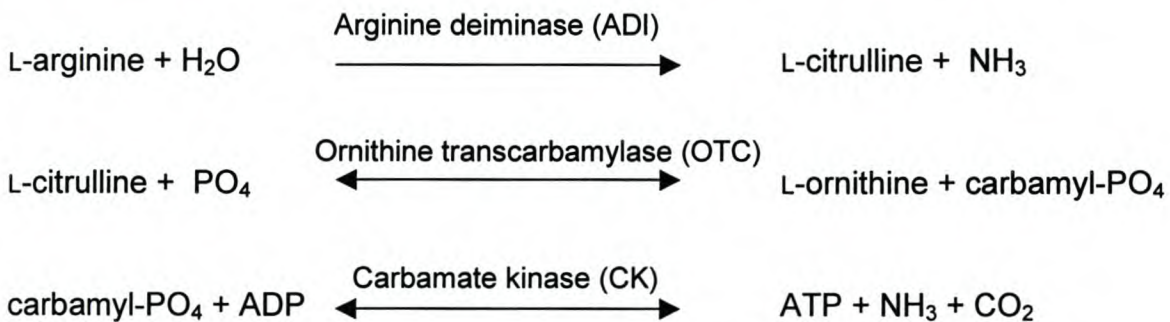
The intermediates (citrulline and carbamyl phosphate) of this pathway react with ethanol to form ethyl carbamate. Citrulline concentrations are at a maximum level when arginine concentrations are at a minimum (Liu *et al.* 1994). In the USA, ethyl carbamate concentrations of 15 ng g⁻¹ are the legal limit in table wines (Liu and Pilone 1998).

Certain wine-associated LAB have the ability to utilise arginine. These include strains of *O. oeni* and heterofermentative lactobacilli (e.g. *Lb. brevis, Lb. buchneri, Lb. hilgardii*). Homofermentative LAB do not catabolise arginine (Pilone *et al.* 1991; Edwards and Jensen 1992; Edwards *et al.* 1993; Liu *et al.* 1994, 1995; Liu and Pilone 1998).

2.9.5 FORMATION OF HAZE AND CO₂

Haze and excessive amounts of CO_2 reduce the quality of wine. The growth of LAB after bottling forms a haze and sediment in the bottle. In addition, heterofermentative LAB produce CO_2 from the metabolism of carbohydrates (Henick-Kling 1988).

2.9.6 GERANIUM TONE

As the name suggests, this off-flavour is reminiscent of crushed geranium leaves and results from the metabolism of sorbic acid. The compound, 2-ethoxyhexa-3,5-diene, is the main impact compound and has a very low aroma threshold of 0·1 μg l⁻¹. Some LAB are able to reduce sorbic acid to sorbyl alcohol (sorbinol) (Wurdig *et al.* 1974). In wine, sorbinol isomerises to 3,5-hexadiene-2-ol, which reacts with ethanol to form 2-ethoxyhexa-

3,5-diene (Crowell and Guymon 1975). Because formation requires the presence of ethanol, the defect is found in wine rather than in juice/must. Sorbic acid can auto-oxidise, forming acrolein, crotonaldehyde, malondialdehyde and formic acid. *Oenococcus oeni,* but not *Pediococcus* or *Lactobacillus* spp., are able to produce the geranium off-flavour (Bartowsky and Henschke 1995). Edinger and Splittstoesser (1986) reported that *Pediococcus* and *Lactobacillus* strains were unable to carry out the preliminary reduction.

Sorbic acid (200 mg l^{-1}) is usually added to prevent yeast growth. It is effective against *Saccharomyces* yeasts, but ineffective against oxidative film-forming yeast, *Brettanomyces* and *Zygosaccharomyces*, LAB and AAB (Zoecklein *et al.* 1995).

2.9.7 MANNITOL-ASSOCIATED TAINT

The reduction of fructose or fructose-6-phosphate by heterofermentative lactobacilli (such as *Lb. brevis*) results in the formation of mannitol (Sponholz 1993). However, a taint may form because mannitol degradation is often accompanied by other defects, such as acetic acid, D-lactic acid, n-propanol, sliminess and diacetyl (Bartowsky and Henschke 1995). The main concern to the winemaker is the increased potential for acetic acid formation. Sponholz (1993) reports that it is associated with high-pH sweet wines.

2.9.8 MOUSY TAINT

Mousy taint off-flavour is perceived by some tasters as reminiscent of mouse urine, and by others as cracker biscuits. These off-flavours are usually detected only after the wine has been swallowed or expectorated (Bartowsky and Henschke 1995). As it is caused by heterocyclic bases (Heresztyn 1986; Herderich *et al.* 1995), it is possible that delayed detection is the result of an increase in palate pH due to the buffering action of saliva, which increases the volatility of these bases (Bartowsky and Henschke 1995). At least three compounds are associated with this taint. The two isomers of 2-acetyltetrahydropyridine have an aroma threshold in wine of 1·6 µg l^{-1}, 2-acetyl-1-pyrroline has an aroma threshold in wine of 0·1 µg l^{-1}, while 2-ethyl-3,4,5,6-tetrahydropyridine has a taste threshold of 150 µg l^{-1} in wine (Costello *et al.* 1993; Herderich *et al.* 1995).

A mousy taint can be produced by LAB (*Lb. hilgardii, Lb. brevis, Lb. cellobiosus,* now known as *Lb. fermentum* and *O. oeni*) and *Dekkera* or *Brettanomyces* yeasts (Costello *et al.* 1993). This taint is most commonly observed in wines after the completion of MLF. Bacterial formation of these compounds is dependent on the presence of ethanol, hence their presence in wines and not in musts (Bartowsky and Henschke 1995). Lysine stimulates the production of a mousy taint (Heresztyn 1986), but it is not known whether this amino acid is the main determinant for the occurrence of the taint. Possible ways to reduce the incidence of mousy taint include the use of LAB strains selected for their inability to form this taint, inhibition of spoilage bacteria by adding SO_2 immediately after

completion of MLF, or reducing the amino acid precursor (lysine) in wine by a suitable yeast (Bartowsky and Henschke 1995).

2.9.9 REDUCED SULPHUR COMPOUNDS

Occasionally, hydrogen sulphide (H_2S) development is observed during MLF and in *tirage* wines containing malolactic bacteria (Monk, 1986; Bartowsky and Henschke 1995). The potential then exists for mercaptan formation. LAB require the sulphur-containing amino acids cysteine and methionine for growth in wine, but some bacteria, including *Lactobacillus* spp., can also degrade sulphur-containing amino acids to H_2S under certain conditions (Bartowsky and Henschke 1995).

2.9.10 REFERMENTATION

In a survey conducted by Fugelsang and Zoecklein (1983), most (66%) of the wine makers considered L-malate levels < 15 mg l^{-1} as indicative of the completion of MLF. The remaining 34% considering < 30 mg l^{-1} as complete and stable with respect to continued LAB activity. Even when MLF has been determined to be complete, there is the potential for secondary LAB growth on other substrates. An example of secondary LAB spoilage is that produced by *Lb. fructivorans*, also known as the cottony bacillus and the "Fresno mould". This wine LAB has extraordinary alcohol tolerance (> 20% v/v). Historically and recently, this LAB has been isolated from high alcohol dessert wines (> 20% v/v). Visually it appears as a mycelial growth in bottled wine. With modern winemaking techniques, especially the use of SO_2 and sanitation, reported incidences of infection have decreased.

2.9.11 ROPINESS AND POLYSACCHARIDE FORMATION

Ropy wines exhibit an oily, slimy, viscous consistency due to the production of extra-cellular polysaccharides, composed of D-glucan (Llaubères *et al.* 1990). The genera *Leuconostoc* and *Pediococcus* have been implicated in ropiness (Sponholz 1993; Du Toit and Pretorius 2000). The production of extracellular polysaccharides by *Ped. damnosus* and *Ped. pentosaceus* isolated from ropy wines was induced by ethanol, and this trait was plasmid mediated. Ropiness only occurs during the alcoholic fermentation or after bottling, when ethanol is present. Ropiness can be controlled by lowering the pH to below 3·5 (Du Toit and Pretorius 2000).

2.10 CONCLUSION

The classification of LAB remains the source of many discussions and is the focus of intense taxonomic studies. There is an urgent plea for a polyphasic approach, involving both phenotypic and phylogenetic characterisation of bacteria (Vandamme *et al.* 1996). The advent of 16S rRNA sequence analysis has made it possible to determine whether taxa are phylogenetically homogenous and, if not, where the misclassified strains belong

within the hierarchical system (Stackebrandt and Goebel 1994). Comparative studies have clearly revealed the limitations of sequence analysis at determining the relationships at strain level. DNA hybridization is acknowledged as a superior method for the elucidation of relationships between closely related taxa, such as strains and species (Stackenbrandt and Goebel 1994). The use of PCR in identification studies has become more evident. The development of species-specific primers has improved the reliability and simplicity of this technique.

The literature on the LAB that occur in base wine and their influence on base wine quality are very limited. Very little research has been done on the LAB species and the evolution of these species in brandy base wines. The contribution of MLF to wine flavour and quality has been the focus of many discussions, but no clear answer is available. The effect of MLF on the flavour or aroma composition of base wine and brandy is unclear and needs to be investigated. Due to the variety of LAB species and strains in grape must and wine and the various factors affecting their growth, it is difficult to determine if MLF will be beneficial or detrimental to quality (Lonvaud-Funel 1999). Other factors, such as viticultural region, grape variety, wine composition, winemaking techniques and styles, will also affect quality. Therefore, all the above-mentioned factors should be considered when wine flavour or quality is investigated.

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CHAPTER 3

RESEARCH RESULTS

The occurrence of malolactic fermentation in brandy base wine and its influence on brandy quality

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3. RESEARCH RESULTS

The Occurrence of Malolactic Fermentation in Brandy Base Wine and its Influence on Brandy Quality

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In this study, we determined the extent to which lactic acid bacteria (LAB) occurred in brandy base wines, their ability to catalyse the malolactic fermentation (MLF) and the effect of MLF on the quality of the base wine and the brandy distillate. Lactic acid bacteria were isolated and enumerated from grape juice, experimental and commercially produced brandy base wines. Spontaneous MLF occurred in approximately 50% of the commercial base wines. The occurrence of MLF had an influence on the quality of the base wines and the resulting distillates. Samples that had undergone MLF differed significantly from samples that had not undergone MLF. There was a loss of fruitiness and aroma intensity in samples where MLF occurred. Volatile compounds, such as iso-amyl acetate, ethyl acetate, ethyl caproate, 2-phenethyl acetate and hexyl acetate, decreased in samples that had undergone MLF, while ethyl lactate, acetic acid and diethyl succinate increased in the same samples. Spontaneous malolactic fermentation does occur in commercial brandy base wines and it generally has a negative influence on the quality of the base wine and brandy. This study showed that MLF influences the quality of the base wine and the resulting distillate and, with this in mind, commercial base wine producers should be able to produce brandy of higher quality.

3.1 INTRODUCTION

The winemaking process comprises an alcoholic fermentation conducted by yeast and a secondary fermentation, performed by lactic acid bacteria (LAB), that is called malolactic fermentation (MLF). The main reaction associated with MLF is the decarboxylation of malic acid to lactic acid and CO_2 due to a malolactic enzyme (Lonvaud-Funel 1995). The yeast and LAB present in the grape must therefore will influence the quality of the end product. Lactic acid bacteria are usually present in low numbers (10^3 cfu g^{-1}) on healthy grapes and in the subsequent must, but the population might increase to 10^4-10^5 cfu g^{-1} if the grapes are infected with fungi and acetic acid bacteria (Lonvaud-Funel 1995, 1999; Fleet 1998). The LAB associated with the winemaking process include species of the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Fleet 1993; Stiles and Holzapfel 1997; Lonvaud-Funel 1999).

Variables of the must, such as pH, temperature, sulphur dioxide (SO_2), ethanol levels and the availability of nutrients, will influence the LAB directly and this, in turn, will affect the malolactic fermentation (Wibowo *et al.* 1985; Britz and Tracey 1990). Malolactic fermentation is performed for three important reasons, namely deacidification, production of flavour compounds and microbial stability (Lonvaud-Funel 1995). The flavours produced from MLF have been described as "lactic", "buttery", "nutty", "yeasty" and "oaky" and it is believed that MLF enhances the fruity character of wine, reduces the intensity of vegetative aromas and improves the mouthfeel (Bartowsky and Henschke 1995; Henick-Kling *et al.* 1993, Henick-Kling 1995). Citric acid metabolism is linked to MLF and the most important metabolite produced is diacetyl, which is perceived as buttery (Martineau and Henick-Kling 1995; Nielsen and Prahl 1997). Wine colour is affected due to the increase in pH and the metabolic activity on phenolic compounds, such as tannins and anthocyanins (Lonvaud-Funel 1995).

Production of an acceptable base wine for use in the distillation of brandy requires that no or little SO_2 is added (Leaute 1990). Sulphur dioxide is the winemaker's most powerful antimicrobial agent, as well as a highly effective antioxidant. Furthermore, the base wines usually have a lower alcohol content (10 - 11%) and are fermented at 18°C. Base wines therefore are more susceptible to microbial spoilage than table wines. Consequently, many base wines might be prone to MLF due to the favourable growth conditions for the natural LAB that are present after the alcoholic fermentation.

The aims of this study were to determine the extent to which LAB occur in South African brandy base wines, their ability to catalyse the MLF and their effect on the quality of the base wine and the resulting brandy distillate.

3.2 MATERIALS AND METHODS

3.2.1 ISOLATION AND CULTURE CONDITIONS

The selective enumeration of LAB contained in grape juice and wine samples was carried out as follows: Sample aliquots of 100 µl from a 10-fold dilution series were plated out on MRS (Merck, Germany) and Acid Grape (AG) (Dicks *et al.* 1990) agar. The MRS and AG media were supplemented with 50 mg l⁻¹ Delvocid (Gist-Brocades B.V., Netherlands) and 25 mg l⁻¹ kanamycin sulphate (Boehringer Mannheim, Germany) for the inhibition of yeast and acetic acid bacteria (AAB) respectively. Each dilution was plated out in triplicate and maintained under facultatively anaerobic conditions (Gas Generating kit, Oxoid Ltd, England) for three to four days at 30°C.

3.2.2 SMALL-SCALE FERMENTATIONS

Clarified grape juice samples of Chenin blanc and Colombard cultivars were collected from the beginning of February to the middle of March during the 1998-2000 harvest seasons. During this period, a total of 46 samples were collected from five base wine-producing cellars. Each clarified grape juice sample was divided into two fractions and subjected to one of the following treatments: (1) Inoculated with wine yeast 228 (Anchor Yeast, South Africa) at 0·3 g l⁻¹ and treated with lysozyme at 0·5 g l⁻¹ (SANOVA, Germany); and (2) Inoculated with wine yeast 228. None of the treatments contained SO_2, as is prescribed for brandy base wines. The alcoholic fermentation (AF) was performed at 15°C and CO_2 production was monitored by weighing the fermentation canisters. After the fermentation was dry, base wines were incubated further until the MLF was completed. Conventional parameters, such as balling, reducing sugars (RS), alcohol, total acids (TA), free and total SO_2 and pH were determined according to standard procedures (Iland *et al.* 1993). Lactic acid bacterial counts were recorded for the juice during AF, after the AF and during MLF. Bottle (2 litres) and small-scale fermentations (15 litres) were performed on grape juice samples that were obtained in 1998. In 1999 and 2000, only small-scale fermentations (15 litres) were conducted.

3.2.3 COMMERCIAL-SCALE FERMENTATIONS

One hundred and eleven commercial base wine samples from base wine producing cellars were assessed for their MLF status from 1998 to 2000. These base wines were sampled either after alcoholic fermentation (AF) at the wine cellar or just prior to their delivery at the distillery.

Furthermore, the effect of MLF on the quality of base wines and brandy was investigated on a commercial scale (35 000 litres) during the 2001 harvest season. The clarified grape juice samples (70 000 litres) were divided into two fractions and subjected to the following treatments: (1) Inoculated with wine yeast VIN13 (Anchor Yeast) and

treated with lysozyme (DSM Food Specialties Oenology, France), which was added in two parts, 0·25 g l^{-1} at the beginning of the fermentation and another 0·25 g l^{-1} at the end of the AF; and (2) Inoculated with wine yeast VIN13.

3.2.4 ANALYSIS OF ORGANIC ACIDS

The organic acid profiles of juice and base wine samples were determined using high-pressure liquid chromatography (HPLC). Analyses were carried out for the juice and wine samples after AF and MLF. A Hewlett Packard HP 1090 Liquid Chromatograph coupled to an HP 3388 Integrator Terminator was used with the following settings: flow: 0·8 ml min^{-1}; %B: 80 (percentage of solvent pumped during elution); maximum pressure: 60 bar; oven temperature: 65°C; injection volume: 5 μl.

The progression of MLF was assessed by evaluating the concentrations of malic and lactic acid by HPLC and paper chromatography (Kunkee 1968). A lactic acid concentration of less than 0·3 g l^{-1} was indicative of no MLF. Base wine samples containing a lactic acid concentration greater than 0·3 g l^{-1} and more than 0·2 g l^{-1} of malic acid were considered to have undergone partial MLF. Finally, if the malic acid concentration was less than 0·2 g l^{-1}, the sample was deemed to have undergone complete MLF. Base wines derived from small-scale and commercial fermentations were double distilled after completion of MLF.

3.2.5 DISTILLATION OF BASE WINES

Distillations of small-scale fermentations were performed in electrically heated 4·5 litre round bottomed flasks. Two thin copper sheets (1 x 50 x 120 mm) and 0·67 g l^{-1} of $CuSO_4.5H_2O$ were added to simulate the conditions of a copper pot still and the addition of boiling stones ensured a homogenous heat distribution during the distillation process. In the first distillation, volatile components were concentrated over a period of approximately 10 h, at a flow rate of 5 ml min^{-1}, to a final alcohol concentration of 30% (v/v). This procedure was followed for the second distillation and the heads fraction (40 ml), which was collected at a flow rate of 1 ml min^{-1}, was discarded. The heart fraction was distilled at a flow rate of 5 ml min^{-1}. Distillation was continued until the distillate contained an alcohol concentration of 70% (v/v). The distillate was stored at 4°C until further analysis. The commercial base wines were pot stilled at a commercial distillery, using the "Charente" method of distillation. The pot stills used had a capacity of 2 000 litres. The first and second distillation was performed using the same pots.

3.2.6 EXTRACTION OF VOLATILE COMPOUNDS AND CHEMICAL ANALYSIS

The analyses of the volatile compounds in the base wines were performed after the MLF was completed. The volatile flavour and aroma compounds in the base wines and their distillates were analysed by gas chromatography using a Hewlett Packard HP 5890 Gas

Chromatograph, coupled to an HP 7673 auto sampler and injector and an HP 3396A integrator, with the following specifications: column type: DB wax column; dimensions: 0·5 µm x 32 mm; carrier gas: hydrogen; detector: FID by 250; injector temperature: 200°C; split ratio: 20 ml min^{-1}; temperature programme: 35°C for 15 min, thereafter increasing at 6°C min^{-1} to 230°C; run time: 75 min. The samples for analysis were prepared as follows: A liquid-liquid extraction procedure was performed using 50 ml of the base wine, 30 ml of diethylether and 4 ml of a 2·2 mg l^{-1} 4-methyl-2-pentanol solution, which served as the internal standard. Continuous liquid-liquid extraction of base wine samples at 60 rev min^{-1} was performed for 30 min and 1 ml of the diethylether layer was removed for subsequent analysis of the volatile components. The volatile fraction (1 ml) was extracted from 5 ml 70% (v/v) distillate samples that contained 0·25 ml of internal standard.

3.2.7 SENSORY EVALUATION

The triangular method of aroma testing was employed for the small-scale base wine and distillate. The panel of 23 judges comprised students, lecturers and brandy experts from major brandy producing companies in South Africa. The judging was carried out between 09h00 and 12h00 at room temperature. Samples were presented in coded, blue glasses in isolated tasting booths. The brandy distillate was diluted to an alcohol concentration of 23% (v/v) prior to the sensory evaluation. An aroma profiling, using the brandy aroma wheel (Jolly and Hattingh 2001), was also performed on the 70% distillates. A trained panel of 12 judges evaluated the samples based upon the aroma descriptors for unaged brandy. In a preliminary round table discussion, a few aroma descriptors (smooth associated, herbaceous, fruity, floral, sweet associated, other positive and negative aromas) were selected. Additional training sessions were conducted to allow the panel to practise the evaluation of the selected descriptors. A standardised scoring sheet using a 10 cm line scale was used for the evaluation.

3.2.8 STATISTICAL ANALYSIS

The brandy profiling experiment was analysed as a randomised block design using the tasters/evaluators as blocks. Multiple Analysis of Variance (MANOVA) (Johnson and Wichern 1992) was performed on the smooth associated, herbaceous, fruity, floral, sweet associated and positive and negative aromas (eg. heads, tails, chemical) to test for the differences between the two treatments, the differences among distillate, as well as the treatment and distillate interaction. Where significant differences were obtained ($P < 0.05$), Fisher's Least Significance Difference (LSD) method was used for pairwise comparisons (Ott 1993). The data was analysed using the Statistical Analysis System (SAS 1990) software. Triangular testing was done on the small-scale base wines and distillates to determine if the two treatments could be distinguished through sensory evaluation. The

results of the triangular tests were analysed using a Triangular-testing programme (Randall 1998), which was designed especially for wine and brandy evaluation.

3.3 RESULTS

3.3.1 PRESENCE OF LACTIC ACID BACTERIA

Lactic acid bacteria were present at concentrations of 7×10^4 to 8×10^5 cfu ml^{-1}, 2×10^2 to 4×10^3 cfu ml^{-1} and 2 to 9×10^4 cfu ml^{-1} in the grape juice in 1998, 1999 and 2000 respectively. Fewer LAB were present in the juice and the commercial base wines of 1999 than those of 1998 and 2000. This excludes commercial base wines that had undergone MLF. The LAB decreased slightly during AF and increased again at the end of AF (representative samples shown in **Fig. 3.1**). The highest LAB count attained for a base wine was 3×10^7 cfu ml^{-1}. In brandy base wines that had undergone MLF, the LAB population was in excess of 1×10^6 cfu ml^{-1}, whereas in samples in which no MLF had taken place, the LAB population was less than 1×10^6 cfu ml^{-1} (representative samples are shown in **Tables 3.1** and **3.2**). This was observed for both the commercially produced and experimental base wine samples in all three harvest seasons.
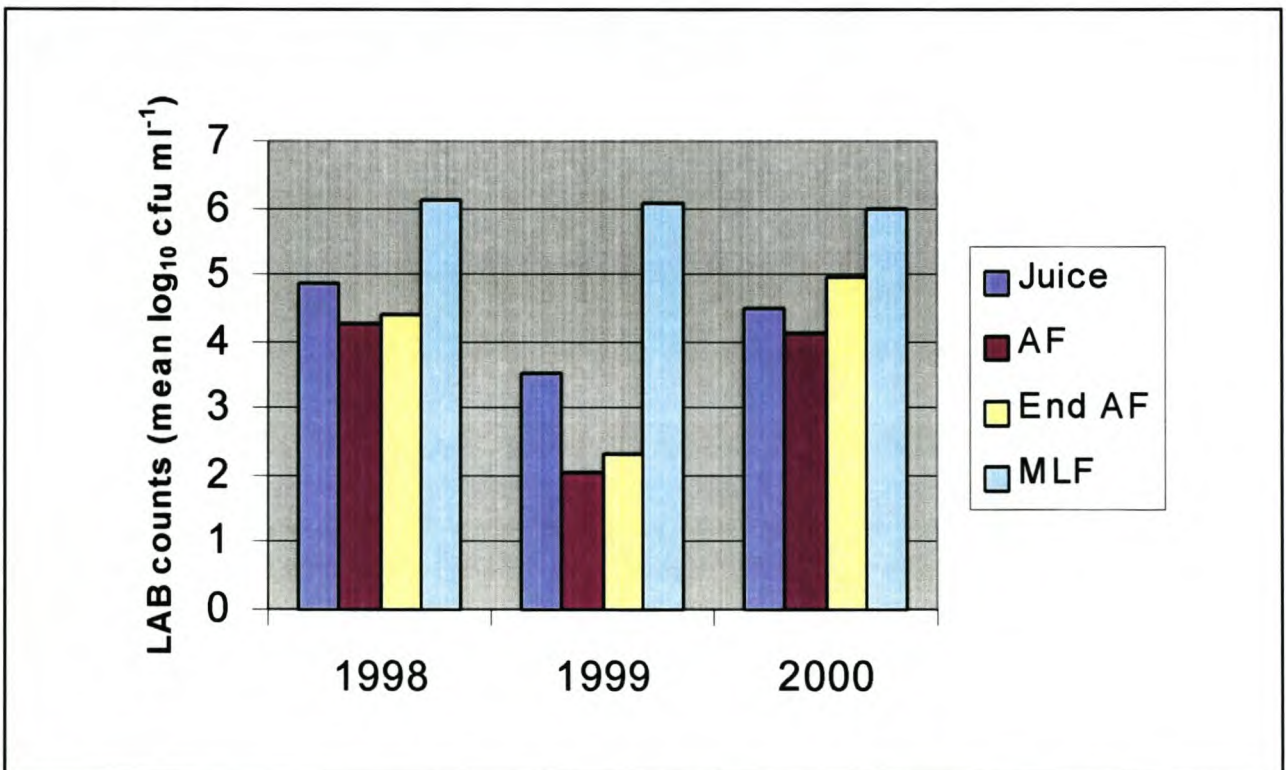

Fig. 3.1 Development of the LAB population in the experimental brandy base wines during the different stages of winemaking: in the juice, at the beginning of alcoholic fermentation (AF), at the end of AF and during malolactic fermentation (MLF).

3.3.2 PROGRESSION OF MLF IN SMALL-SCALE BASE WINES

The progression of MLF in small-scale base wine samples varied for the different years. In 1998, the time to completion of MLF was 10 to 33 days after the AF. In 1999, MLF was delayed for 40 to 75 days after the AF. The MLF occurred 34 to 70 days after AF in the small-scale base wines in 2000.

The metabolism of malic acid in small-scale base wines usually, but not always, was accompanied by the utilisation of citric acid. **Table 3.1** lists representative small-scale base wines to illustrate the progression of MLF. In 39% of the small-scale base wines that had undergone MLF, the citric acid was completely utilised. In most of the small-scale samples that had undergone MLF, the pH was considerably higher than in the control treatment.

Table 3.1 Organic acids and LAB population of small-scale base wines in 1999

| Sample | Treatment | g l⁻¹ | | | | pH | cfu ml⁻¹ |
		Citric acid	Tartaric acid	Malic acid	Lactic acid		LAB population
BT55	Juice	0·28	6·78	5·63	0·00	3·07	6×10^3
	1*	0·27	4·13	3·88	0·00	3·07	4×10^2
	2	0·03	3·94	0·05	3·32	3·27	1×10^6
ST12	Juice	0·46	5·15	4·01	0·00	3·44	1×10^4
	1	0·37	2·13	2·22	0·12	3·49	3×10^4
	2	0·00	2·19	0·00	2·76	3·82	1×10^6
RT501	Juice	0·33	3·22	4·53	0·00	3·61	8×10^4
	1	0·27	1·40	2·21	0·00	3·65	1×10^4
	2	0·25	1·41	0·00	2·22	3·91	2×10^6
BT54	Juice	0·69	5·24	3·70	0·00	3·31	2×10^4
	1	0·22	2·17	2·15	0·00	3·34	1×10^3
	2	0·04	2·21	0·04	2·35	3·58	2×10^6

*Treatment 1: MLF inhibited.

Treatment 2: Spontaneous MLF occurred.

Analysis of treatments 1 and 2 were performed five to 12 weeks after juice analysis.

3.3.3 MONITORING OF COMMERCIAL BASE WINES

In 1998, 14% of the commercial brandy base wines monitored had undergone complete MLF prior to their distillation. A further 50% of these commercial base wines had undergone partial malic acid degradation. In 1999, 3% of the commercial base wines had undergone complete MLF on delivery at the distillery. Another 39% had undergone partial

MLF on delivery at the distillery. In 2000, 10% of the commercial base wines had undergone complete MLF and 45% had undergone partial MLF on delivery at the distillery. **Table 3.2** lists the concentrations of the different organic acids in representative commercial samples that had undergone no, partial and complete MLF. The LAB counts for these samples can also be seen in **Table 3.2**.

Table 3.2 Organic acids and LAB populations of commercial brandy base wines in 1998 and 1999

| Sample | g l^{-1} | | | | Extent of MLF | cfu ml^{-1} |
	Citric acid	Tartaric acid	Malic acid	Lactic acid		LAB population
BT67	0·43	2·56	3·66	0·16	No MLF	7 x 10^3
ST186	0·27	1·88	2·90	0·30	No MLF	3 x 10^4
ST26	0·45	3·02	3·01	0·27	No MLF	4 x 10^3
RT632	0·26	2·54	2·99	1·25	Partial	2 x 10^6
MT112	0·29	1·96	0·60	2·05	Partial	3 x 10^7
BT59	0·24	1·47	1·24	1·93	Partial	3 x 10^6
ST149	0·00	2·48	0·00	2·79	Complete	1 x 10^6
MT144	0·27	1·90	0·20	2·71	Complete	7 x 10^6
MT148	0·32	2·00	0·16	2·71	Complete	3 x 10^6

*Samples collected prior to delivery at the distillery.

3.3.4 ANALYSIS OF VOLATILE COMPOUNDS

Gas chromatographic analysis of small and commercial-scale base wines that had undergone MLF generally displayed a significant increase in the levels of ethyl lactate and acetic acid and a slight increase in diethyl succinate. In contrast, the levels of iso-amyl acetate, ethyl acetate, ethyl caproate, hexyl acetate and 2-phenethyl acetate decreased in the same samples (representative samples are shown in **Table 3.3**). The other quantified compounds were not influenced by MLF.

In the 70% (v/v) brandy distillate of the samples that had undergone MLF, the ethyl lactate and diethyl succinate concentrations were still significantly higher than in the samples that had not undergone MLF (**Table 3.4**). The iso-amyl acetate, ethyl acetate, ethyl caproate and 2-phenethyl acetate concentrations were still lower in the samples that had undergone MLF. The hexyl acetate concentrations were lower in the commercial-scale distillates that had undergone MLF. The small-scale distillates showed higher hexyl acetate concentrations in the samples that had undergone MLF than in the control samples.

Table 3.3 Influence of MLF on volatile compounds in brandy base wines

Sample	Treatment	Ethyl acetate	Iso-amyl acetate	Ethyl caproate	Ethyl lactate	Ethyl caprilate	Ethyl caprate	Hexyl acetate	2-Phenethyl acetate	Diethyl succinate	Propanol	Iso-amyl alcohol	Acetic acid
						mg l^{-1}							
SL (SS)*	1†	57·59	2·11	2·06	1·28	1·43	1·11	0·13	0·27	ND	115·05	102·67	554·94
	2	54·90	1·81	1·89	28·08	1·51	1·19	0·12	0.24	ND	112·81	100·42	863·24
R (SS)	1	76·42	4·00	2·67	1·82	1·61	1·41	0·29	0·23	ND	161·79	117·06	443·21
	2	70·91	3·28	2·38	15·28	1·63	1·31	0·24	0·18	ND	144·87	113·91	619·44
B (SS)	1	70·62	5·47	2·55	0·70	1·56	1·32	0·66	0·44	ND	115·33	124·15	614·07
	2	61·02	5·12	2·28	7·81	1·62	1·24	0·61	0·19	ND	110·92	120·68	784·59
BT (CS)	1	270·82	21·87	ND‡	ND	8·79	1·35	2·40	0·93	1·10	57·08	141·51	584·43
	2	215·72	11·46	ND	40·45	9·15	1·37	0·42	0·65	1·31	59·30	161·75	937·14
GT (CS)	1	176·16	11·04	ND	1·49	5·31	0·98	0·70	0·34	1·09	43·03	177·56	317·21
	2	139·43	6·12	ND	20·27	4·23	0·86	0·33	0·22	1·15	46·47	191·46	271·32

*(SS) Small-scale and (CS) Commercial-scale samples.

†1: MLF inhibited.

2: Spontaneous MLF occurred.

‡Not detected.

Table 3.4 Influence of MLF on volatile compounds in 70% brandy distillates

Sample	Treatment	Ethyl acetate	Iso-amyl acetate	Ethyl caproate	Ethyl lactate	Ethyl caprilate	Ethyl caprate	Hexyl acetate	2-Phenethyl acetate	Diethyl succinate	Propanol	Iso-amyl alcohol	Acetic acid
							mg l^{-1}						
SL (SS)*	1†	106·31	2·52	11·09	9·46	12·91	27·54	0·23	1·04	1·02	179·90	752·04	17·54
	2	97·25	2·24	9·94	88·17	12·76	26·17	0·24	1·07	6·46	177.39	752·05	45·16
R (SS)	1	125·20	8·95	12·50	9·03	16·14	43·57	0·40	0·67	1·66	233.94	831·75	22·00
	2	107·90	6·64	11·96	37·34	15·93	36·58	0·35	0·39	3·09	224.28	862·90	48·11
B (SS)	1	111·39	13·67	11·95	3·18	11·44	23·29	0·97	1·66	1·23	155.75	816·42	40·22
	2	98·02	13·45	10·97	39·36	13·46	30·30	1·27	2·06	2·35	161.54	822·39	44·87
BT (CS)	1	511·75	40·03	8·79	2·81	19·09	46·78	4·33	0·98	ND‡	316·65	933·44	37·83
	2	372·61	26·47	6·94	31·17	18·71	48·46	1·51	0·88	1·29	345.08	1012·78	36·52
GT (CS)	1	472·68	31·22	7·66	4·27	12·61	12·87	2·89	1·51	0·67	234·46	1219·43	22·54
	2	263·95	13·74	4·55	28·19	9·84	14·64	1·00	0·32	4·36	225·09	1050·90	10·82

*(SS) Small-scale and (CS) Commercial-scale samples.

†1: MLF inhibited.

2: Spontaneous MLF occurred.

‡ Not detected.

3.3.5 SENSORY EVALUATION OF SMALL-SCALE BASE WINES AND DISTILLATES

The evaluation panel for the triangular testing was able to distinguish between base wines that had undergone MLF and those that had not (**Table 3.5**). The significance level (for $P \leq 0.05$) indicated that there were significant differences between the two treatments. The significance level was higher than 95% for most of the groups (**Table 3.5**). The only group for which the base wine that had undergone MLF could not be distinguished from the base wine that had not, was group 6. In most cases, the panel preferred the base wines that had not undergone MLF. Many of the base wines that had undergone MLF exhibited a loss of aroma and a lack of freshness.

Table 3.5 Triangular differentiation of base wines that had undergone MLF

Group	Evaluators	Correct	Significance level*	Favourite	Percentage †
1	10	8/10	99·66	MLF	75%
2	10	7/10	98·03	MLF	67%
3	10	10/10	99·99	no MLF	80%
4	10	6/10	92·34	no MLF	80%
5	10	8/10	99·66	no MLF	75%
6	10	0/10	0·01	no MLF	67%
7	10	10/10	99·99	no MLF	67%
8	10	10/10	99·99	MLF	67%
9	10	7/10	98·03	no MLF	100%
10	10	9/10	99·96	no MLF	75%
11	10	10/10	99·99	no MLF	100%
12	10	6/10	92·34	MLF	100%

*A level > 95% ($P < 0.05$) is significant.
† Percentage that preferred the favourite.

The triangular evaluation of the 70% (v/v) brandy distillate displayed similar tendencies to that of the base wines (**Table 3.6**). The distillates that had not undergone MLF were preferred. The panel was again able to distinguish between the 70% distillate in which MLF had occurred and the distillate in which no MLF had occurred. However, there were more groups of distillates in which the samples that had undergone MLF could not be distinguished from the samples that had not.

Table 3.6 Triangular differentiation for 70% distillate in which MLF had occurred

Group	Evaluators	Correct	Significance level*	Favourite	Percentage †
1	23	15/23	99 83	no MLF	63%
2	23	9/23	65·14	no MLF	88%
3	23	21/23	99·99	no MLF	78%
4	23	13/23	98·40	MLF	71%
5	23	16/23	99·94	no MLF	67%
6	23	5/23	7·58	MLF	63%
7	23	18/23	99·99	no MLF	100%
8	23	15/23	99·83	MLF	67%
9	23	21/23	99·99	no MLF	89%
10	23	14/23	99·38	no MLF	67%
11	23	12/23	95·2	no MLF	89%
12	23	8/23	48·07	no MLF	63%

*A level > 95% ($P < 0.05$) is significant.

† Percentage that preferred the favourite.

The aroma profile of the small-scale distillates that had and had not undergone MLF is illustrated in **Fig. 3.2**. The smooth associated, herbaceous and fruity flavours were slightly more intense in the sample in which no MLF occurred, while sweet associated flavours, such as chocolate and caramel, were more intense in samples that had undergone MLF. The negative aromas (eg. solvent/chemical) were also more prominent in distillates that had undergone MLF.
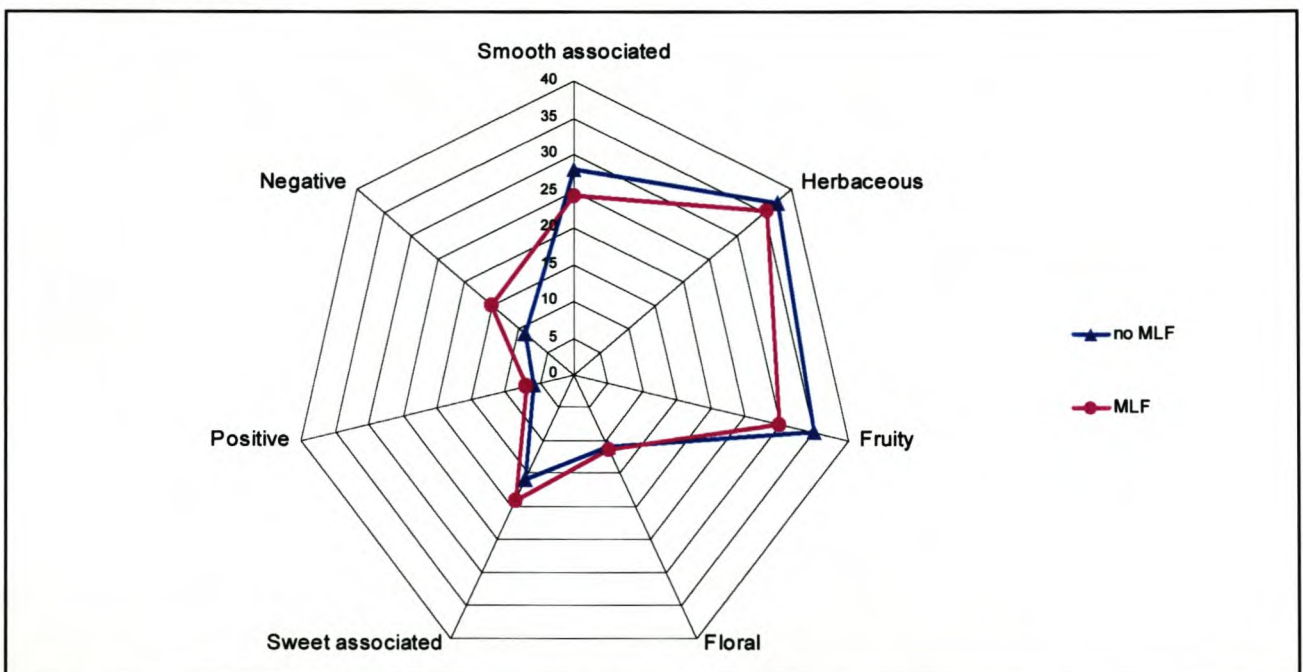

Fig. 3.2 A comparison of the aroma profiles of 70% brandy distillate that had undergone MLF and that had not undergone MLF.

Multivariate analysis of variance (MANOVA) for the different treatments revealed significant differences at $P \leq 0.1$ (**Table 3.7**). Multivariate analysis of variance indicated that the aroma profiles were significantly different for all the distillates ($P = 0.0006$). Multivariate and univariate analysis of variance revealed no significant interaction between the distillate and the treatment. Analysis of variance (ANOVA) for the sensory evaluation indicated significant differences ($P \leq 0.05$) amongst the judges for the aroma descriptors (**Table 3.8**). The MLF had a significant influence ($P \leq 0.05$) on fruitiness and negative aromas. Each distillate was also significantly different ($P < 0.05$) in terms of fruity, floral and sweet associated aromas.

Table 3.7 Probability (P) values for the different variables

Source of variation	Wilks Lamda value	F-value	$P*$
Treatment	0.9558	1.7635	0.0948
Distillate	0.6368	1.5921	0.0006
Treatment + Distillate	0.7885	0.844	0.8292

*P values derived from multivariate analysis of variance.

Table 3.8 Effect of different variables on the aroma compounds

Source of variation	Probability (P) values*						
	Smooth associated	Herbaceous	Fruity	Floral	Sweet associated	Positive	Negative
Evaluators	0·001	0·001	0·001	0·001	0·001	0·001	0·001
Treatment	0·169	0·439	0·043	0·874	0·557	0·544	0·024
Distillate	0·863	0·008	0·001	0·019	0·015	0·768	0·817
Distillate + Treatment	0·747	0·918	0·734	0·717	0·284	0·264	0·933

* Probability values derived from analysis of variance.

3.4 DISCUSSION

Naturally-occurring LAB, which are present in the juice and base wine, were responsible for all spontaneous MLF. The LAB population present in the grape juice and base wine varied in cell numbers from 1998 to 2000. The lower LAB population in 1999 could be due to climatical conditions.

The lower LAB population in the grape juice of 1999 resulted in the delayed onset of MLF. There is a definite correlation between the size of the LAB population and the occurrence of MLF. In samples in which MLF occurred, the LAB population needed to be in excess of 1×10^6 cfu ml^{-1}.

In small-scale base wine samples that had undergone complete spontaneous MLF, the malic acid was sufficiently degraded by the LAB. In most of these samples, citric acid was also utilised. The metabolism of citric acid might explain the higher acetic acid concentrations in these samples. In these particular samples, the acetic acid concentration was higher than in samples in which citric acid was not utilised (results not shown).

The occurrence of MLF in samples ST12 and RT501 resulted in a significant increase, of approximately 0·3 pH units, in the pH. The occurrence of MLF in high pH base wines could result in the development of other spoilage bacteria. **Table 3.1** shows that the control wines did not undergo any MLF and that the pH values differ only slightly from that of the juice. The use of lysozyme to inhibit the growth of LAB and the occurrence of MLF was effective for most of the control base wines. Similar results were obtained by Gerbaux *et al.* (1997, 1999). There were exceptions where the control base wines underwent partial MLF. This could be because the LAB that performed the MLF might be less sensitive to the activity of the lysozyme or because there was a loss in the activity of the lysozyme as time progressed.

Although only a limited number of volatile compounds were quantified in this study, it is already clear that MLF does influence the composition and quality of brandy base wines and the resultant distillates. The occurrence of spontaneous MLF in base wines was associated with a decrease in the concentration of the fruity esters, such as iso-amyl acetate, ethyl caproate, hexyl acetate and 2-phenyl acetate. The decrease in the concentrations of these fruity esters might explain the reduced fruitiness of the base wines that had undergone MLF. Laurent *et al.* (1994) observed an increase in iso-amyl acetate in samples that had undergone MLF, although the fruity aroma in these wines was reduced. In contrast, Henick-Kling *et al.* (1993) found that fruitiness increased in wines that had undergone MLF. Bartowsky and Henschke (1995) demonstrated that the aroma/flavour attributes imparted to the wine depend on the LAB strain that performs the MLF.

Ethyl acetate concentrations decreased in base wine samples that had undergone MLF, contrary to the results obtained by Davis *et al.* (1985). As expected, ethyl lactate, acetic acid and diethyl succinate increased in the base wine samples that had undergone MLF. The small-scale fermentations showed that propanol and iso-amyl alcohol were higher in base wines that had undergone MLF. Contradicting results were obtained for propanol and iso-amyl alcohol in commercial-scale base wines. Hexyl acetate, 2-phenethyl acetate and acetic acid concentrations in distillates that had undergone MLF were contradictory for the small-scale and commercial-scale distillates. This might be due to the differences in the distillation process and equipment.

For the triangular testing, the significance level was calculated at $P \leq 0.05$ and was used to determine if there were significant differences between the two treatments. A level higher than 95% ($P \leq 0.05$) is considered to be statistically significant. According to the significance levels of the triangular tests, the two treatments were shown to be significantly different (**Tables 3.5** and **3.6**). The sensory evaluation also showed that, in most cases, the occurrence of spontaneous MLF had a negative effect on the quality of the base wines evaluated. For most of the samples, the evaluators preferred the small-scale base wines that had not undergone MLF. There were exceptions where the base wine that had undergone MLF was actually preferred. This might be due to the different LAB that performed the MLF, and is part of an ongoing investigation.

Due to the negative effect of MLF on the quality of base wines, winemakers should control the growth of LAB by storing base wines at low temperatures (10°C) after the fermentation is completed, or by adding lysozyme at the beginning of or after the alcoholic fermentation.

The results also showed that the distillation process has a significant effect on the final product. Some base wine groups that were favoured less performed very well during the sensory evaluation of the distillates. When the small-scale distillates are compared to commercial-scale distillates, it is clear that the distillation process has a definite influence on the concentrations of compounds and will have an effect on the quality of the final product. The significance levels for the 70% distillates indicated that it was possible to distinguish between the distillates in which MLF had occurred and the distillates in which no MLF had occurred. The results also showed that the samples that had not undergone MLF were still preferred. There were, however, changes in the preferred treatment for different groups, as seen in groups 1, 2, 4, 6 and 12 (**Tables 3.5** and **3.6**). The preferred treatment for the base wines was different to the preferred treatment for the distillates.

Multiple analysis of variance for the two treatments was significant for $P \leq 0.1$ (**Table 3.7**). In other words, there is a 90% probability that the base wine that had not undergone MLF is different from the base wine that had undergone MLF. The MANOVA reveals that the distillate was the most significant ($P \leq 0.05$) source of variation for all of the aroma descriptors (smooth associated, herbaceous, fruity, floral, sweet associated, other positive aromas and negative aromas) evaluated. Every distillate had a significantly different aroma profile. It is logical that the composition of the distillate will be different for every sample, as cut-off points and liquid-vapour equilibrium compositions will vary for each distillation due to the varying composition of each wine. There was no significant interaction between the treatment and the distillate. The analysis of variance revealed significant differences among the judges for all the descriptors (**Table 3.8**). The scoring of the different individuals was the most significant factor. This implies that every person smells and perceives the aroma compounds differently. Evaluation differences are commonly reported in the literature (Guinard and Cliff 1987). Analysis of variance for the treatments revealed that MLF did not have a significant influence on all the aroma

descriptors (**Table 3.8**), but showed that MLF had a significant influence on fruitiness and negative aroma.

This study showed that lactic acid bacteria are present at high numbers in juice and brandy base wines and are able to perform the malolactic fermentation. This study also showed that spontaneous MLF does indeed occur in South African brandy base wines and that it does influence the composition and perceived sensory quality of the base wine and the resulting brandy distillate.

More detailed work must be performed at a commercial scale and on volatile aroma-contributing compounds that may be influenced by MLF. The effect of wood maturation on MLF distillates should also be investigated.

3.5 ACKNOWLEDGEMENTS

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CHAPTER 4

RESEARCH RESULTS

Identification of lactic acid bacteria isolated from South African brandy base wines

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Identification of lactic acid bacteria isolated from South African brandy base wines

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By law, no sulphur dioxide is allowed in brandy base wine and is therefore ideal for the proliferation of lactic acid bacteria. A total of 54 strains were isolated from grape juice and at different stages of brandy base wine production. The strains were identified using numerical analysis of total soluble cell protein patterns, 16S rRNA sequence analyses and PCR using species-specific primers. The predominant species was *Oenococcus oeni* (22 strains), but *Lactobacillus brevis* (8 strains), *Lactobacillus paracasei* (8 strains) and *Lactobacillus plantarum* (6 strains) also were isolated frequently. Many of the *O. oeni* strains were isolated from brandy base wines after completion of spontaneous malolactic fermentation (MLF). The *Lactobacillus* spp. were isolated from all the different stages of brandy base wine production. *Lactobacillus plantarum* was the dominant species in the juice, but disappeared during the later stages of production. *Lactobacillus hilgardii*, *Lb. brevis* and *Lb. paracasei* were also isolated from base wines after spontaneous MLF. Strains identified as *Lactobacillus vermiforme* were isolated during the alcoholic fermentation and after MLF had been completed. Total soluble cell protein patterns grouped *O. oeni* strains into two phenotypic groups. Two phenotypic clusters were also identified for *Lb. brevis* strains. All the *Lb. paracasei* isolates could be grouped into one phenotypic cluster. There was a good correlation between the clustering of the protein profiles and the results obtained by PCR using species-specific primers. This is the first report of the presence of *Lb. paracasei* and *Lb. vermiforme* in brandy base wines.

4.1 INTRODUCTION

Lactic acid bacteria (LAB) occur naturally on grapes and their ability to grow in grape juice and wine is well documented (Davis et al., 1985; Wibowo et al., 1985; Ribéreau-Gayon et al., 2000). The LAB associated with grapes or the winemaking process belong to the genera *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus* (Lonvaud-Funel, 1999; Du Toit and Pretorius, 2000). Lactic acid bacteria can be beneficial or detrimental to the quality of wine, depending on the species and even on the strain, and on the stage at which they occur in the vinification process (Lonvaud-Funel, 1999). Winemakers generally use LAB starter cultures to induce malolactic fermentation (MLF) in wine, but spontaneous MLF may also occur. During MLF, L(+)-malic acid is converted to L(+)-lactic acid and CO_2 (Kunkee, 1991; Lonvaud-Funel, 1999). The use of commercial starter cultures gives the winemaker more control over the occurrence of and the LAB species performing MLF. The growth of LAB in wine is influenced by factors such as pH, alcohol concentration, sulphur dioxide (SO_2), temperature and nutrient availability (Wibowo et al., 1985; Fugelsang, 1997).

Eight or nine species of LAB can be identified in grape must. *Lactobacillus* spp., *Pediococcus* and *Leuconostoc mesenteroides* progressively disappear during the alcoholic fermentation. In most cases, *Oenococcus oeni* is the only species identified when the fermentation is completed (Lonvaud-Funel et al., 1991). The following species have been found in wine: *Lactobacillus brevis* (Vaughn, 1955; Du Plessis and Van Zyl, 1963; Pilone et al., 1966; Chalfan et al., 1977; Sharpe, 1981; Dicks and Van Vuuren, 1988; Edwards et al., 1993), *Lactobacillus buchneri* (Vaughn, 1955; Du Plessis and Van Zyl, 1963; Pilone et al., 1966; Sharpe, 1981), *Lactobacillus casei, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus fermentum* (Vaughn, 1955), *Lactobacillus fructivorans* (Amerine and Kunkee, 1968; Edwards et al., 1993), *Lactobacillus hilgardii* (Douglas and Cruess, 1936; Vaughn, 1955; Du Plessis and Van Zyl, 1963; Dicks and Van Vuuren, 1988; Edwards et al., 1993), *Lactobacillus jensenii, Lactobacillus kunkeei* (Edwards et al., 1998), *Lactobacillus nagelii* (Edwards et al., 2000), *Lactobacillus plantarum* (Wibowo et al., 1985; Edwards et al., 1993) and *Lactobacillus sakei* (Kandler and Weiss, 1986). *Pediococcus parvulus, Pediococcus damnosus* and *Pediococcus pentosaceus* have been associated with wine (Wibowo et al., 1985; Edwards and Jensen, 1992; Fugelsang, 1997; Gindreau et al., 2001). The only species from the genus *Leuconostoc* that has been isolated from wine is *Leuconostoc mesenteroides* (Lafon-Lafourcade et al., 1983; Lonvaud-Funel, 1999; Ribéreau-Gayon et al., 2000). *Oenococcus oeni* has been isolated from various wines (Lafon-Lafourcade et al., 1983; Wibowo et al., 1985; Fugelsang, 1997; Ribéreau-Gayon et al., 2000).

Little is known about the bacterial population of brandy base wines. Lafon-Lafourcade et al. (1983) studied the evolution of LAB in cognac base wines. These authors isolated *Lc. mesenteroides* and *O. oeni* from freshly produced must and, in addition, *Lb. casei* and *Lb. brevis* were found in the pressings along with *O. oeni*. On the first day of the alcoholic

fermentation, *Lc. mesenteroides*, *Lb. plantarum* and *O. oeni* were isolated. After the third day of alcoholic fermentation, *O. oeni* was the only species recovered from the base wine. The base wine used for the distillation of brandy differs from normal white wine in terms of a lower pH and a lower alcohol concentration. Very little or no SO_2 is used during production. The conditions in brandy base wines therefore are favourable to promote the growth of LAB.

The aims of this study were to identify the naturally occurring LAB in South African brandy base wines and to determine which species are responsible for the spontaneous MLF of the base wines, as observed by Du Plessis et al. (2002). The isolates were identified using numerical analysis of total soluble cell protein patterns, 16S rRNA sequence analysis and a PCR method using species-specific primers.

4.2 MATERIALS AND METHODS

4.2.1 ISOLATION AND CULTIVATION OF BACTERIAL STRAINS

Strains were isolated from a previous study on South African brandy base wines (Du Plessis et al., 2002). Strains were grown on MRS (Merck) and acidic grape (AG) medium (Dicks et al., 1990) at 30°C under anaerobic conditions (Gas Generating kit, Oxoid Ltd). Purified cultures were maintained at –80°C in 30% (v/v) glycerol. The type and reference strains included in this study are listed in **Table 4.1**.

4.2.2 PRELIMINARY IDENTIFICATION

Isolates were selected on the basis of Gram reaction, morphology and catalase activity. Catalase-negative and Gram-positive rods and cocci were selected and screened for the production of CO_2 from glucose and gluconate. Cultures were inoculated in an adapted MRS broth (MRS without beef extract and citrate). The same medium was used to detect CO_2 production from sodium gluconate. Ten millilitres of the modified broth was allocated per test tube, containing a Durham tube. The isolates were incubated for two to seven days at 30°C. Arginine hydrolysis was performed on certain strains using the method of Schillinger and Lücke (1987).

4.2.3 NUMERICAL ANALYSIS OF TOTAL SOLUBLE CELL PROTEIN PATTERNS

The strains were cultured on MRS or AG agar for 48 h at 30°C. The method used for the preparation of total cell protein extracts, SDS-PAGE and preparation of the gels for numerical analysis were as described by Pot et al. (1994). The software package, GEL COMPAR (version 4.0) of Applied Maths (Kortrijk, Belgium), was used to analyse the protein profiles. This programme recorded the normalised electrophoretic protein

Table 4.1

Reference and type strains included in this study

Strains	Source[a]	Origin[b]	Comments
Lactobacillus brevis	ATCC 14869[T]	Faeces	Type strain
Lactobacillus brevis subsp. *gravesensis*	ATCC 27305[T]	Wine	Type strain
Lactobacillus buchneri	DSM 20057[T]	Tomato pulp	Type strain, the same as, ATCC 4005[T]
Lactobacillus casei	DSM 20011[T]	Cheese	Type strain, the same as, ATCC 393[T]
Lactobacillus hilgardii	DSM 20176[T]	Wine	Type strain
Lactobacillus nagelii	ATCC 700692[T]	Wine	Type strain
Lactobacillus paracasei subsp. *paracasei*	DSM 5622[T]		Type strain, the same as, ATCC 25302[T]
Lactobacillus plantarum	ATCC 14917[T]	Pickled cabbage	Type strain, the same as DSM 20174[T]
	ATCC 8014	Corn silage	Previously *Lactobacillus arabinosus*
Lactobacillus pentosus	DSM 20314[T]	Unknown	Type strain
Lactobacillus vermiforme	NCDO 962	Sugar beet factory	Previously *Betabacterium vermiforme*
Lactobacillus zeae	DSM 20178[T]	Corn steep liquor	Type strain the same as, ATCC 15820[T]
Oenococcus oeni	DSM 20252[T]	Wine	Type strain the same as, ATCC 23279[T]
	NCDO 1894 NCDO 2122	Friesa wine	
	DSM 7008	wine	Malolactic fermentation starter culture
	DSM 12923	wine	Malolactic fermentation starter culture

[a] ATCC, American Type Culture Collection, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; and NCDO, National Collection of Dairy Organisms, UK.

[b] Information obtained from culture collection catalogues and relevant literature.

patterns of the densitometric traces, grouped the isolates by the Pearson product moment correlation coefficient (*r*) and performed cluster analysis by the unweighted average pair-group (UPGMA) method.

4.2.4 16S rRNA SEQUENCING

Representative strains from the protein clusters were selected for 16S rRNA analysis. The method described by Collins et al. (1991) was used. The polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene using conserved primers close to the 3' and 5' ends of the gene. The PCR products were purified with a Prep-A-Gene kit (Biorad, USA) and sequenced using a Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems Inc. USA) and an automatic DNA sequencer. The closest relatives of the unknown isolates were determined through sequence database searches and the sequence of closely related strains was retrieved from GenBank or Ribosomal Database Project Libraries. Retrieved sequences were aligned with the newly determined sequences using the PILEUP programme.

4.2.5 COLONY PCR WITH SPECIES-SPECIFIC PRIMERS

Preparation of bacteria for the PCR reactions involved growing the bacteria on MRS or AG agar plates at 30°C until colonies could be distinguished clearly. A BIOMETRA TRIO-Thermoblock machine was used for all PCR reactions. A multiplex PCR assay was performed, using specific primers designed by Torriani et al. (2001) for the identification of *Lactobacillus paraplantarum, Lb. plantarum* and *Lb. pentosus*. The multiplex PCR mixture (50 μl) contained 1.5 mM of $MgCl_2$, the primers paraF, pentF and pREV (0.25 μM each), 0.12 μM of primer plan F, 0.2 mM of deoxynucleotide mix (Boehringer Mannheim), 0.5 U of Taq DNA polymerase (Bioline), 5 μl of 10 x NH_4 buffer and a single colony. The initial denaturation was at 94°C for 3 min, 30 cycles of denaturation at 94°C (30 sec), annealing at 56°C (10 sec), elongation at 72°C (30 sec), and final extension at 72°C for 5 min. The PCR products were separated on a 2% (w/v) agarose gel. The species-specific primers used for the identification of *Lb. paracasei* by Ward and Timmins (1999) were used in this study. The 50 μl reaction mix consisted of 0.3 μM of each primer, 0.2 mM of the nucleotide mix, 1.5 mM of $MgCl_2$, 5 μl of 10x-reaction buffer and one bacterial colony. The mixture was then heated for 10 min at 100°C. After the heat treatment, 0.5 units of Taq DNA polymerase (Bioline) was added to the mix. The PCR conditions were: one cycle at 94°C for 3 min, 30 cycles of 94°C for 45 sec, 48°C for 45 sec and 72°C for 1 min. Final extension was carried out at 72°C for 5 min. The PCR products were separated on a 2% (w/v) agarose gel. Colony PCR was also performed on the *O. oeni* strains. The PCR-specific reaction for *O. oeni* was performed with the primers On1 and On2, which were designed by Zapparoli et al. (1998). The 50 μl PCR reaction mix consisted of 0.25 μM of each primer, 0.2 mM of the deoxynucleotide mix, 1 mM of $MgCl_2$, 5 μl of 10x-reaction buffer and one bacterial colony. The mixture was then heated for 10 min at 100°C. After

the heat treatment, 0.5 units of Taq DNA polymerase (Bioline) was added to the mix. The PCR conditions were: one cycle at 94°C for 1 min, 30 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1min 30 sec. A final extension of 72 °C for 10 min was also included. The PCR products were separated on a 1% (w/v) agarose gel.

4.2.6 BIOCHEMICAL TESTS

Biochemical tests were performed on a selection of obligately heterofermentative strains, using the API 50CH system (API-Biomerieux) according to the instructions of the manufacturer. Test preparations were incubated at 30°C and the reactions were recorded after 24h and 48h.

4.3 RESULTS

4.3.1 PRELIMINARY IDENTIFICATION

A total of 54 Gram-positive and catalase-negative rods and cocci-bacilli were isolated from grape juice and brandy base wine (**Fig. 4.1, 4.2** and **4.3**). Seven strains were isolated from grape juice, 15 strains during the alcoholic fermentation (AF), 20 strains during the malolactic fermentation and 12 strains after completion of MLF. Seventeen of the isolated strains produced CO_2 from gluconate, but not from glucose, and were classified as facultatively heterofermentative. Seven of these strains were isolated from the juice, four strains were isolated during the alcoholic fermentation, four strains were isolated while the base wine was undergoing MLF and two strains were isolated two weeks after the completion of the MLF. Thirty-seven of the strains produced CO_2 from glucose and were classified as obligately heterofermentative. Fifteen of these obligately heterofermentative strains were rods. Three of these obligately heterofermentative rods were isolated during the alcoholic fermentation, seven were isolated during MLF and five were isolated after MLF had been completed. Twenty-two of the obligately heterofermentative strains had cocci-bacilli morphology. They grew slowly and none of them were able to degrade arginine. One strain was isolated from the juice, six strains were isolated during the AF, 10 strains were isolated during the MLF and five strains were isolated after MLF had been completed.

4.3.2 NUMERICAL ANALYSIS OF TOTAL SOLUBLE CELL PROTEIN PATTERNS

The phenotypic relatedness of the facultatively heterofermentative strains is shown in **Fig. 4.1**. Three clusters were delineated at $r = 0.80$. Cluster I comprised eight strains at $r \geq 0.81$, with reference strains of *Lb. paracasei* DSM 5622^T and *Lb. casei* DSM 20011^T. Three strains formed cluster II at $r \geq 0.83$ with *Lb. pentosus* DSM 20314^T. Six strains in cluster III grouped at $r \geq 0.84$ with *Lb. plantarum* ATCC 14917^T and ATCC 8014.

Lactobacillus nagelii ATCC 700692^T and *Lactobacillus zeae* DSM 20198^T did not cluster, confirming their species status.

Five clusters were delineated at r = 0.78 for the obligately heterofermentative rods (**Fig. 4.2**). Cluster I comprised four strains at $r \geq 0.78$ with the type strain *Lb. brevis* ATCC 14869^T. Cluster II comprised four strains at $r \geq 0.81$ with the type strain *Lb. brevis* subspecies *gravesensis* ATCC 27305^T. The third cluster comprised two strains, which clustered at $r \geq 0.84$ with the type strain *Lb. hilgardii* DSM 20176^T. Cluster IV contained isolate V 1 and the type strain *Lb. buchneri* DSM 20057^T at r = 0.96. Five strains formed cluster V at $r \geq 0.65$ with *Lb. vermiforme* NCDO 962.

Two clusters were delineated at r = 0.73 for the cocci-bacilli-shaped strains (**Fig. 4.3**). Five strains formed cluster I at $r \geq 0.79$ with *O. oeni* DSM 12923 (MLF starter culture, Chr. Hansen). Cluster II comprised 17 strains, which clustered at $r \geq 0.82$ with *O. oeni* DSM 20252^T, NCDO 1894, NCDO 2122 and DSM 7008 (MLF starter culture, Chr. Hansen).

4.3.3 16S rRNA SEQUENCING AND BIOCHEMICAL TESTS

Three isolates (C 17, J 26 and L 43) were identified as *Lb. paracasei* by 16S rRNA sequence analysis (**Fig. 4.1**). Isolate K 57 was identified as *Lb. plantarum*, K 22 was identified as *Lb. pentosus* by 16S rRNA sequencing analyses, and three obligately heterofermentative strains (W 16, U 75, W 1) were identified as *Lb. vermiforme*.

Isolate J 15 was identified as *Lb. brevis* and isolate V 1 was identified as *Lb. buchneri* from the results obtained from the biochemical tests.

4.3.4 PCR USING SPECIES-SPECIFIC PRIMERS

A PCR method using species-specific primers was used to confirm the identity of *Lb. paracasei* isolates. This PCR reaction generated a PCR fragment of 290 bp. All the isolates and the type strain of *Lb. paracasei* in cluster I (**Fig. 4.1**) generated a PCR fragment of this size (**Fig. 4.4**). A multiplex PCR assay using species-specific primers for *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* was used to confirm the identity of the isolates in clusters II and III (**Fig. 4.1**). The expected sizes of the PCR fragments were 318 bp for *Lb. plantarum*, 218 bp for *Lb. pentosus* and 107 bp for *Lb. paraplantarum*. The reference strains of *Lb. plantarum* and all the isolates generated the expected 318 bp fragment (**Fig. 4.5**). The type strain of *Lb. pentosus* generated a PCR fragment of 218 bp, but the isolates of cluster III generated PCR fragments of 318 bp.

Strains identified as *O. oeni* using SDS-PAGE were confirmed with the *O. oeni* specific primers (**Fig. 4.6a** and **b**). This PCR reaction generated a 1025 bp fragment. All the isolates and the type and reference generated the 1025 bp fragment. No products were obtained when the primers were used on the *Lactobacillus* species, which served as controls.
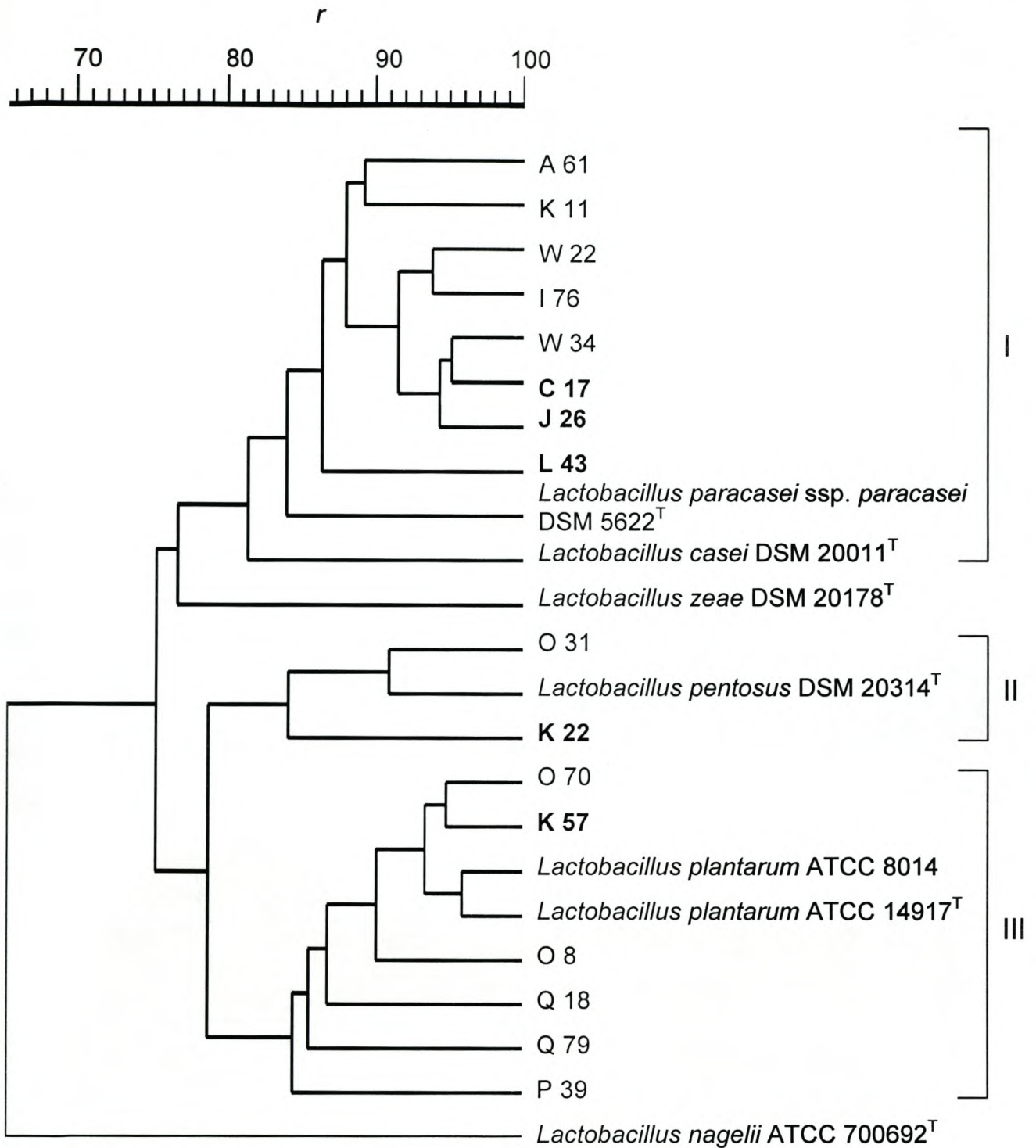
Fig. 4.1. Dendrogram showing the clustering of facultatively heterofermentative lactobacilli strains obtained by numerical analysis of total soluble cell protein patterns. Clustering analysis was performed by the unweighted average pair-group (UPGMA) method. Strains indicated in bold were selected for 16S rRNA sequencing.
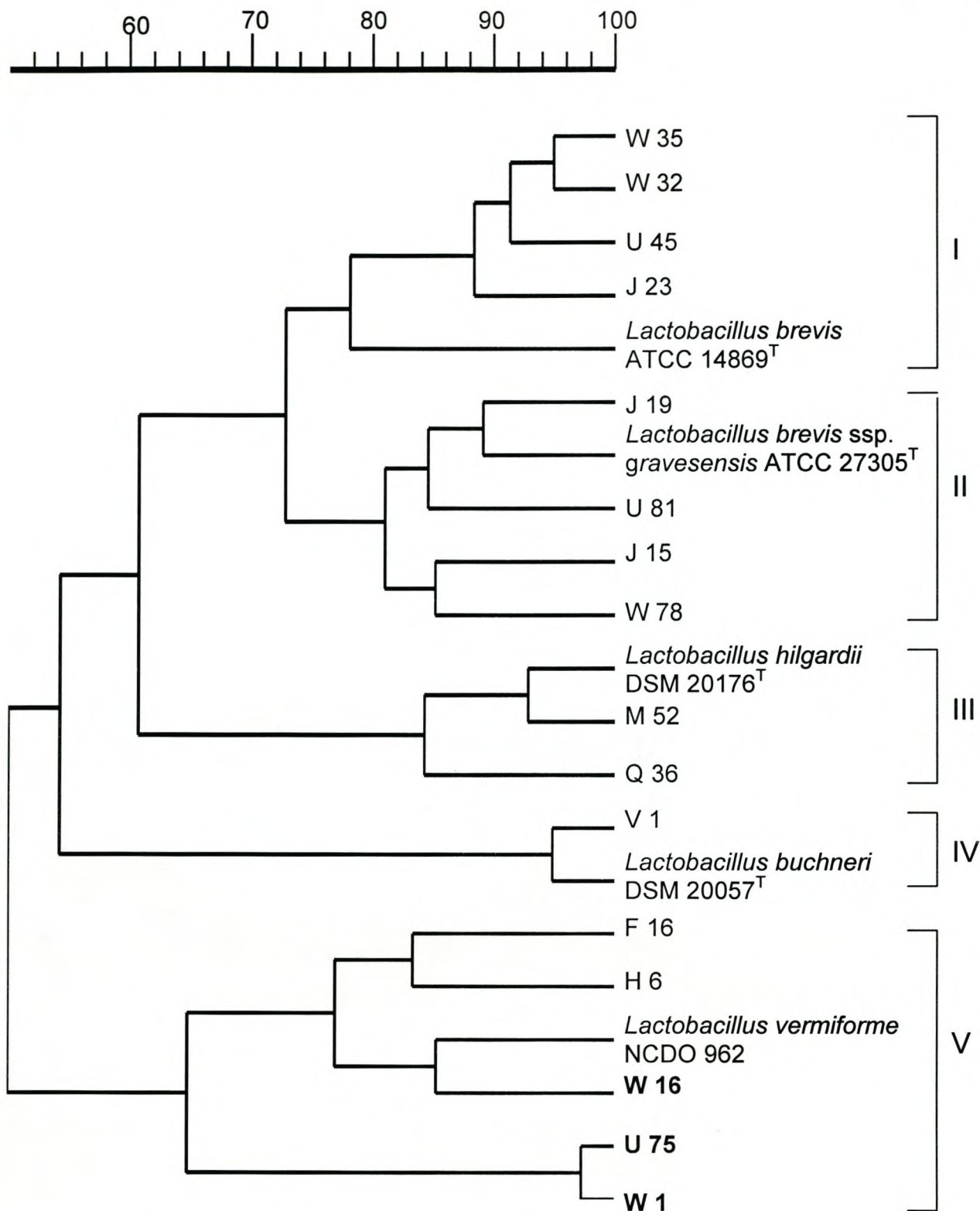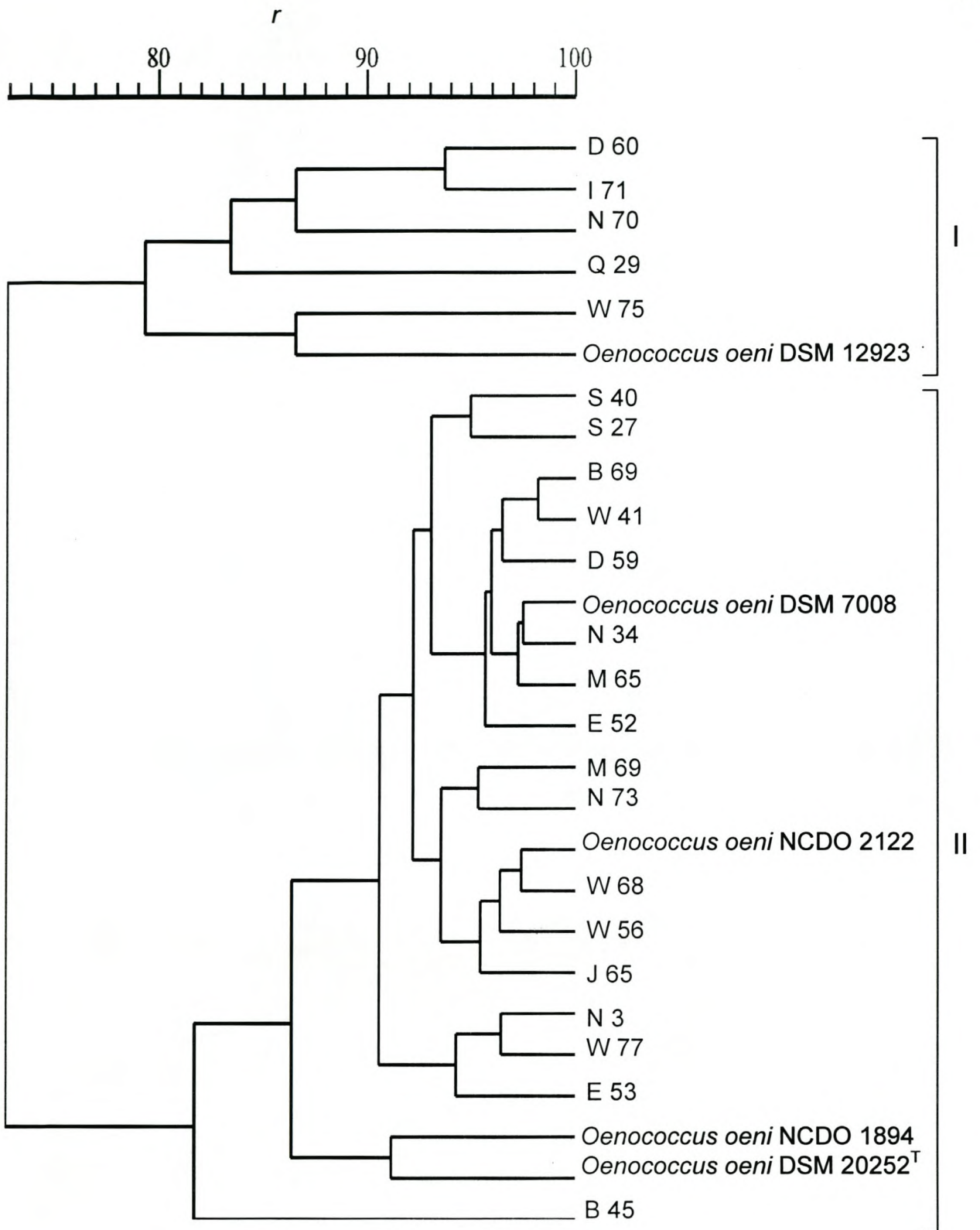
Fig. 4.2. Dendrogram showing the clustering of obligately heterofermentative *Lactobacillus* strains obtained by numerical analysis of total soluble cell protein patterns. Clustering analysis was performed by the unweighted average pair-group (UPGMA) method. Strains indicated in bold were selected for 16S rRNA sequencing.
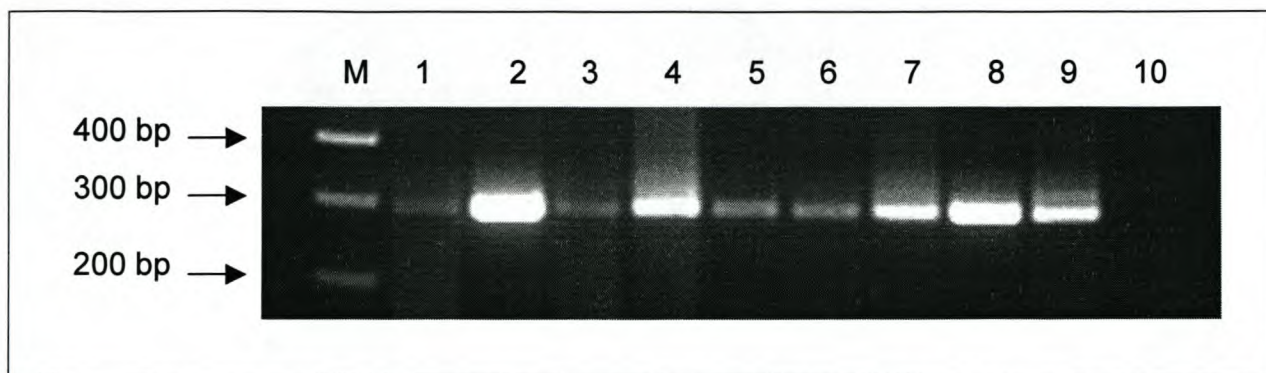
Fig. 4.3. Dendrogram showing the clustering of *Oenococcus oeni* strains obtained by numerical analysis of total soluble cell protein patterns. Clustering analysis was performed by the unweighted average pair-group (UPGMA) method.

Fig. 4.4. Agarose gel of species-specific PCR products obtained from *Lactobacillus paracasei* strains. M: DNA molecular marker XIV. Lane 1: K 11, lane 2: A 61, lane 3: W 22, lane 4: I 76, lane 5: W 34, lane 6: C 17, lane 7: J 26, lane 8: L 43, lane 9: *Lb. paracasei* DSM 5622^T and lane 10: *Lactobacillus casei* DSM 20011^T.
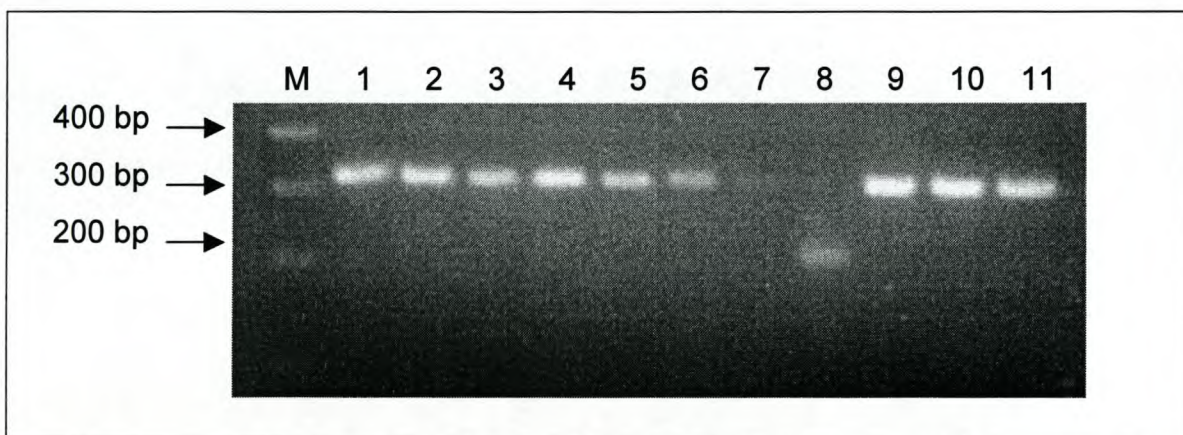

Fig. 4.5. Agarose gel of species-specific PCR products obtained from *Lactobacillus plantarum* and *Lactobacillus pentosus* strains. M: DNA molecular weight marker XIV. Lane 1: *Lb. plantarum* ATCC 14917^T, lane 2: O 70, lane 3: K 57, lane 4: O 8, lane 5: Q 18, lane 6: Q79, lane 7: P39, lane 8: *Lb. pentosus* DSM 20314^T, lane 9: K 22, lane 10: O 31 and lane 11: *Lb. plantarum* ATCC 8014.
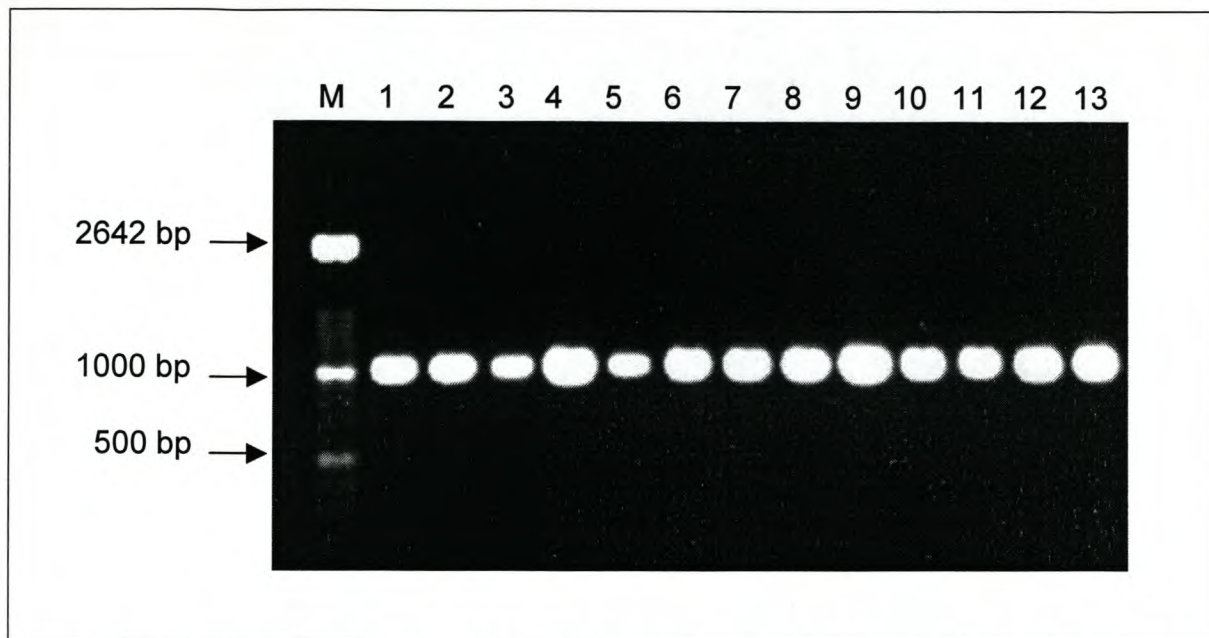
Fig. 4.6a. Agarose gel of species-specific PCR products obtained from *Oenococcus oeni* strains. M: DNA molecular weight marker XIV. Lane 1: B 45, lane 2: B 69, lane 3: D 59, lane 4: D 60, lane 5: E 52, lane 6: E 53, lane 7: I 71, lane 8: J 65, lane 9: M 65, lane 10: M 69, lane 11: N 3, lane 12: N 34 and lane 13: N 70.
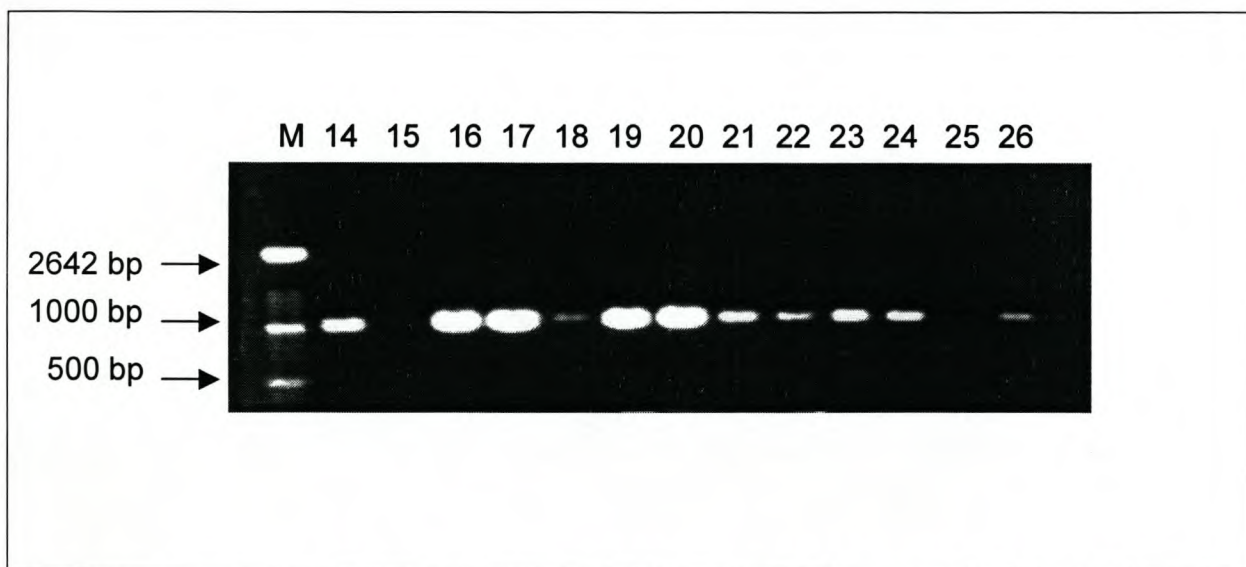

Fig. 4.6b. Agarose gel of species-specific PCR products obtained for *Oenococcus oeni*. M, DNA molecular weight marker XIV. Lane 14: *O. oeni* DSM 20252[T], lane 15: *Lactobacillus plantarum* ATCC 14917[T], lane 16: N 73, lane 17: Q29, lane 18: S 27, lane 19: S 40, lane 20: W 41, lane 21: W 56, lane 22: W 68, lane 23: W 75, lane 24: W 77; lane 25: *Lb. brevis* ATCC 14869[T] and lane 26: *Oenococcus oeni* DSM 7008.

4.4 DISCUSSION

The facultatively heterofermentative lactobacilli formed two well-separated protein profile clusters (**Fig. 4.1**). The identity of isolates identified as *Lb. paracasei* by protein profiles was confirmed by 16S rRNA sequencing and species-specific PCR primers. The results obtained confirmed previous findings (Dicks et al., 1996), i.e. that *Lb. paracasei* and *Lb. zeae* are genetically not closely related. Strains O 70 and K 57 formed a tight phenotypic cluster with the type and reference strains of *Lb. plantarum* (ATCC 14917^T and 8014). The clustering of *Lb. plantarum* and *Lb. pentosus* at r = 0.79 indicated that these species are phenotypically closely related, as reported in previous findings (Collins et al., 1991; Van Reenen and Dicks, 1996). *Lb. plantarum* and *Lb. pentosus* are genotypically closely related, as indicated by a 16S rRNA sequence similarity of > 99% (Collins et al., 1991). In cases where the species are very closely related, rRNA sequencing may not be sufficiently discriminative (Quere et al., 1997). The classifications of the isolates of cluster III (**Fig. 4.1**) as *Lb. plantarum* were also confirmed by the multiplex PCR results (**Fig. 4.5**). The results obtained with the multiplex PCR assay do not confirm the clustering of the isolates in cluster II (**Fig. 4.1**). According to the protein profile clustering and 16S rRNA sequencing, isolate K 22 belongs to *Lb. pentosus*. However, PCR results indicated that both O 31 and K 22 are actually members of *Lb. plantarum*. These strains might be very closely related to *Lb. pentosus*. *Lb. nagelii* formed a separate cluster at $r \geq 0.75$, indicating that it is phenotypically distinct from the other facultatively heterofermentative lactobacilli.

Strains of *Lb. brevis* are phenotypically (Dicks and Van Vuuren, 1987) and genetically (Farrow et al., 1986) heterogeneous. *Lb. brevis* is closely related to *Lb. buchneri* and *Lb. hilgardii*. *Lb. buchneri* is phenotypically distinguished from *Lb. brevis*, mainly by its ability to ferment melezitose (Sharpe, 1981). However, Kandler and Weiss (1986) reported that some strains identified as *Lb. brevis* could ferment melezitose. Strains of *Lb. brevis* and *Lb. hilgardii* are phenotypically similar and only differ in their ability to ferment arabinose. *Lb. brevis* ferments arabinose, whereas *Lb. hilgardii* cannot (Kandler and Weiss, 1986). Sohier et al. (1999) used fingerprinting and rDNA sequencing to classify arabinose-fermenting strains as *Lb. hilgardii*. Two distinct clusters were observed for *Lb. brevis* ATCC 14869^T and ATCC 27305^T (cluster I and II, **Fig. 4.2**) and this confirmed results obtained through DNA homology studies (Farrow et al., 1986).

Clusters V and VI show low correlation values and are well separated from the other clusters. Results obtained from 16S rRNA sequence analysis clearly show that the strains in cluster VI are members of *Lb. vermiforme*. Farrow et al. (1986) found high DNA homology (72 to 90 %) among three *Lb. vermiforme* strains and the type strain of *Lb. hilgardii* (NCDO 264^T). As a result of these findings, the species name *Lb. vermiforme* was rejected (Kandler and Weiss, 1986). Recently, *Lb. vermiforme* strains were also identified from South African fortified wines (Stratiotis and Dicks, 2002). The same authors suggested the revival of the name *Lb. vermiforme*.

The obligately heterofermentative cocci-bacilli formed two separate but related clusters (**Fig. 4.3**). The strains in cluster I grouped with an *O. oeni* strain that is used as a commercial starter culture for MLF. The strains in cluster II formed tight sub-clusters with the *O. oeni* reference strains and another MLF starter culture strain. The identity of the *O. oeni* strains was verified using the species-specific PCR reaction. *O. oeni* is phenotypically heterogeneous and can be grouped into two subgroups on the basis of pentose fermentation reactions (Garvie, 1967). The species *O. oeni* is genomically and phylogenetically homogeneous (Dicks et al., 1990). The protein pattern of the strains in cluster I differed from the strains in cluster II, indicating that *O. oeni* is a phenotypically heterogeneous species. Clustering analysis of protein patterns revealed no correlation between the distribution of strains and their geographical origin.

This study concludes that the strains isolated most frequently from the base wines were *O. oeni* and *Lb. brevis*. In many of the experimental base wine samples, *Lb. plantarum* species were the dominating species in the grape juice, but their viability decreased significantly during the alcoholic fermentation. *O. oeni* was found to be the dominating species in 15 of the 23 brandy base wine samples that had undergone spontaneous MLF. Nine of the 23 samples were commercially-produced brandy base wines. The remaining 14 samples were experimental base wines. In six of the commercial base wine samples, *O. oeni* was the dominant species. *Lb. hilgardii* and *Lb. paracasei* were identified in the three other samples. In nine of the 14 experimental base wines, *O. oeni* was responsible for the occurrence of MLF. *Lb. hilgardii* and *Lb. brevis* strains were prominent in these samples. *Lactobacillus* species were isolated at all of the different stages of experimental base wine production, but usually in low numbers. In some experimental base wine samples, *Lb. brevis* and *Lb. paracasei* developed after MLF had been completed. Isolates identified as *Lb. vermiforme* were isolated during the AF and after MLF were completed. The development of the lactobacilli in base wine samples was linked to the decrease in the quality of the base wine and the distillate. Only *O. oeni* occurred in the base wine samples preferred by the evaluation panel. No homofermentative *Lactobacillus* or *Pediococcus* species were isolated or identified in any of the base wines.

4.5 CONCLUSION

This study showed that *O. oeni* is the predominant species in South African brandy base wines. However, it is not the only species responsible for the occurrence of spontaneous MLF in South African base wines. Several *Lactobacillus* spp. were also isolated. This is the first time that *Lb. paracasei* and *Lb. vermiforme* have been reported in brandy base wine. The presence of the *Lactobacillus* spp. could be correlated to the decrease in quality of the base wine and distillate. *O. oeni* were found to have a more favourable influence on base wine and distillate quality.

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS
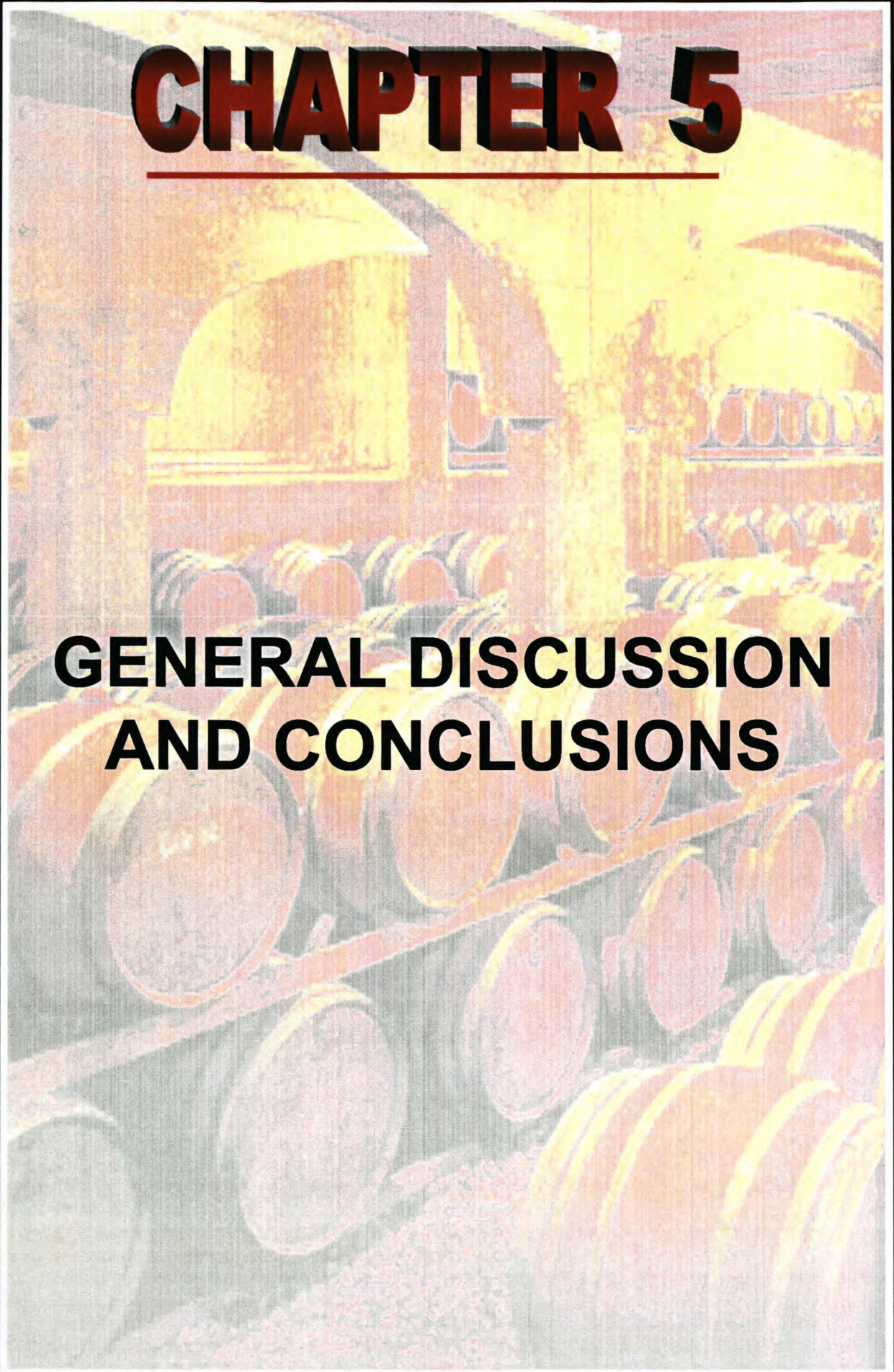
5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

Wine flavour is determined by numerous biological and physiochemical events that occur from the vineyard to the bottle, several of which may involve microbial activity. Lactic acid bacteria (LAB) exert their biggest effect on wine flavour after the completion of alcoholic fermentation (AF), specifically during and after the malolactic fermentation (MLF) (Bartowsky and Henschke 1995). Lactic acid bacteria are Gram-positive, catalase-negative cocci or rods. Species from the genera *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus* are associated with the winemaking process (Lonvaud-Funel 1999). A wine can acquire a greater complexity of harmonious flavours or, at the other extreme, is rendered unmarketable when spoilt. Various authors differ in their view of the contribution of MLF to the organoleptic quality of wine (Davis *et al.* 1985; Bartowsky and Henschke 1995). The flavour attributes imparted by MLF are, to some extent, determined by the malolactic strain. Several papers have been published which proved that different strains of LAB may increase or decrease the intensity of certain wine aroma or flavour attributes.

It is known that the ability of lactic acid bacteria to grow in wines after the alcoholic fermentation is determined by wine pH, alcohol concentration, temperature and SO_2 (Wibowo *et al.* 1985; Britz and Tracey 1990). These parameters determine the LAB population present and even which species dominates. The conditions in base wine vary from those found in normal white wine with respect to pH and the use of SO_2 (Leaute 1990). Very little or no research has been done on the LAB population in brandy base wines, especially in South African base wines. South Africa produces about 250 million litres of base wine annually. Brandy is South Africa's favourite spirit, with sales of about 45 million litres per annum. Brandy remains the backbone of the South African wine industry and a major force in the local and national economy. There are more than 4·5 million brandy drinkers in South Africa and about 50 brandy trademarks. A wide variety of brandies are found on the market, from fine estate brandies, high priced boutique brandies, to those consumed mainly with mixers (The South African Brandy Foundation).

In Chapter 1, the importance of this study was highlighted. The main aims of this study were to determine if spontaneous MLF occurred in South African brandy base wines, how it influenced the base wine and distillate quality and to identify the LAB that were responsible for the occurrence of MLF.

Chapter 3 describes the occurrence of LAB and spontaneous MLF in South African brandy base wines. This study revealed that LAB are present at significant numbers and are able to induce spontaneous MLF. Spontaneous MLF occurred in about 50% of the commercial base wines sampled. It was also shown that the incidence of MLF varied from year to year and that this could be due to the differing climatic conditions and the quality of the grapes. This study also showed that spontaneous MLF had an effect on the chemical composition of the base wine. The concentrations of certain esters, such as iso-amyl

acetate, ethyl acetate and 2-phenethyl acetate, decreased as a result of MLF. The concentrations of acetic acid, lactic acid and diethyl succinate were found to increase during MLF. These changes in the chemical composition had a significant effect on the sensory quality of the base wine and of the resultant brandy distillate. The base wines that had undergone MLF could be distinguished from the base wines that had not undergone MLF. In most of the samples evaluated, the panel members preferred the sample that had not undergone MLF. However, there were exceptions where the samples that had undergone MLF were preferred. This suggests that the specific strains performing MLF may play a role. The results obtained from the sensory evaluation varied from person to person, especially among the brandy makers from the different companies. During the sensory analysis of the distillates, it was found that the samples that had undergone MLF had a slightly lower intensity of smooth associated, herbaceous and fruity flavours. Sweet associated flavours, such as chocolate and caramel, were more intense in these samples. The negative flavours, such as solvent and chemical flavours, were also higher in MLF wines. The LAB strains, grape cultivar and the distillation technique could have influenced the aroma profile obtained for the different samples. In most of the base wine samples, the selected grape cultivar was Chenin blanc. Wines produced from this cultivar are sometimes neutral and lack a specific cultivar-associated aroma. Delicate and fruity wines do not always benefit from MLF, as could have been the case for these samples. Unfortunately the samples were not aged, so the effect of the wood and ageing on the MLF samples could not be determined. Wood maturation plays an important role in brandy production and the changes that occur during this phase have a big impact on the quality of the final product. The fact that the brandy distillate that had undergone spontaneous MLF was favoured less than the distillate that had not undergone MLF does not mean that the same tendencies will be observed with regard to the brandies. The results prove that MLF can significantly influence the quality of the base wine and distillate. The effect of MLF on the final product is not that clear. Brandy producers have different opinions about what constitutes a good brandy distillate and even a good brandy. This can be attributed to the fact that different styles of brandy are produced to meet the demands of divergent consumer tastes and preferences. Malolactic fermentation might allow the brandy makers to experiment with different brandy styles or attributes and even to improve on current styles.

Chapter 4 discusses the identification of the bacteria isolated in this study. Lactic acid bacteria were isolated at different stages of brandy production and were identified by total soluble protein patterns, 16S rRNA sequence analysis and PCR using species-specific primers. The facultatively heterofermentative LAB were identified as *Lactobacillus paracasei*, *Lb. plantarum* and *Lb. pentosus* using protein patterns. The *Lb. paracasei* isolates all clustered in one phenotypic cluster. Two distinct protein clusters were obtained for *Lb. plantarum* and *Lb. pentosus* isolates. These species are genotypically closely related and show highly similar types (Dellaglio *et al.* 1975). However, the isolates

identified as *Lb. pentosus* using protein patterns and 16S rRNA sequencing were later identified as *Lb. plantarum* with the PCR method using species-specific primers. The identification of isolate K 22 as *Lb. pentosus* through 16S rRNA sequence analysis illustrates the limitations of 16S rRNA sequencing of closely related species, as shown by Fox *et al.* (1992).

The obligately heterofermentative isolates could be separated into two distinct morphological groups. Fifteen of the obligately heterofermentative strains were rods and were identified preliminary as *Lactobacillus* strains. The remaining 22 obligately heterofermentative strains were cocci-bacilli shaped and were considered to belong to the genus *Leuconostoc* or *Oenococcus*. The heterofermentative lactobacilli were identified as *Lb. brevis, Lb. hilgardii, Lb. buchneri* and *Lb. vermiforme*. Two distinct phenotypic clusters were identified for *Lb. brevis* isolates, indicating a heterogeneous collection of strains. This study identified five strains *as Lb. vermiforme* and they were phenotypically distinct from *Lb. hilgardii* DSM 20176^T. Previously, the species name *Lb. vermiforme* was rejected because Farrow *et al.* (1986) found high DNA homology among three strains of *Lb. vermiforme* (NCDO 961, NCDO 962 and NCDO 1965) and the type strain *Lb. hilgardii* NCDO 264^T. Stratiotis and Dicks (2002) recently also identified *Lb. vermiforme* strains in South African fortified wine. The taxonomic position of *Lb. vermiforme* should be elucidated. The *Lactobacillus* isolates were isolated mainly from the juice, during the AF and after MLF were completed. The occurrence of the *Lactobacillus* strains after the MLF might explain the off-flavours in some of the base wines and distillates.

Twenty-two obligately heterofermentative isolates were identified as *Oenococcus oeni*. The *O. oeni* strains formed two distinct protein clusters, with phenotypic differences among the strains. *O. oeni* was the species isolated most frequently and was also responsible for the occurrence of spontaneous MLF in most of the base wines. No correlation could be made between the geographical origin and the clustering of strains.

Spontaneous MLF might have a negative influence on the quality of base wine, therefore the growth of LAB should be controlled by storing base wines at low temperatures (10°C) after the fermentation is completed, or by adding lysozyme at the beginning of or after the alcoholic fermentation. The distillation of the base wines as soon as the alcoholic fermentation is completed will eliminate the occurrence of spontaneous MLF.

This study concludes that spontaneous MLF does occur in brandy base wines and that it has an influence on the composition and the quality of the base wine and the resulting distillate. Base wines and distillates that had undergone spontaneous MLF differed significantly from base wines and distillates that had not undergone MLF. It was found that *O. oeni* was the predominant species, but *Lb. brevis, Lb. buchneri, Lb. hilgardii, Lb. paracasei, Lb. plantarum* and *Lb. vermiforme* were also present in the base wines. It was found further that *O. oeni* had a beneficial effect and that the *Lactobacillus* spp. had a detrimental effect on the quality of the base wine and distillate.

Future work should concentrate more on commercial-scale fermentations and on the influence of MLF in brandy base wines in which commercial starter cultures conduct the MLF. The information generated from such experiments would then clearly indicate the most beneficial treatment to ensure the quality of brandy base wine. The changes that occur during wood maturation should also be investigated before any definite conclusions can be drawn on the effect of MLF on brandy quality or style.

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