An investigation into lactic acid bacteria as a possible cause of bitterness in wine

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.

20 Februarie 2003

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SUMMARY

Spoilage, be it due to microbial actions, chemical reactions or both, poses a serious threat to the food and beverage industries. Not only can spoilage lead to great economic losses, but it can also cause industries to lose their competitive edge in the economic and consumer market. Considering all the modern technologies and the range of preservation techniques that are available, it is surprising that spoilage is still an economic problem. Wine spoilage due to unpalatable bitterness, and the role of lactic acid bacteria (LAB) in causing this bitterness, have received much attention over the years, but no definite understanding has yet emerged.

The first objective of this study was to isolate, enumerate and identify the LAB from three red grape varieties, viz. Pinotage, Merlot and Cabernet Sauvignon. The LAB populations on the grapes of all three varieties ranged from 10^2 to 10^4 cfu/ml during the 2001 and 2002 harvest seasons. The Cabernet Sauvignon grapes had slightly higher numbers than the Pinotage and Merlot. The LAB population in the Cabernet Sauvignon, Pinotage and Merlot wines after completion of the alcoholic fermentation ranged from 10^2 to 10^5 cfu/ml, while during 2002 the numbers in wine undergoing malolactic fermentation (MLF) ranged from 10^4 to 10^8 cfu/ml. The isolated LAB were divided into the three metabolic groups, with 59% belonging to the facultatively heterofermentative group, 26% to the obligately heterofermentative group and 15% to the obligately homofermentative group. The isolates were identified by means of species-specific primers as *Leuconostoc mesenteroides* (4), *Oenococcus oeni* (28), *Lactobacillus brevis* (15), *Lb. hilgardii* (15), *Lb. plantarum* (98), *Lb. pentosus* (12), *Lb. paraplantarum* (3), *Lb. paracasei* (28), *Pediococcus acidilactici* (2) and *Pediococcus* spp. (35). The most predominant species isolated was *Lb. plantarum*, followed by *Pediococcus* spp. The results suggest that Pinotage carries a more diverse LAB population in comparison to Merlot and Cabernet Sauvignon.

The second objective of this study was to determine the presence of the glycerol dehydratase gene in the LAB strains by using the GD1 and GD2 primers. Twenty-six strains tested positive, namely *Lb. plantarum* (15), *Lb. pentosus* (1), *Lb. hilgardii* (5), *Lb. paracasei* (2), *Lb. brevis* (2) and a *Pediococcus* spp. (1). Interestingly, 62% of these strains were isolated from Pinotage. The strains all had the ability to degrade glycerol by more than 90%, and no significant differences were observed between the species. The GD-possessing strains exhibited varying degrees of inhibition towards Gram-positive and Gram-negative bacteria, and the results suggest that this inhibition activity may be similar to that of reuterin, which is produced by *Lb. reuteri*.

This study can form the foundation for unravelling the causes of bitterness in red wines. Combining the results of this study with analytical, sensory and molecular data may very well provide the industry with valuable tools with which to combat the occurrence of bitterness.

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OPSOMMING

Bederf as gevolg van mikrobiese aksies, chemiese reaksies of beide, hou 'n groot bedreiging vir die voedsel- en drankbedrywe in. Nie net kan bederf lei tot groot ekonomiese verliese nie, maar dit kan ook veroorsaak dat bedrywe hul kompeterende voordeel in die ekonomiese en verbruikersmarkte verloor. As die moderne tegnologie en die reeks preserveringstegnieke wat beskikbaar is, in ag geneem word, is dit verbasend dat bederf steeds 'n ekonomiese probleem is. Wynbederf as gevolg van oormatige bitterheid en die rol van melksuurbakterieë (MSB) in die ontwikkeling van hierdie bitterheid het oor die jare heen baie aandag geniet, maar geen definitiewe verklaring is nog daarvoor gevind nie.

Die eerste doelwit van hierdie studie was om MSB vanaf drie rooidruifvariëteite, nl. Pinotage, Merlot en Cabernet Sauvignon, te isoleer, te kwantifiseer en te identifiseer. Die MSB-populasies op die druiwe van al drie variëteite het gedurende die 2001- en 2002-parsseisoene tussen 10^2 en 10^4 kvu/ml gevarieer. Die Cabernet Sauvignon-druiwe het effens hoër getalle as die Pinotage- en Merlot-druiwe gehad. Die MSB-populasies in die Cabernet Sauvignon-, Pinotage- en Merlot-wyne aan die einde van die alkoholiese fermentasie het tussen 10^2 en 105^5 kvu/ml gevarieer. Gedurende 2002 het die MSB-getalle in die wyne waarin appelmelksuurgisting (AMG) aan die gang was tussen 10^4 en 10^8 kvu/ml gevarieer. Die geïsoleerde MSB was onderverdeel in die drie metaboliese groepe, met 59% wat behoort aan die fakultatiewe, heterofermentatiewe groep, 26% aan die obligate, heterofermentatiewe groep en 15% aan die obligate, homofermentatiewe groep. Die isolate is geïdentifiseer as *Leuconostoc mesenteroides* (4), *Oenococcus oeni* (28), *Lactobacillus brevis* (15), *Lactobacillus hilgardii* (15, *Lactobacillus plantarum* (98), *Lactobacillus pentosus* (12), *Lactobacillus paraplantarum* (3), *Lactobacillus paracasei* (28), *Pediococcus acidilactici* (2) en *Pediococcus* spp. (35) deur middel van spesie-spesifieke inleiers. Die mees algemeen geïsoleerde spesies was *Lb. plantarum*, gevolg deur *Pediococcus* spp. Die resultate impliseer dat Pinotage 'n meer uiteenlopende MSB-populasie in vergelyking met Merlot en Cabernet Sauvignon dra.

Die tweede doelwit van hierdie studie was om die teenwoordigheid van die gliseroldehidratase-geen in die MSB-isolate deur middel van die GD1- en GD2-inleiers te bepaal. Ses-en-twintig isolate was positief, nl. *Lb. plantarum* (15), *Lb. pentosus* (1), *Lb. hilgardii* (5), *Lb. paracasei* (2), *Lb. brevis* (2) en 'n *Pediococcus* spp. (1). 'n Interessante resultaat was dat 62% van hierdie isolate vanaf Pinotage geïsoleer is. Die isolate was almal in staat om meer as 90% van die gliserol te gebruik en geen noemenswaardige verskille is tussen die isolate waargeneem nie. Die GD-bevattende isolate het verskillende grade van inhibisie teenoor Gram-positiewe en Gram-negatiewe bakterieë getoon, en die resultate impliseer dat hierdie inhiberende aktiwiteit dieselfde is as dié van reuterin wat deur *Lb. reuteri* geproduseer word.

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Hierdie studie kan dus die basis vorm vir die ontrafeling van die oorsake van bitterheid in rooiwyne. Deur die resultate van hierdie studie met analitiese, sensoriese en molekulêre data te kombineer, kan die wynbedryf voorsien word van waardevolle metodes om die voorkoms van bitterheid mee te bekamp.

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This thesis is dedicated to my mother, Hedy Krieling, and my grandmother, Agnes Krieling.

Hierdie tesis is aan my moeder, Hedy Krieling, en my ouma, Agnes Krieling, opgedra.

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BIOGRAPHICAL SKETCH

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal *International Journal of Food Microbiology*, to which Chapter 3 will be submitted for publication.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**
Bitterness in Foods and Beverages

Chapter 3 **RESEARCH RESULTS**
Isolation, identification and characterisation of glycerol-degrading lactic acid bacteria from South African red wines

Chapter 4 **GENERAL DISCUSSION AND CONCLUSIONS**

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CONTENTS

CHAPTER 1. GENERAL INTRODUCTION AND PROJECT AIMS 1

1.1 GENERAL INTRODUCTION 1

1.2 PROJECT AIMS 3

1.3 LITERATURE CITED 3

CHAPTER 2. LITERATURE REVIEW: BITTERNESS IN FOODS AND BEVERAGES 5

2.1 INTRODUCTION 5

2.2 PHYSIOLOGICAL ASPECTS OF BITTERNESS 6

2.3 BITTER COMPOUNDS 8

2.3.1 Amino acids and peptides 9
2.3.2 Phenols and polyphenols 9
2.3.3 Methylxanthines 12
2.3.4 Acrolein 12

2.4 BITTERNESS IN FOODS 15

2.4.1 Fruits 16
2.4.1.1 Citrus 16
2.4.2 Diary products 17
2.4.2.1 Cheese 17

2.5 BITTERNESS IN BEVERAGES 19

2.5.1 Wine 19
2.5.2 Apple ciders 20
2.5.3 Tea 21
2.5.4 Coffee 22
2.5.5 Beer 22

2.6 METHODS TO REMOVE BITTERNESS 23

2.6.1 Debittering of citrus juice by biological means 23
2.6.2 Removal of bitterness from citrus products by adsorption techniques 24
2.6.3 Decaffeination of coffee 24
2.6.3.1.1 Traditional or European process 24
2.6.3.1.2 Swiss water process 24

2.7 CONCLUSION 25

2.8 LITERATURE CITED 26

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 3. RESEARCH RESULTS 30

Isolation, identification and characterisation of glycerol-degrading lactic acid bacteria from South African red wines

3.1 INTRODUCTION 31

3.2 MATERIALS AND METHODS 33

3.2.1 Collection of samples 33
3.2.2 Isolation and enumeration of lactic acid bacteria 33
3.2.3 Preliminary identification of lactic acid bacteria 33
3.2.4 Identification of the bacteria to species level 34
3.2.5 Screening of the isolates for the presence of the glycerol dehydratase enzyme 34
3.2.6 Isolation of genomic DNA 34
3.2.7 DNA sequence analysis 35
3.2.8 Glycerol metabolism 35
3.2.9 Preliminary screening of the GD-possessing isolates for antimicrobial acitivity 36

3.3 RESULTS 37

3.3.1 Enumeration of LAB 37
3.3.2 Preliminary identification 37
3.3.3 Identification to species level 37
3.3.4 Screening of the isolates for the presence of the glycerol dehydratase enzyme 44
3.3.5 Glycerol metabolism 46
3.3.6 Preliminary antimicrobial activity 47

3.4 DISCUSSION 49

3.5 CONCLUSION 51

3.6 ACKNOWLEDGEMENTS 51

3.7 LITERATURE CITED 51

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS 54

4.1 GENERAL DISCUSSION AND CONCLUDING REMARKS 54

4.2 LITERATURE CITED 56

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The wine industry faces the challenge of competing in a market that is becoming increasingly competitive and with consumers who are increasingly health and environmentally conscious (Juriaanse, 1999; Bisson et al., 2002; Pretorius and Bauer, 2002). Producers are thus faced with the challenge of delivering high quality products to ensure consumer satisfaction and financial gain. Quality in this sense includes the flavour and aroma profile, and also relies on consumer preference (Bisson et al., 2002; Pretorius and Bauer, 2002). Wine spoilage can therefore cause the industry to lose its competitive edge and lead to huge economical losses (Loureiro, 2000). Regardless of this, and despite modern technology and the range of preservation techniques that are available, spoilage is still a problem that is not controlled adequately. Most often, spoilage is the result of microbial activity, chemical reactions or combinations of the two (Huis in't Veld, 1996; Loureiro, 2000). Over the years, the various types of spoilage mechanisms with regard to winemaking have been well documented and characterised yet, "grey areas" with regard to certain types of spoilage still exist. Unpalatable bitterness in wine and the role of lactic acid bacteria (LAB) in causing this bitterness is one such area.

LAB form part of the natural microflora found on grapes and their ability to grow in grape juice and wine is well documented (Davis et al., 1985; Wibowo et al., 1985; Lonvaud-Funel, 1999; Du Toit and Pretorius, 2000). Like many other microorganisms, LAB can be either beneficial or detrimental to the quality of wine, depending on the circumstances. Several species belonging to the four commonly found LAB genera in wine, namely *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus,* have been isolated from wine and are known to influence the sensory and also the hygienic quality of wine (Lonvaud-Funel, 1999; Du Toit and Pretorius, 2000). Sensory faults caused by LAB include off-flavours, such as mousiness, ester taint, phenolic, vinegary, buttery and geranium tone, and bitterness. The production of biogenic amines and the precursors of ethyl carbamate does not spoil the sensory profile of wine, but pose health risks for the consumer by affecting the hygienic quality or wholesomeness of the wine (Lonvaud-Funel, 1999; Du Toit and Pretorius, 2000).

Glycerol, one of the major products of yeast metabolism during the alcoholic fermentation of wine, can be metabolised by certain LAB. When grapes are infected with *Botrytis cinerea*, significant amounts of glycerol can also be found in the grape must before fermentation (Nieuwoudt et al., 2002). The degradation of glycerol will not only lead to a change in the sensorial quality of the wine, but it may also lead to the formation of acrolein (Rentschler and Tanner, 1951; Schütz and Radler, 1984a; Axelsson et al., 1989; Scanes et al., 1998; Claisse and Lonvaud-Funel, 2000; Sauvageot et al., 2000). Rentschler and Tanner (1951) showed that acrolein can

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combine with the phenolic groups of tannins to form a bitter complex. Acrolein is not a direct product of glycerol metabolism, but occurs in chemical equilibrium with 3-hydroxypropionaldehyde (3-HPA) in an aqueous solution, with a shift towards acrolein in conditions of acid and heat (Pressman and Lucas, 1942; Slininger et al., 1983; Claisse and Lonvaud-Funel, 2000; Du Toit and Pretorius, 2000; Sauvageot et al., 2000).

Sobolov and Smiley reported the anaerobic metabolism of glycerol by *Lactobacillus* species as early as 1959. Since then, the enzyme, glycerol dehydratase (GD), of two species, i.e. *Lactobacillus reuteri* and *Lactobacillus collinoides*, has been purified and characterised. Other species able to degrade glycerol include *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans* (Kandler, 1983; Schütz and Radler, 1984a, b; Davis et al., 1986; Talarico and Dobrogosz, 1990; Sponholz, 1993; Du Toit and Pretorius, 2000; Gorga et al., 2002, Sauvageot et al., 2002). These LAB all lack the oxidative pathway for glycerol degradation and therefore cannot use glycerol as a sole carbon source, but only as an external electron acceptor. The coenzyme B_{12}-dependent GD converts the glycerol to 3-HPA via the reductive branch. This 3-HPA is subsequently reduced to 1,3-propanediol (1,3-PDL), with 1,3-PDL:NAD oxidoreductase as the catalyst (Lüthi-Peng et al., 2002; Sauvageot et al., 2002). Talarico and Dobrogosz (1989) found that, unlike the other species, *Lb. reuteri* had the ability to accumulate 3-HPA and excrete it into the fermentation medium. This equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA has potent antimicrobial activities and was termed "reuterin". Several studies have shown that the co-fermentation of glucose with glycerol leads to better growth of the above-mentioned lactobacilli. It was also found that both glucose and lactate have a stimulating effect on glycerol metabolism. Accumulation of 3-HPA was favoured at a molar ratio of glucose to glycerol of 0.33 and, at a ratio of glucose to glycerol greater than 1.6, the main product of glycerol degradation is 1,3-PDL in *Lb. reuteri* (Talarico et al., 1990; Veiga da Cunha and Foster, 1992; El-Ziney et al., 1998; Lüthi-Peng et al., 2002).

Since the enzyme GD is strain dependent, it is important to determine what percentage of strains occurring in wine fermentations possess this enzyme. Several studies have been done on cider and one specific LAB species has been isolated in all these studies, namely *Lb. collinoides* (Carr and Davies, 1972; Claisse and Lonvaud-Funel, 2000; Sauvageot et al., 2000). Primers and a DNA probe have been developed to detect the presence of glycerol-degrading LAB in ciders (Claisse and Lonvaud-Funel, 2001). To date, no studies have been conducted specifically on wine-isolated LAB to determine the presence of this GD enzyme and the influence of wine conditions on the activity of the enzyme. In order to design adequate strategies to prevent bitterness in wine, it is necessary to first identify the indigenous LAB associated with grapes and wine to understand the underlying mechanisms of the

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specific bacteria involved and their interactions with other microorganisms (yeasts) or in chemical reactions.

1.2 PROJECT AIMS

This study forms part of a research project funded by WINETECH to unravel the causes of bitterness in wine. The specific focus of this study was on the role played by LAB in the bitterness of wine. To understand the underlying mechanisms of this specific spoilage, it is advantageous to know the identity of the organisms usually associated with the raw material (the grapes) and the final product (the wine).
The specific aims of this study were:

1. to isolate LAB from three red grape varieties, namely Pinotage, Merlot and Cabernet Sauvignon, from five different wineries in South Africa;
2. to isolate LAB from the same varieties at different stages of the fermentation process;
3. to identify the isolated LAB by selected biochemical tests and species-specific primers;
4. to screen the isolated LAB for the presence of the glycerol dehydratase enzyme with PCR;
5. to evaluate GD-possessing isolates for their ability to metabolise glycerol; and
6. to screen these isolates for possible antimicrobial activity.

1.3 LITERATURE CITED

Axelsson, L.T., Chung, T.C., Dobrogosz, W.J., Lindgren, L.S., 1989. Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. Microb. Ecol.Health D. 2, 131-136.

Bisson, L.F., Waterhouse, A.L., Ebeler, S.E., Walker, M.A., Lapsley, J.T., 2002. The present and future of the international wine industry. Nature 418, 696-699.

Carr, J.G., Davies, P.A., 1972. The ecology and classification of strains of *Lactobacillus collinoides* nov. spec. : a bacterium commonly found in fermenting apple juice. J. Appl. Bacteriol. 35, 463-471.

Claisse, O., Lonvaud-Funel, A., 2000. Assimilation of glycerol by a strain of *Lactobacillus collinoides* isolated from cider. Food Microbiol. 17, 513-519.

Claisse, O., Lonvaud-Funel, A., 2001. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. J. Food Protect. 64, 833-837.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H., Fleet, G.H., 1985. Practical implications of malolactic fermentation: A review. Am. J. Enol. Vitic. 36, 290-301.

Davis, C.R., Wibowo, D.J., Lee, T.H., Fleet, G.H., 1986. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. Appl. Environ. Microbiol. 51, 539-545.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, 74-96.

El-Ziney, M.G., Arneborg, N., Uyttendaele, M., Debevere, J., Jakobsen, M., 1998. Characterisation of growth and metabolite production of *Lactobacillus reuteri* during glucose/glycerol cofermentation in batch and continuous cultures. Biotech. Lett. 20, 913-916.

Stellenbosch University http://scholar.sun.ac.za

Gorga, A., Claisse, O., Lonvaud-Funel, A., 2002. Organisation of the encoding glycerol dehydratase of *Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans*. Sci. Aliments. 22, 151-160.

Huis in't Veld, J.H.J., 1996. Microbial and biochemical spoilage of foods: an overview. Int. J. Food Microbiol. 33, 1-18.

Juriaanse, A.C., 1999. Changing pace in food science and technology: Examples from dairy science show how descriptive knowledge can be transferred into predictive knowledge. Trends Food Sci. Tech. 10, 303-306.

Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria [Fermentation, food technology]. Antonie v. Leeuwenhoek 49, 209-224.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76, 317-331.

Loureiro, V., 2000. Spoilage yeasts in foods and beverages: Characterisation and ecology for improved diagnosis and control. Food Res. Int. 33, 247-256.

Lüthi-Peng, Q., Dileme, F.B., Puhan, Z., 2002. Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri*. Appl. Microbiol. Biotech. 59, 289-296.

Nieuwoudt, H.H., Prior, B.A., Pretorius, I.S., Bauer, F.F., 2002. Glycerol in South African table wines: An assessment of its relationship to wine quality. S. Afr. J. Enol. Vitic. 23, 22-30.

Pressman, D., Lucas, H.J., 1942. Hydration of unsaturated compounds. XI. Acrolein and acrylic acid. J. Am. Chem. Soc. 64, 1953-1957.

Pretorius, I.S., Bauer, F.F., 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. 20, 426-432.

Rentschler, H., Tanner, H., 1951. Das Bitterwerden der Rotweine. Mitt. Lebensm. Unters. Hygiene 42, 463-475.

Sauvageot, N., Muller, C., Hartke, A., Auffray, Y., Laplace, J., 2002. Characterisation of the diol dehydratase *pdu* operon of *Lactobacillus collinoides*. FEMS Microbiol. Lett. 209, 69-74.

Sauvageot, N., Gouffi, K., Laplace, J.M., Auffray, Y., 2000. Glycerol metabolism in *Lactobacillus collinoides*: production of 3-hydroxypropionaldehyde, a precursor of acrolein. Int. J. Food Microbiol. 55, 167-170.

Scanes, K.T., Hohmann, S., Prior, B.A., 1998. Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. S. Afr. J. Enol. Vitic. 19, 17-24.

Schütz, H., Radler, F., 1984a. Anaerobic reduction of glycerol to propanediol-1,3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. System. Appl. Microbiol. 5, 169-178.

Schütz, H., Radler, F., 1984b. Propanediol-1,2-dehydratase and metabolism of glycerol of *Lactobacillus brevis*. Arch. Microbiol. 139, 366-370.

Slininger, P.J., Bothast, R., Smiley, K., 1983. Production of 3-hydroxypropionaldehyde from glycerol. Appl. Environ. Microbiol. 46, 62-67.

Sobolov, M., Smiley, K.L., 1959. Metabolism of glycerol by an acrolein-forming *Lactobacillus*. J. Bacteriol. 79, 261-266.

Sponholz, W.R. 1993. Wine spoilage by microorganisms. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology, Harwood Academic Publishers, Chur, pp. 395-420.

Talarico, T.L., Dobrogosz, W.J., 1989. Chemical characterisation of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. 33, 674-679.

Talarico, T.L., Axelsson, L.T., Novotny, J., Fiuzat, M., Dobrogosz, W.J., 1990. Utilization of glycerol as a hydrogen acceptor by *Lactobacillus reuteri*: purification of 1,3-propanediol: NAD^+ oxidoreductase. Appl. Environ. Microbiol. 56, 943-948.

Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterisation of glycerol deyhydratase from *Lactobacillus reuteri*. Appl. Environ. Microbiol. 56, 1195-1197.

Veiga da Cunha, M., Foster, M.A., 1992. Sugar-glycerol cofermentations in lactobacilli: The fate of lactate. J. Bacteriol. 174, 1013-1019.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.L., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.

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CHAPTER 2

LITERATURE REVIEW

Bitterness in foods and beverages

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2. LITERATURE REVIEW

2.1 INTRODUCTION

When choosing foods and beverages, people are governed predominantly by the taste of the chosen product. Sweet-tasting, as opposed to bitter-tasting foods and beverages are generally preferred by the consumer. Bitterness, however, is not always a negative attribute of foods and beverages and, in some foodstuffs, a certain degree of bitterness is required. Coffee, tea, beer and red wine, for instance, rely on a limited degree of bitterness to help balance their flavour profile. Other products, such as tonics or grapefruit juice, are considered unacceptable if they do not exhibit distinguishable bitterness. Drewnowski (2001) stated that interactions among mixtures of sweet, bitter, and sour tastes, and between taste and volatile flavour elements, add to the complexity and to the enjoyment of tea, coffee, chocolate, fruit juice, and other beverages. However, the occurrence of excessive bitterness in some food products can cause sensory defects and cause that product to be unacceptable for the consumer market.

A number of studies have shown that the ability to detect bitterness varies widely within the general population. Certain individuals can detect bitterness only at very high concentrations, while others are extremely sensitive to even minute traces of bitterness. The aversion to bitterness may be the result of a survival trait in early man. Abnormal bitterness was often equated with danger, since many alkaloids and toxins are very bitter (Maga, 1990). The ability to perceive bitterness can also be an inheritable trait (Bartoshuk, 1994).

Bitterness can be expressed in many ways and the compounds that are responsible for bitterness are extremely varied. The wide range of bitter-tasting compounds include, amongst others, amino acids, peptides, phenols, polyphenolic compounds, methylxanthines and acrolein. The ability to predict bitterness from chemical composition remains rather limited, except for a few structural generalisations that have been made (Rouseff, 1990; Drewnowski, 2001).

A number of diverse procedures exist to remove bitterness from or reduce bitterness in foods and beverages. Solid foods pose a more difficult task for the removal of bitterness than liquid foods, since most procedures involve some form of liquid-solid extraction.

This section will cover the broader aspects of bitterness, reflecting briefly on the physiological aspects of bitterness, compounds eliciting bitterness in selected foods and beverages, and techniques for removing bitterness from certain foods and beverages.

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2.2 PHYSIOLOGICAL ASPECTS OF BITTERNESS

Bitterness is one of the four primary tastes, the others being sweet, sour and salty. The first step in taste perception occurs at the apical end of taste receptor cells that are found in the taste buds in the mouth. Each taste bud is composed of 50 to 100 cells and is found in the papillae on the tongue, as well as on the palate and on the wall of the throat. Three kinds of taste papillae – circumvallate, fungiform and foliate – can be distinguished on the basis of morphology and location (**Fig. 2.1**) (Bakker, 1998; Hoon et al., 1999; Montmayeur and Matsunami, 2002).

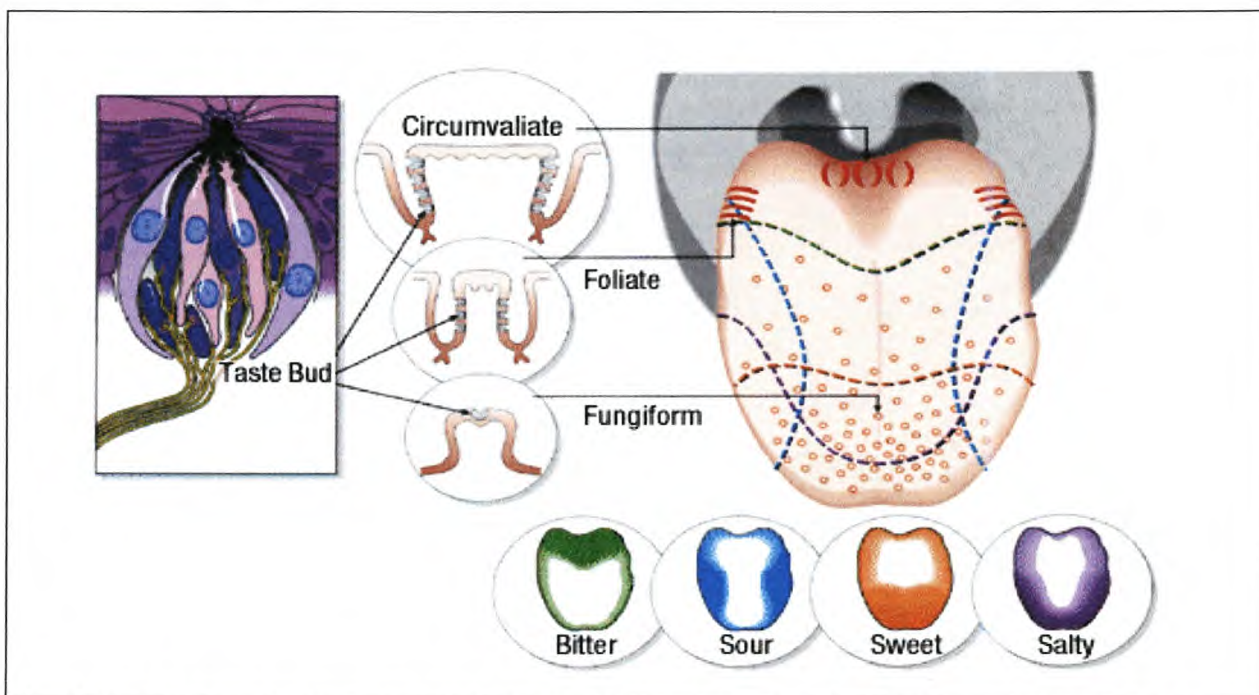

Fig. 2.1. Diagram of a human tongue, highlighting the regional preferences for sweet, sour, bitter and salty stimuli. Note that, while different areas of the tongue display strong preferences for certain taste modalities, there is significant overlap between the various regions. Also shown, in expanded scale, are the three different types of taste papillae and their corresponding topographic distribution (Adapted from Hoon et al., 1999)

An in-depth understanding of taste transduction has emerged in the past three years and this has helped to clarify the molecular processes for bitterness (Brand, 2000). Adler et al. (2000) and Matsunami et al. (2000) reported the identification of a large family (40 to 80 members) of G-protein-linked receptors that is expressed exclusively in taste receptor cells and is co-expressed with the taste cell G-protein, $G\alpha_{gust}$. It was also shown that these receptors recognise and respond to bitter stimuli (Chandrashekar et al., 2000). Bitterness perception via a receptor-mediated second messenger system and the linked cellular transduction sequence has been characterised (**Fig. 2.2**) and this information provides intracellular molecular targets to modify bitterness.

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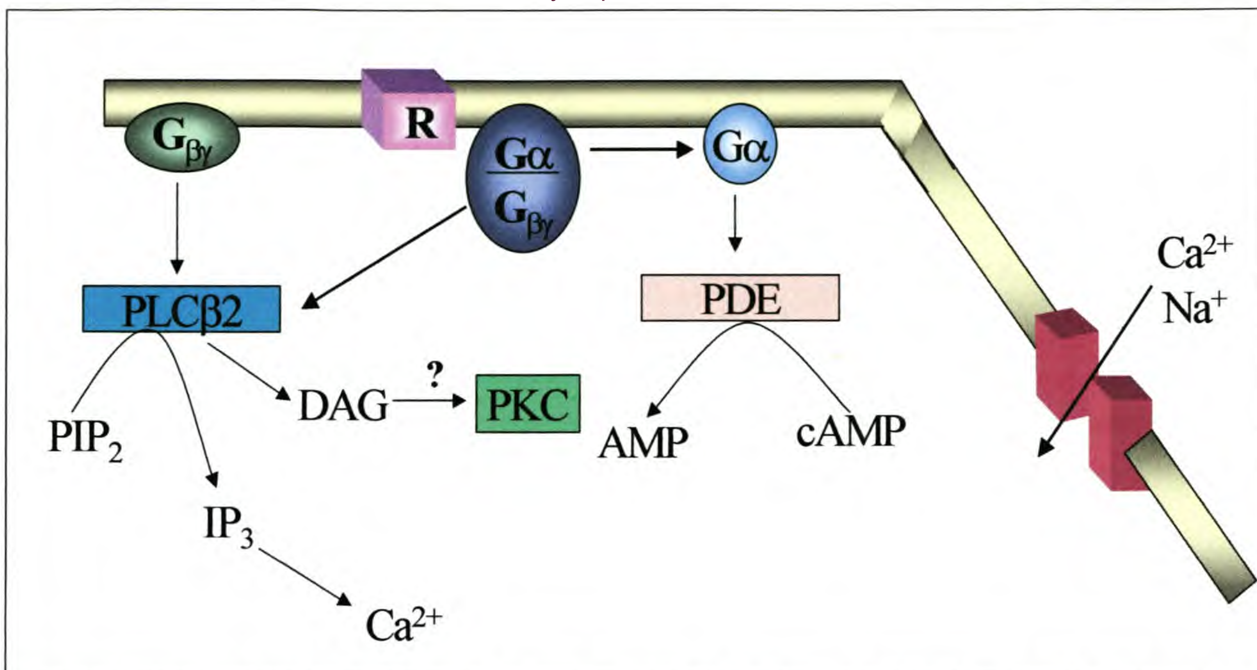

Fig.2.2. Pathways for bitter taste. Binding of a bitter stimulus to a receptor (R) activates a G protein, which then degrades into two subunits. The G_α (gustducin) subunit stimulates a phosphodiesterase (PDE) to break down cyclic nucleotides (eg. cAMP). This decrease in cyclic nucleotide levels may activate a cyclic nucleotide inhibitable channel, leading to an increase in intracellular positive charge. The Gβγ subunit activates a phospholipase Cβ2 (PLCβ2) enzyme, which metabolises phosphatidylinositol 4,5-bisphosphate (PIP_2) into the two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG may activate kinases (PKC), whereas IP_3 releases calcium from intracellular storage depots. Cellular depolarisation and the release of neurotransmitter result in the bitter message being carried to the central nervous system (Brand, 2000).

The ability to perceive bitter taste and the sensitivity towards it vary greatly across individuals and, in some cases, it can be an inheritable trait (Bartoshuk, 1994). Some people perceive very little bitter taste, others are considered "supertasters", with an acute sensation to bitter taste, and there are also those who have damaged chemosensory organs that hamper their ability to taste bitter substances (Drewnowski and Rock, 1995). The detection thresholds for bitter tastes are extremely low and bitter compounds, including extremely toxic poisons, are detected by humans in micromolar amounts. Bitter quinine, for example, was detected at concentrations of 25 µmol/l, while sucrose had a detection threshold of 10 000 µmol/l (Glendenning, 1994; Drewnowski and Gomez-Carneros, 2000). The perception of bitter intensity declines as people grow older, and therefore age may influence the liking for bitter tasting foods and beverages (Drewnowski, 2001).

For the food and beverage industries, it is important to be able to measure taste intensity. In most cases, the main measurement method is the human sensory test. Taking all the above-mentioned factors that influence taste perception in humans into consideration, it is understandable that a need for taste-sensing devices has arisen. A multi-channel taste sensor (i.e. electronic tongue), whose transducer is composed of an array of lipid/polymer membranes of different characteristics, has been developed (**Fig. 2.3**) (Hayashi et al., 1990; Toko, 1998). The output of this taste-

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sensing system is not the amount of specific taste substances, but the taste quality and intensity. This is based on the fact that different electric output patterns are obtained for chemical substances producing different taste qualities, such as sourness and saltiness. The taste-sensing system could quantify the taste of beer, mineral water, coffee and milk (Toko, 2000, Takagi et al., 2001). Toko (2000) used the taste sensor together with an electronic nose to study wine flavour. They found that, by using both systems, they could effectively discriminate between four wines (two red and two white), and that they could also discriminate between differently aged samples of the same red wine. In another study, the suppression effect of phospholipids, such as phosphatidic acid, on bitterness was evaluated by means of the electronic tongue. The responses to quinine hydrochloride and L-tryptophan, which have a bitter taste, were measured in a phospholipid cocktail containing various kinds of taste substances. As the phospholipid concentration increased, the responses to the quinine hydrochloride and L-tryptophan decreased, while the responses to the other substances were not affected. The results obtained by using the electronic tongue were similar to that of the human sensory test (Takagi et al., 2001). The electronic tongue or taste-sensing system may prove to be a new, automated method to measure the intensity of bitterness and replace the human sensory test. It may also contribute to the clarification of the mechanism of taste perception.

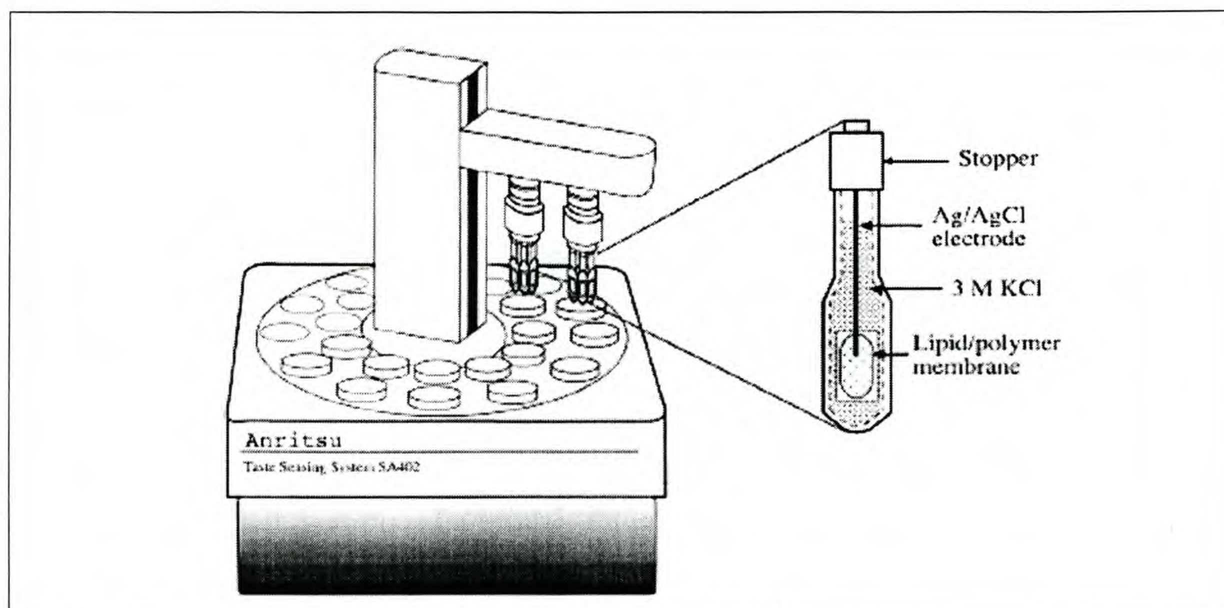

Fig.2.3. Taste-sensing system SA402, Anritsu (Toko, 2000).

2.3 BITTER COMPOUNDS

Bitterness can be elicited from a diverse collection of chemical compounds with varying sizes and functional groups. Almost any type of molecule can exhibit bitterness, but a bitter molecule can be rendered non-bitter by small modifications to its structure. The ability to predict bitterness from chemical composition is therefore

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limited. Some structural generalisations have been established, however; for example, bitterness can be correlated with the degree of hydrophobicity of the molecule (Rousseff, 1990). Bitterness is a property associated with a highly diverse range of chemical structures and this section will focus on a few of the compounds commonly found in foods and beverages.

2.3.1 Amino acids and peptides

The structural relationships of amino acids have been well studied and it is known that amino acids can taste either sweet or bitter (Solms, 1969; Schiffman et al., 1981; Maga, 1990). An amino acid must have an L-structure and an ammonium group, present for it to have a bitter taste. In the presence of the ammonium group the compound can be modified without altering the bitter taste significantly. A branched alkane chain also gives a more bitter taste than the corresponding number of straight chain carbon atoms. Amino acids with either two alkane chains or three methyl groups at the α-carbon atom are also not bitter. The amino acids L-phenylalanine and L-tryptophane are bitter, since aromatic side chains increase the intensity of bitter taste. The addition of amino, hydroxy, carboxylic or carboxamide groups usually decreases the bitter taste of amino acids (Maga, 1990).

Ney (1979) showed that side chain hydrophobicity and, in turn, total amino acid or peptide hydrophobicity, are directly related to bitter taste. A hydrophobicity value, Δf (free energy of transfer of the side chains of amino acids), exists for each amino acid, based on its solubility properties (**Table 2.1**).

By adding the Δf values for all amino acids in a peptide, then dividing by the number of residues, the Q value ($Q=\Sigma\Delta f/n$) for a peptide can be calculated, where Q = average hydrophobicity of a peptide, Δf = hydrophobicity of an individual amino acid and n = number of amino acid residues. At least one hydrophobic side chain is required for a peptide to taste bitter, with the kind and number of hydrophobic groups influencing the bitter taste thresholds (Maga, 1990). Ney (1979) found that bitterness occurred only when the Q value of a peptide (MW > 6000 Da) exceeded 1400, and that such Q values occur only in peptides that are predominantly hydrophobic. In general, proline-containing peptides taste bitter, even though free proline is only slightly bitter. However, for a proline peptide to taste bitter, the other associated peptide has to be more than five carbon atoms longer (Belitz and Wiester, 1985). Shinoda et al. (1985) found that N-terminal arginine is indispensable for bitterness, while Ishibashi et al. (1987) concluded that the number of leucine residues at the C-terminal in a peptide also determined its bitterness.

2.3.2 Phenols and polyphenols

Phenolic compounds elicit bitterness, as well as astringency, in many foods and beverages (Delcour et al., 1984; Bravo, 1998; Drewnowski and Gomez-Carneros, 2000). The bitterness of wine and cider, for example, is caused mainly by phenolic

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compounds and, in particular, by oligomeric proanthocyanidins (Lea and Arnold, 1978; Arnold et al., 1980; Macheix et al., 1990). Some bitter-tasting phenolic compounds are shown in **Table 2.2**.

Table 2.1
Hydrophobicity values (Δf) of side chains of amino acids (Ney, 1979)

Amino acid	Abbr.	Δf cal/mol)
Glycine	Gly	0
Serine	Ser	40
Threonine	Thr	440
Histidine	Thr	500
Aspartic acid	Asp	540
Glutamic acid	Glu	550
Argenine	Arg	730
Alanine	Ala	730
Methionine	Met	1300
Lysine	Lys	1500
Valine	Val	1690
Leucine	Leu	2420
Proline	Pro	2620
Phenylalanine	Phe	2650
Tyrosine	Tyr	2870
Isoleucine	Ile	2970
Tryptophan	Trp	3000

The amount of phenolic compounds in plant-derived foods and the level of bitterness are influenced by genetic factors and by environmental conditions. The phenolic content of plants is also influenced by the type of cultivar, germination, degree of ripeness, processing and storage conditions (Bravo, 1998; Drewnowski, 2001). Dietary phenolic compounds are divided into more than 15 classes, with one of the most important ones being the flavonoids. The flavonoid group includes flavanones, flavonols, flavones, isoflavones, flavans (catechins) and anthocyanins (Drewnowski, 2001). The basic structures of these flavonoids are shown in **Fig. 2.4** (Bravo, 1998).

Generally, the low molecular weight phenolic compounds tend to be bitter, while the high molecular weight (MW >500 Da) polyphenols (e.g. tannins) are more likely to be astringent (Noble, 1994; Drewnowski, 2001).

The non-flavonoid phenolic compounds, which also elicit bitterness, form part of the hydroxycinnamic acid derivatives. This group includes p-coumaric, caffeic and ferulic acids (Macheix et al., 1990).

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Table 2.2
Selected bitter-tasting phenolic compounds in common plant-derived foods and beverages[1]

Phenolic compounds	Typical component	Taste quality	Food source	Amount present
Flavanones	Naringin	Bitter	Grapefruit pith	13285-17603 mg/kg
			Grapefruit seeds	296-2677 mg/kg
			Immature grapefruit	97920-144120 mg/kg
			Grapefruit juice	300-750 mg/kg
Flavones	Tangeretin	Bitter	Orange fruit	0-30 mg/kg DW
			Orange juice	0.6 mg/L
			Juice from concentrate	0.2-0.5 mg/L
	Nobiletin	Bitter	Orange fruit	14-112 mg/kg DW
			Orange juice	2.7-2.9 mg/L
			Juice from concentrate	1.8-2.3 mg/L
Flavonols	Quercetin	Bitter	Grapefruit juice	4.9 mg/L
			Lemon juice	7.4 mg/L
			Fresh hops	700 mg/kg FW
			Wine	4.1-16 mg/L
			Black tea infusion	10-25 mg/L
			Oolong tea infusion	13 mg/L
			Green tea infusion	14-23 mg/L
Flavans	Catechin	Bitter	Red wine	11.1 mg/L
			Green tea infusion	13.0-19.1 mg/L
			Oolong tea infusion	6.0-6.4 mg/L
			Black tea infusion	9.2-15.6 mg/L
	Epicatechin	Bitter	Red wine	7.7 mg/L
			Low fat cocoa powder	940-2470 mg/kg
			Instant cocoa powder	180-320 mg/kg
			Green tea infusion	105.0-118.0 mg/L
			Oolong tea infusion	63.5-68.0 mg/L
			Black tea infusion	16.8-35.0 mg/L
Phenolic flavonoids	Catechin mono- and polymers MW < 500	Bitter	Red wine	1000-3500 mg/L
			Rosé wine	200 mg/L
	Catechin polymers MW > 500 (tannins)	Astringent	Red wine	
			Apple cider	
	Polyphenols	Astringent and bitter	Low fat cocoa powder	8380-31000 mg/kg
			Instant cocoa powder	1370-4460 mg/kg

[1]MW, molecular weight; DW, dry weight; FW, fresh weight.

Adapted from Drewnowski and Gomez-Carneros (2000).

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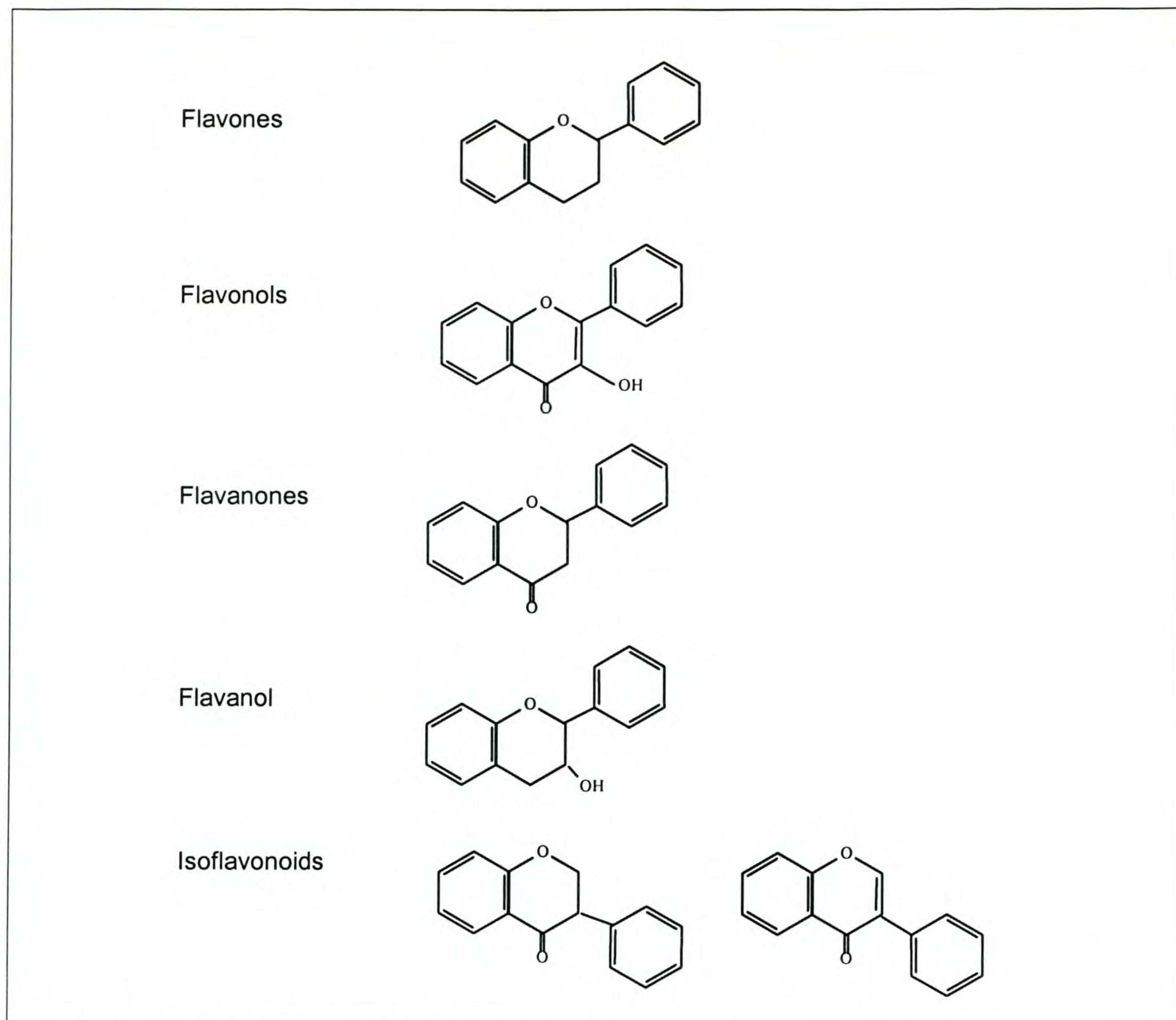

Fig. 2.4. Basic structure of the flavonoids (Adapted from Bravo, 1998).

2.3.3 Methylxanthines

The best-known example of a methylxanthine is probably caffeine, which is one of the most widely used psychoactive substances in the world (Ramalakshmi and Raghavan, 1999). Caffeine is produced in the leaves and seeds of many plants, and is present in tea leaves, coffee beans, chocolate, some soft drinks and pain relievers (**Table 2.3**). The chemical structure of caffeine is shown in **Fig. 2.5** (Yamanishi, 1990). Caffeine is intensely bitter and its detection threshold in water is approximately 3 ppm. It is moderately soluble in water, but is also hydrophobic enough to pass through biological membranes. Thus, it is rapidly and completely absorbed from the gastrointestinal tract (Ramalakshmi and Raghavan, 1999).

2.3.4 Acrolein

Unlike the compounds discussed above, acrolein as a single component does not possess a bitter taste. It is, however, included in this discussion, since it is known

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that acrolein combined with phenolic groups exhibits an intensely bitter taste in wine. Acrolein (**Fig. 2.6**) is a volatile and highly toxic unsaturated aldehyde.

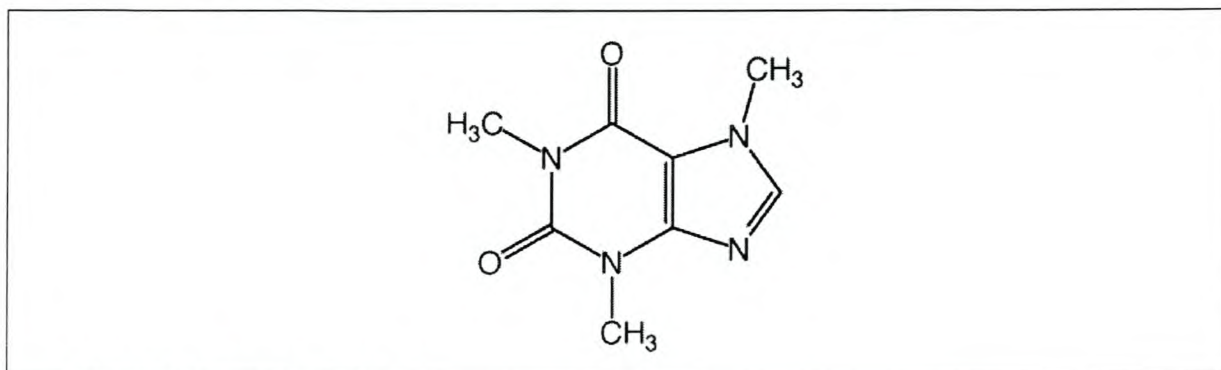

Fig. 2.5. Structure of caffeine.

Table 2.3
Caffeine content of foods and beverages (Drewnowski, 2001)

Item (serving size)	Caffeine content (mg)	
	Typical	Range
Coffee (250 ml)		
Brewed, drip	100	60-180
Instant	65	30-120
Decaffeinated	3	1-5
Espresso (30 ml)	40	30-50
Tea (250 ml)		
Brewed tea – green, black, oolong	60	25-110
Instant	28	24-31
Iced	25	9-50
Soft drinks (250 ml)		
Cola and citrus beverages	24	20-40
Cocoa beverages (250 ml)	6	3-32
Dark chocolate (30 g)	20	5-35
Milk chocolate (30 g)	6	1-15

Glycerol is one of the most important byproducts produced by yeasts during the alcoholic fermentation. A number of bacterial species, including *Klebsiella pneumoniae, Citrobacter intermedium, Citrobacter freundii, Clostridium pasteurianum, Propionibacterium freundenreichii, Salmonella enterica*, *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus reuteri, Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans*, are able to metabolise glycerol. However, the ability to degrade glycerol is definitely strain dependent (Kandler, 1983; Schütz and Radler, 1984a; Davis et al., 1986; Sponholz, 1993; Du Toit and Pretorius,

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2000; Sauvageot et al., 2000; Gorga et al., 2002). The strains able to degrade glycerol all have a common enzyme, glycerol dehydratase, that dehydrates glycerol to 3-hydroxypropionaldehyde (3-HPA). This 3-HPA can subsequently be reduced to 1,3-propanediol by an NADH-linked oxidoreductase (Schütz and Radler, 1984b; Talarico and Dobrogosz, 1990; Veiga da Cunha and Foster, 1992; Sauvageot et al., 2000; Gorga et al., 2002).

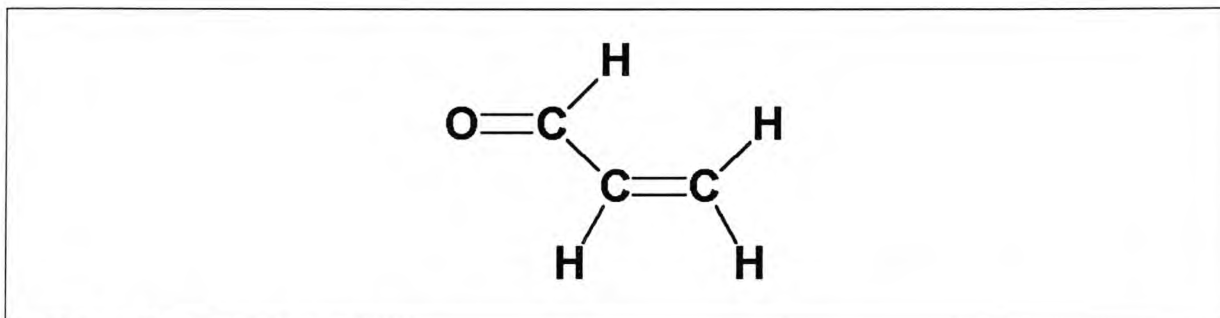

Fig. 2.6. Structure of acrolein.

The enzymes, glycerol and diol dehydratases, are isofunctional and have the same subunit composition, but differ in their substrate specificities. The diol dehydratase has a higher affinity for 1,2-propanediol, while the glycerol dehydratase has a higher affinity for glycerol (Daniel et al., 1999). The glycerol/diol dehydratase genes of *C. pasteurianum* and of enteric bacteria such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *C. freundii* and *S. enterica* serovar *typhimurium* have been cloned and sequenced. The characterised dehydratases have an $\alpha_2\beta_2\gamma_2$ composition, where α, β and γ are the large, medium and small subunits respectively. These subunits are encoded by three contiguous open reading frames (ORFs) (Daniel et al., 1999). The glycerol dehydratase of three *Lactobacillus* species, namely *Lb. reuteri*, *Lb. collinoides* and *Lb. diolivorans*, has been purified and characterised, (Talarico and Dobrogosz, 1990; Gorga et al., 2002; Sauvageot et al., 2002). The dehydratase of *Lb. reuteri* was found to have a molecular mass similar to that of the dehydratases of other genera, but it is composed of four identical subunits of 52 kDa each. Sauvageot et al. (2002) found that the dehydratase of *Lb. collinoides* has a heterotrimer composition, with the subunits having molecular masses of 63, 25 and 19 kDa for the α, β and γ subunits respectively. Recently, the genomes of a few LAB, i.e *Leuc. mesenteroides*, *Lb. gasseri*, *Oenococcus oeni* and *Ped. pentosaceus*, were sequenced and, according to the draft sequence, none of these species (or rather the specific strains) possesses the GD enzyme (http://www.jgi.doe.gov/JGI_microbial/html).

Acrolein is not a product of glycerol metabolism, but occurs in chemical equilibrium with 3-HPA in aqeous solution, with this equilibrium favouring acrolein in conditions of acid and heat (Pressman and Lucas, 1942; Slininger et al., 1983; Claisse and Lonvaud-Funel, 2000; Du Toit and Pretorius, 2000) (**Fig. 2.7**).

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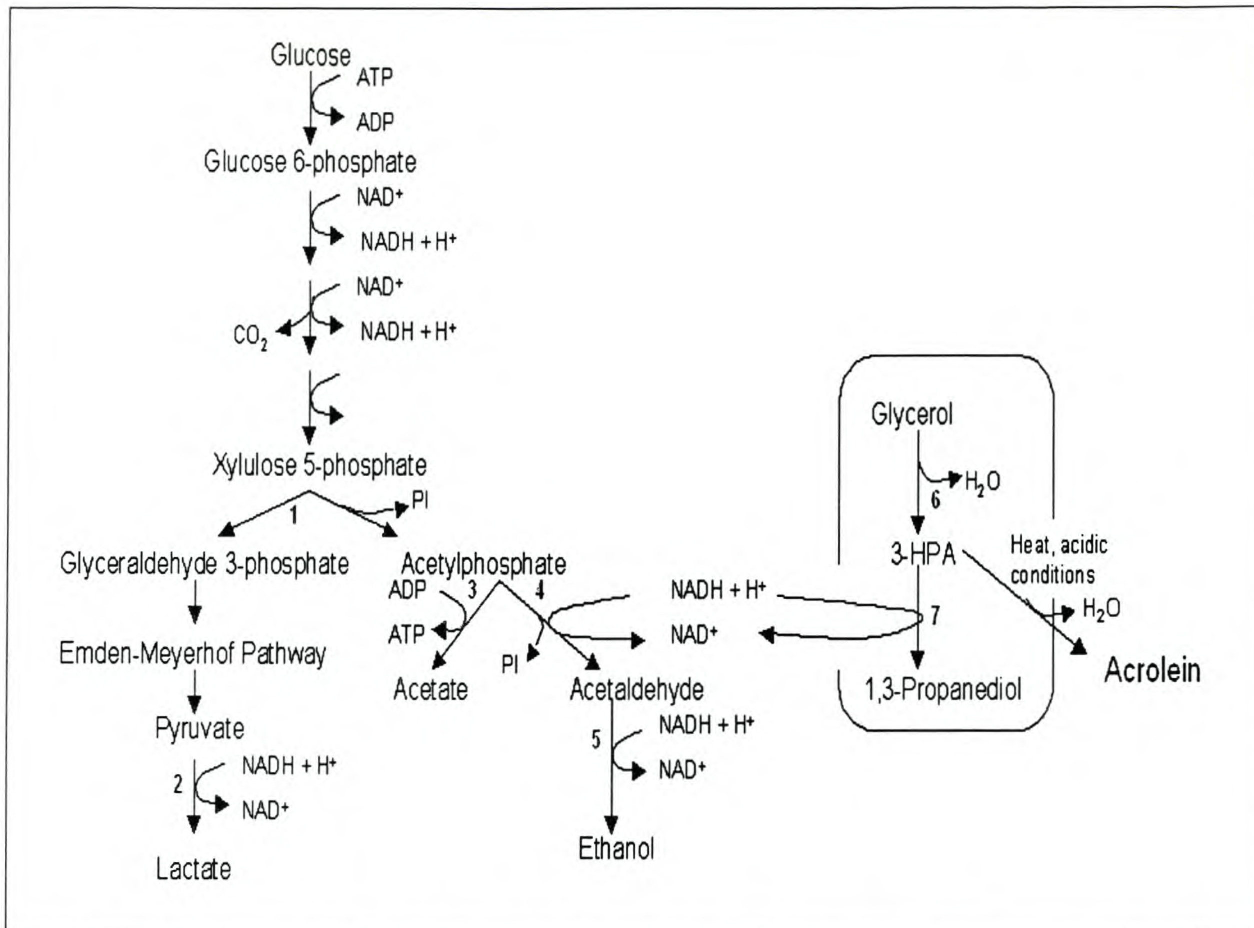

Fig. 2.7. Key reactions of glucose and glycerol metabolism by *Lb. reuteri* under anaerobic conditions. Enzymes involved: 1) Phosphoketolase, 2) lactate dehydrogenase, 3) acetate kinase, 4) phosphotransacetylase, 5) alcohol dehydrogenase, 6) glycerol dehydratase, 7) 1,3-propanediol:NAD oxidoreductase. The enzymes of the phosphogluconate pathway and the Emden-Meyerhof glycolytic pathway are not described here (Adapted from Lüthi-Peng et al., 2002).

Characterisation of the enzyme, glycerol dehydratase, showed that this enzyme is active only when glucose concentrations are very low, and that a glycerol:glucose ratio of 3:1 is optimal for enzyme activity (Veiga da Cunha and Foster, 1992; El-Ziney et al., 1998; Sauvageot et al., 2000, 2002).

2.4 BITTERNESS IN FOODS

Bitterness is present in many foods and, depending on the type of food and the amount of bitterness, it can be a desirable property or considered as an off-flavour. The sources of compounds exhibiting a bitter taste can be divided into biologically formed and/or naturally occurring compounds that are present in the raw material, or compounds that are formed through the interaction of thermally active precursors, or the combined action of varying amounts of heat, time, precursors and enzymes (Maga, 1990).

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2.4.1 Fruits

Citrus, apples, olives and avocados are only a few examples of fruit cultivars in which bitterness has been reported. Various phenolic compounds are most commonly responsible for bitterness in fruits (Macheix et al., 1990; Rouseff, 1990).

2.4.1.1 Citrus

In sweet oranges (*Citrus sinensis*), bitterness is a flavour defect, while in grapefruit (*Citrus paradisi*) it is the most important flavour characteristic. The bitterness in citrus is due to two distinctly different classes of chemical compounds: the limonoids and the flavanone neohesperidosides. Limonin and naringin are generally the major limonoid and flavanone neohesperidoside respectively. Instantaneous bitterness is caused by naringin, while limonin is responsible for delayed bitterness in citrus products (Macheix et al., 1990; Rouseff, 1990; Drewnowski and Gomez-Carneros, 2000).

Naringin is the major flavanoid of grapefruit and pomelo. Naringin is the neohesperidose derivative of the aglycone, naringenin. The chemical structure of naringin is presented in **Fig. 2.8**. Neither naringenin nor neohespiridose are bitter, while naringin is intensely bitter, with a detection threshold of about 20 ppm in water, although 1.5 ppm may also be detected (Puri and Banerjee, 2000). The nature of the aglycone and the sugar moiety therefore is important in the degree of bitterness (Macheix et al., 1990; Puri, 1990; Robins et al., 1990).

The delayed bitterness of limonin in citrus is due to the fact that there is no limonin in the intact fruit, but rather a non-bitter precursor, limonoate A-ring lactone (**Fig. 2.8**). During juice extraction, this non-bitter precursor is gradually converted to limonin under acidic conditions. This conversion is accelerated by heat, but it even occurs during extended frozen storage (Hasegawa and Maier, 1990; Puri, 1990). In water, the detection threshold of limonin is 1 ppm, and in orange juice the detection threshold is 6 ppm (Puri, 1990).

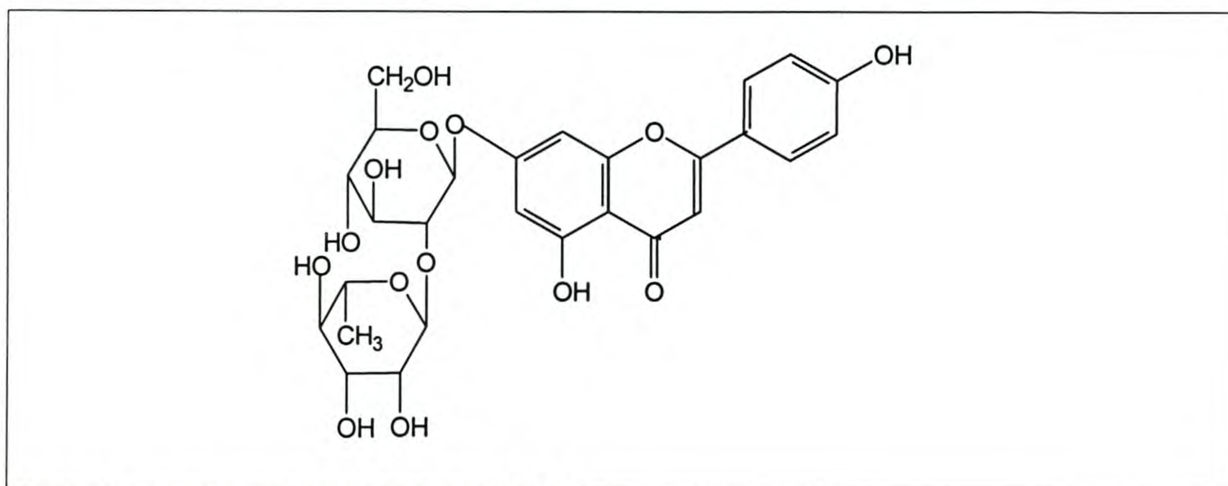

Fig. 2.8. Structure of naringin (Shaw, 1990).

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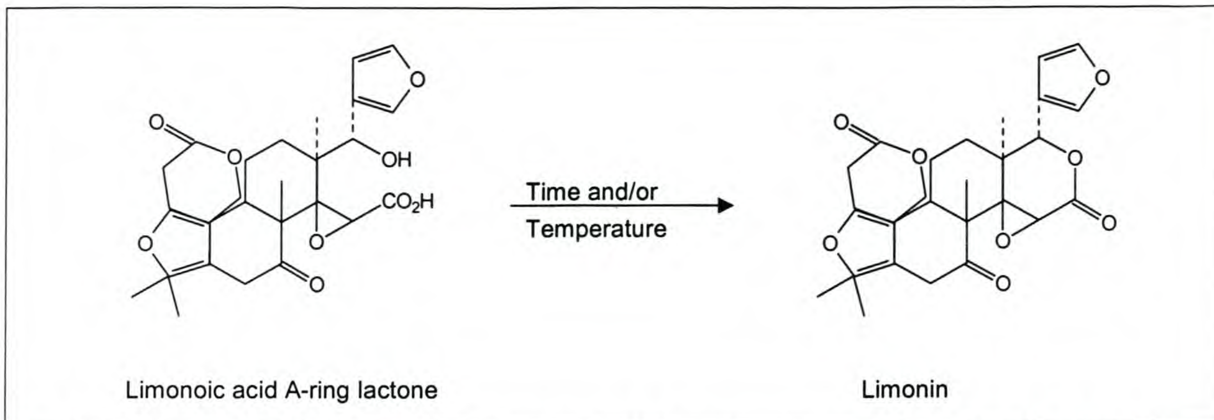

Fig. 2.9. Conversion of the non-bitter precursor to bitter limonin in citrus juice (Puri, 1990).

Limonin and naringin are known to act synergistically on perceived bitterness. The levels of both limonin and naringin are influenced by several factors. The maturity of the fruit at harvest is one of the most important factors. Fruit harvested late in the season have lower levels of limonin and naringin, as opposed to fruit harvested early in the season. Other factors include the pressure applied on the fruit during the extraction process, horticultural practices and climatic conditions (Puri, 1990).

2.4.3 Dairy products

As with all other food products, the mechanisms causing bitterness in dairy products are diverse and complex. However, there seems to be consensus that bitter amino acids and peptides are the major causes of bitterness in dairy products (Ney, 1979; Schmidt, 1990). Other sources include bitter-flavoured fatty acids from milk fat lipolysis and certain alkaloids. The bitter peptides associated with ripened hard cheese products are the most extensively studied bitter components and will be discussed in this section.

2.4.3.1 Cheese

The characteristic flavour of ripened hard cheeses is the result of a complex proteolytic enzyme system. This proteolytic enzyme system is, however, also linked with the bitterness defect frequently developing in cheese (**Fig. 2.10**). The proteolytic enzymes involved in the cheese-making process may include: 1) the proteinases associated with rennet, plant or microbial proteinases used in milk coagulation, 2) the proteinase-peptidase system of starter lactic acid bacteria, 3) the proteinase-peptidase system of non-starter lactic acid bacteria, and 4) the endogenous proteinase of milk (Schmidt, 1990; Habibi-Najafi and Lee, 1996).

The proteolytic systems of the lactic streptococci, e.g. *Streptococcus lactis* and *Streptococcus cremoris*, are mostly used as starters in cheese manufacturing (Lee et al., 1996; Broadbent et al., 1998; Smit et al., 2000; Guldfeldt et al., 2001). It has been shown that there is a relationship between the strains of streptococci used in cheese making and the bitterness of the cheese, and that these organisms can be

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divided into bitter and non-bitter strains. Bitter strains are thought to either have a deficiency of peptidases in which the cleavage of bitter peptides is not achieved, or to possess greater proteolytic activity than non-bitter strains. These strains generally are faster growers, and are able to reach higher numbers under normal cheese-making conditions. A general conclusion has drawn stating that there potentially is more peptidase activity for formation of bitter peptides in bitter strains compared to non-bitter strains (Schmidt, 1990).

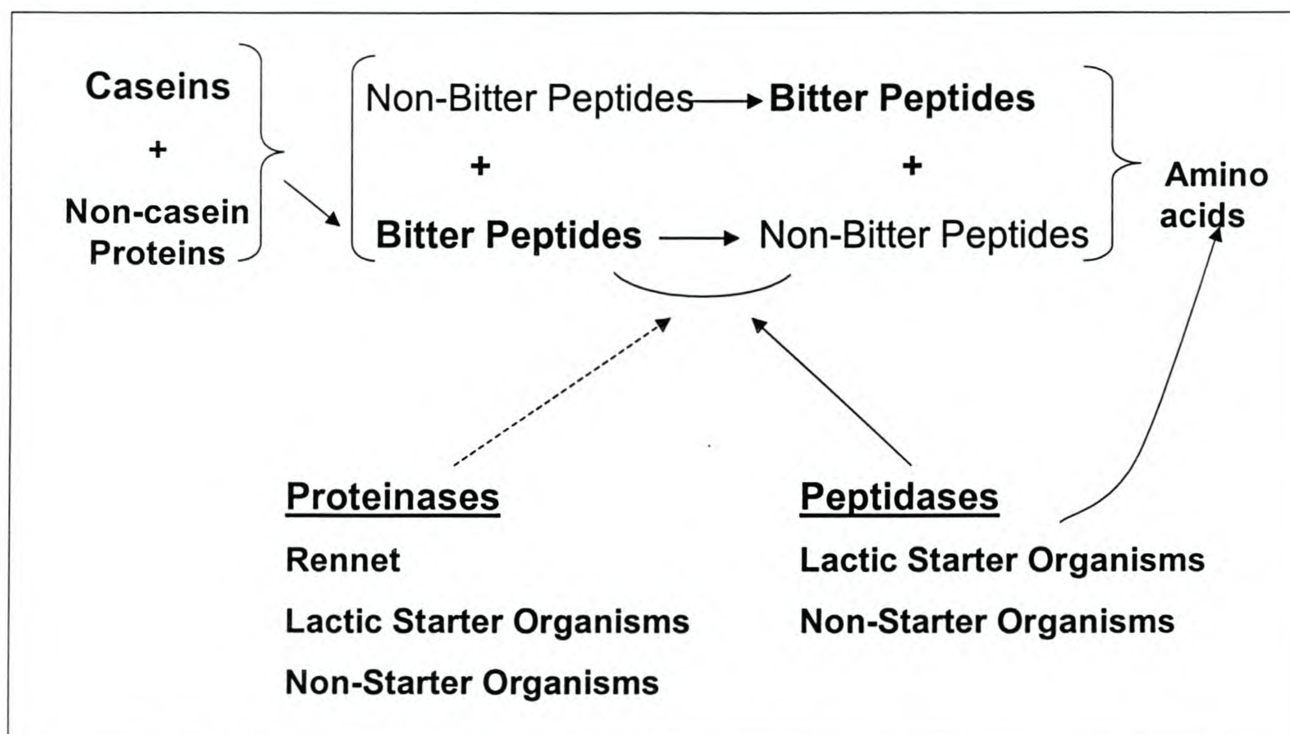

Fig. 2.10. Proteolytic reactions involved in bitter peptide formation in ripened hard cheese products (Schmidt, 1990).

Rennet (chymosin) acts on caseins (α_{s1}, β, κ) in three phases and it has been shown that rennet action alone can produce a bitter flavour (Habibi-Najafi and Lee, 1996). Rennet proteolysis results in the formation of large peptide moieties from casein, which may be non-bitter, but become precursors to bitter peptides via further proteolytic cleavage. Bitter peptides can be formed directly in cheese if rennet or rennet substitutes are used at excessive levels or are retained in the curd at high levels.

The non-starter microflora of the cheese-making process include lactobacilli (*Lb. plantarum*, *Lb. casei*) and pediococci (*Ped. pentosaceus*). These non-starter lactic acid bacteria also produce peptidases that convert larger peptides to bitter peptides (**Fig. 2.10**) (Schmidt, 1990; Habibi-Najafi and Lee, 1996).

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2.5 BITTERNESS IN BEVERAGES

As with many other foodstuffs, bitterness forms an integral part of the overall flavour complexity of many beverages. While a certain degree of bitterness may be expected in many beverages, an excessive amount thereof can be considered a flavour defect. The compounds eliciting bitterness in beverages are also highly varied and a few will be discussed in this section.

2.5.1 Wine

Bitterness and astringency are two important attributes of wine flavour (Robichaud and Noble, 1990). While the naturally occurring flavonoid phenols are considered to be primarily responsible for bitterness in wine, the occurrence of excessive bitterness in red wines is most probably due to a combination of the phenols and the bacterial degradation of glycerol, leading to the formation of acrolein (Rentchler and Tanner, 1951; Sponholz, 1993; Bartowsky and Henschke, 1995; Claisse and Lonvaud-Funel, 2000; Delfini and Formica, 2001).

Flavonoid compounds in wine range from monomeric low molecular weight catechins and epicatechins to polymeric high molecular weight condensed tannins. The flavonoids are found mainly in the seeds, skins and stems of grapes (Noble, 1990; Robichaud and Noble, 1990; Noble, 1994; Drewnowski and Gomez-Carneros, 2000). It has been shown that all cider and grape phenolic fractions are bitter and astringent, with the flavonoid monomers being more bitter than astringent. However, as the size of the molecule increases from one to four units, astringency increases relative to the bitterness. All fractions are still more bitter than astringent (Arnold et al., 1980; Robichaud and Noble, 1990; Noble, 1994). It was also found that fractions containing polymers of six or more flavonoid units were 25 to 30 times more astringent and bitter than the monomeric fractions, but that the ratio of bitterness to astringency was highest for the monomers (Arnold et al., 1980). This explains why young red wines that have more small flavonoids (less than four units) than larger flavonoids, usually are more bitter and astringent. As wines age, they contain more polymerised flavonoids (eight to ten or more units) and are considered to be less bitter (Noble, 1994). Epicatechin was also found to be more bitter and astringent than catechin (Drewnowski and Gomez-Carneros, 2000). Fischer and Noble (1994) found that an increase of 3% v/v ethanol in wine with 100 mg/l catechin significantly increased the bitterness, and that increasing the pH from 2.9 to 3.2 also led to an increase in the bitterness.

Pasteur originally suggested that the bitterness in wines was due to the degradation of glycerol. It was only later that Voisenet associated the acrolein taint with bitterness (Sponholz, 1993). Very little is known about the acrolein content of wine, and this can probably be ascribed to the fact that no routine and standardised method is available to detect acrolein in wine. It appears, however, that approximately 10 mg/l is sufficient to cause bitterness (Margalith, 1981). Acrolein is

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also associated with a peppery and bitter off-flavour in apple ciders, beer, brandy, rum and whiskies (Greenhoff and Wheeler, 1981; Postel and Adam, 1983; Sauvageot et al., 2000).

Since acrolein formation is probably due to bacterial action, as discussed in Section 2.3.4, the presence and levels of undesirable species directly influence the amount of acrolein that can be present in wine. Factors that will affect the bacterial population on grapes and in wine play an important role in the bitterness of wines. These factors include the sugar concentration of the grapes at harvest, the resulting pH of the wine and the levels of SO_2 used during the winemaking process. To date, however, no study has been done to specifically isolate and identify lactic acid bacteria that are able to degrade glycerol from grapes and wine. Various studies have identified strains of *Lactobacillus collinoides* that are able to degrade glycerol in spoiled ciders (Claisse and Lonvaud-Funel, 2000; Sauvageot et al., 2000; Claisse and Lonvaud-Funel, 2001). Glycerol dehydratase (GD), an enzyme of this species, has been studied and a specific DNA probe for detecting the GD enzyme has been designed.

2.5.2 Apple ciders

Four classes of phenolic compounds can be distinguishediln apple ciders: 1) phenolic acids, 2) phloretin derivatives, 3) catechins, and 4) procyanidins. However, only the procyanidins (**Fig. 2.11**) have any significant bitterness and astringency at the levels in which they are found in ciders (Lea, 1978). The procyanidins consist mainly of (-)-epicatechin units, with a small proportion of (+)-catechin as a terminal unit.

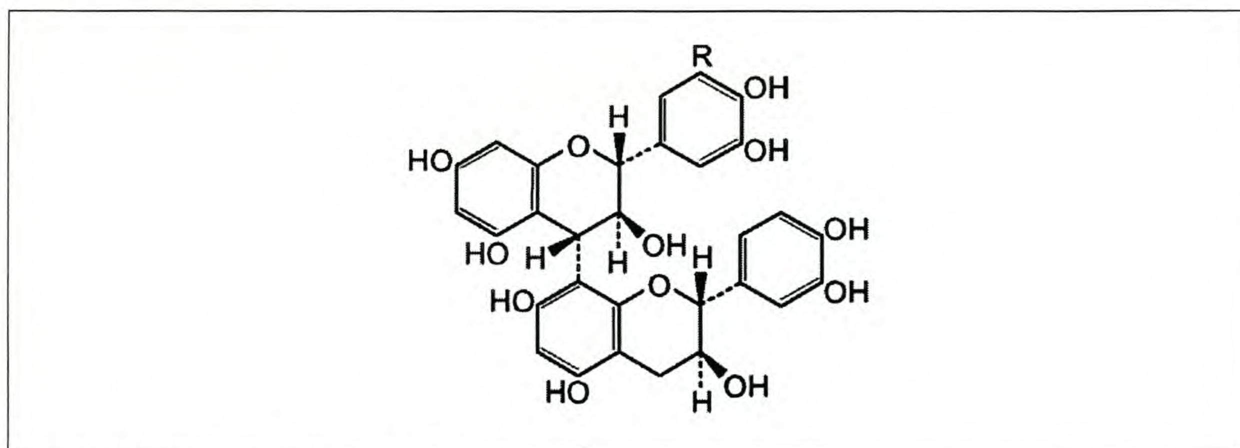

Fig. 2.11. Common structure of a procyanidin.

Sanoner et al. (1999) found that the proportion of polyphenol classes varies considerably between different apple varieties, but that the procyanidins are always the predominant class. The phenolic composition, especially of the procyanindins, of a specific cider cultivar depends on various horticultural as well as processing factors. Horticultural aspects include, amongst others, the age of the trees, the climatic conditions, the nutrient status of the soil and the maturity of the fruit. The

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degree of oxidation and the effect of fining or clarification agents can also have a considerable effect on the levels of procyanidins. These factors together can thus have a significant effect on the bitterness and astringency of the final product.

2.5.3 Tea

Tea is an aromatic stimulant containing various polyphenols, essential oils and caffeine. It is a beverage with a unique taste and the bitterness of tea is elicited by catechins, caffeine, saponin and some amino acids. There are three categories of tea, i.e. fermented or black tea, semi-fermented or oolong tea and non-fermented or green tea. The amounts of the compounds responsible for bitterness in the final beverages will depend on the cultivar used and the manufacturing process (Rouseff, 1990; Yamanishi, 1990; Wang et al., 2000).

The most predominant flavour component in tea is catechin. The catechin content varies between cultivars and the proportions of the individual catechins also vary, but they normally form 20 to 30% of the dry weight of green tea (Wang et al., 2000). The catechins most commonly found in tea include (-)-epicatechin, (-)-epigallocatechin and their gallate esters, while smaller amounts of (+)-catechin and (+)-gallocatechin are also found (**Fig. 2.12**). It has been shown that the taste, colour and aroma of manufactured tea are associated directly or indirectly with modifications to the catechins, for example, degalloation of ester catechins to non-ester catechins can result in a decrease in the bitterness and astringency of green tea (Wang et al., 2000).

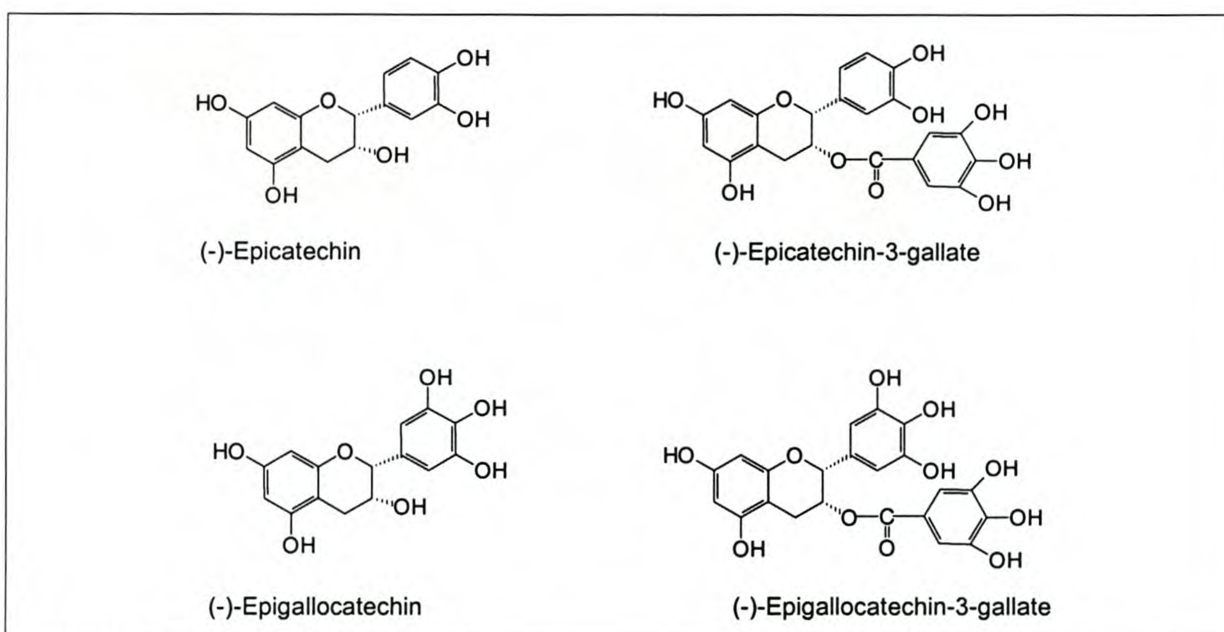

Fig. 2.12. Common catechins in tea.

Caffeine plays an important role in determining the taste and "briskness" of the tea beverage. The concentrations of caffeine in freshly harvested tea shoots range between 2.5 to 5.5% (dry weight basis), while in tea it ranges from 2 to 4%. The amount of caffeine also increases during the manufacturing of black tea (fermented

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tea), partly because of its release from complex molecules that contain the caffeine molecule as part of their structure (Yamanishi, 1990).

Saponins are glycosidic compounds that taste bitter and acrid. Theofolisaponin is found in tea and is generally considered to be detrimental to the overall flavour of tea.

The concentration of amino acids found in tea leaves is 6 to 7% on a dry weight basis. Of the 19 amino acids found in tea leaves, six are known to be mildly bitter. These are valine, leucine, methionine, tryptophane, lysine and histidine. However, the bitterness of these amino acids is thought to have a positive effect on the overall taste of tea.

2.5.4 Coffee

Bitterness is sometimes a negative, but omnipresent, aspect of coffee. When present at low levels, bitterness helps to balance the acidity and adds a desirable taste to the coffee brew. At high levels, bitterness can overpower the other components in the coffee and have a detrimental effect on the taste (Ramalakshmi and Raghavan, 1999).

Chlorogenic acids and caffeine are generally considered to be responsible for bitterness in coffee. Caffeine is present at levels about ten times its threshold value, but it contributes only 10% to the overall bitterness. Coffee also contains high concentrations of chlorogenic acids (caffeic and ferulic esters of quinic acid). The two major coffee cultivars, *Coffea arabica* and *Coffea canephora*, which commonly are known as arabica and robusta respectively, differ in the relative amounts of these compounds. Robusta beans generally contain higher levels of chlorogenic acids and caffeine than arabica beans. The processing methods, e.g. the roasting time of the beans, also affect the amount of bitterness found in coffee (Belitz and Wiester, 1985; McCamey et al., 1990).

2.5.5 Beer

Hops (*Humulus lupulus*) contains many components, but those of particular interest to the brewer are the main α- and β-acids. These compounds are the precursors of beer bitterness. The principle α-acid is humulone. Hops also contains smaller amounts of cohumulone and adhumulone. During the boiling process of the wort, the α-acids isomerise to form the bitter iso-α-acids (**Fig. 2.13**). These isomerised compounds exist as equally bitter *cis*- and *trans*-isomers and are responsible for up to 70% of the observed bitterness in beer (Cowles et al., 1990; Robins et al., 1990).

The β-acids contribute very little to the flavour of beer due to their low solubility in wort and beer. However, during kettle boiling the β-acids (colupulone, lupulone and adlupulone) are partially converted to soluble hulupones that can also impart bitterness to beer (Cowles et al., 1990).

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It was found that a concentration of ±20 mg/l of iso-α-acids is usually considered as not very bitter, while very bitter beers could contain upwards of 40 mg/l. However, the acceptance of bitterness may sometimes depend more on the expectations of the consumer than on the absolute level of bitter compounds (King and Moreau, 1996).

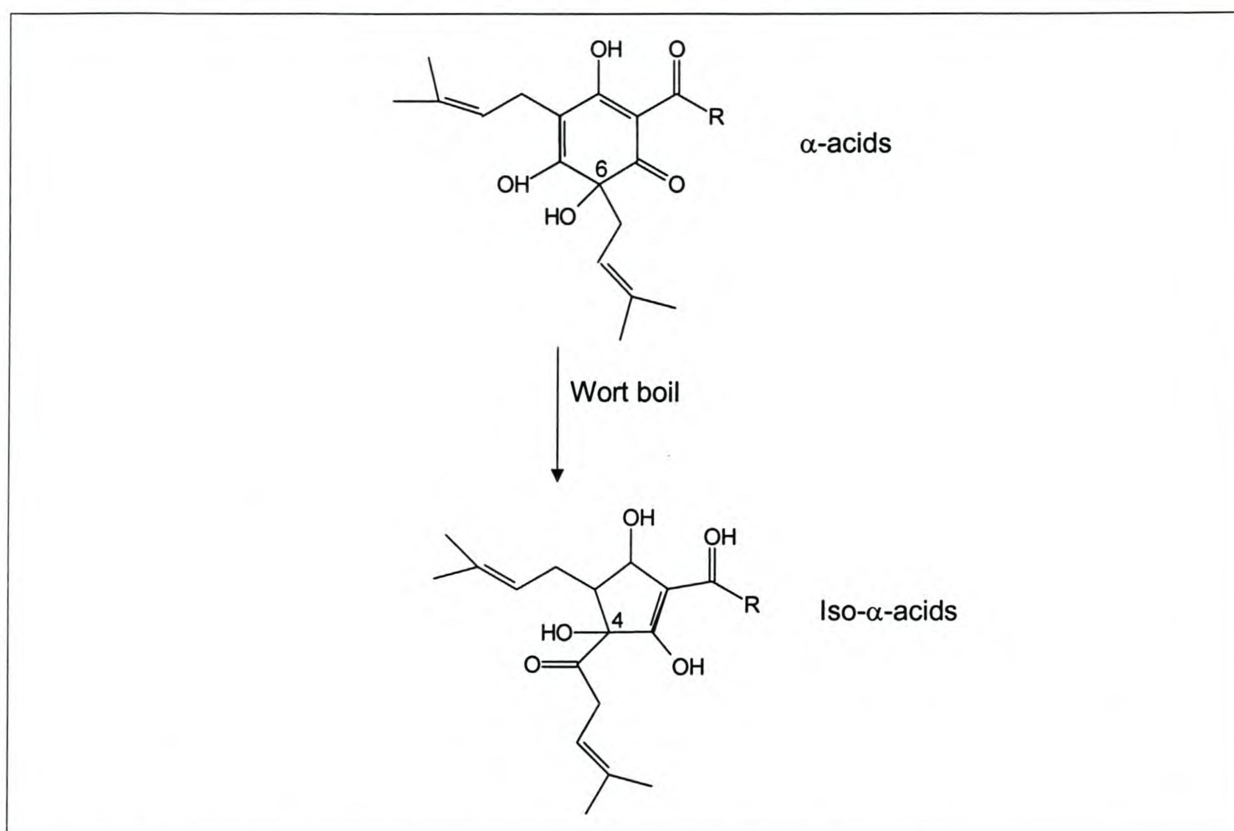

Fig. 2.13. Isomerisation reaction of hops α-acids (Cowles et al., 1990).

2.6 METHODS TO REMOVE BITTERNESS

Excessive bitterness has a negative effect on the quality of many foods and beverages and therefore can have a great economic impact. Several methods have been developed to remove bitter compounds from food products without introducing new substances or removing desirable components.

2.6.1 Debittering of citrus juices by biological means

The bacteria that are able to metabolise limonoids have been identified as *Arthrobacter globiformis*, *Pseudomonas* 321-18, *Acinetobacter*, *A. globiformis* II, *Corynebacterium fascians* and Bacterium No. 342-152-1. Limonoate dehydrogenase from *A. globiformis* was the first enzyme used to prevent the development of limonin bitterness in navel orange juices (Hasegawa et al., 1973; Hasegawa and Maier, 1990; Hasegawa and Pelton, 1983). The dehydrogenase prevents the conversion to bitter limonin by converting limonoate A-ring lactone to 17-dehydrolimonoate A-ring lactone. This process requires a high concentration of enzyme, because this enzyme

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requires a high pH for optimal activity and is unstable at low pH (Hasegawa and Pelton, 1983).

The use of isolated enzymes was followed by using whole bacterial cells. Bacterial cells of the various species possessing the requisite enzymes were immobilised in acrylamide gel, blended and packed in a column. This method proved to be effective in converting bitter compounds to non-bitter compounds. There also was no detrimental effect on the quality of the juice (Hasegawa and Maier, 1990).

2.6.2 Removal of bitterness from citrus products by adsorption techniques

Puri (1990) used Duolite S-861, a polystyrene divinylbenzene cross-linked co-polymeric adsorbent resin, to remove naringin and limonin from citrus juices. It was found that the adsorbent resin was effective in debittering a variety of citrus juices without removing desirable compounds from the juices. This process has been commercialised and is patented by The Coca-Cola Company. Shaw et al. (1984) also reported the use of β-cyclodextrin polymer in a continuous fluid-bed or batch process to reduce levels of limonin, nomilin and naringin in citrus juices. This method proved to be effective in removing the bitter components without affecting the flavour of the juice negatively.

2.6.3 Decaffeination of coffee

Coffee can be decaffeinated by using a variety of processes. However, not all of these processes are favourable towards the flavour and aroma of the final product. The processes used most commonly will be discussed.

2.6.3.1 Traditional or European Process

The traditional of European process is sometimes also referred to as the solvent process, and uses a solvent that binds selectively with caffeine. Common solvents used include methylene chloride, ethyl acetate and highly compressed CO_2 (http://www.coffeereview.com, http://www.worldwidemart.com). Basically, the process involves soaking the coffee beans in water that is almost boiling, extracting the flavour oils and caffeine, and then separating the water into a tank where it is treated with the solvent. The solvent binds to the caffeine and is removed from the flavour oils, while the beans are again added to the mixture and can regain most of their flavour oils. Very small amounts of solvent residues may exist in the beans after decaffeination, but these residues are dismissable with regard to health impacts (http://www.coffeeresearch.com). This method will remove 96 to 98% of the caffeine content (http://www.ineedcoffee.com).

2.6.3.2 Swiss water process

The Swiss water process involves soaking the green beans in hot water to remove the caffeine and most of the flavour compounds. The first batch of beans is then

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discarded, while the caffeine is stripped from the solution by means of activated carbon filters. The remaining solution is saturated with flavour compounds and is then used to soak a new batch of green beans. Due to the solution being saturated with all the components that are soluble in water other than caffeine, only the caffeine in the bean is allowed to escape. The rest of the compounds are in equilibrium, resulting in the beans retaining most of their flavour compounds. However, since the saturated solution is used repeatedly, the flavour components of various batches may become intermixed. This method can remove 94 to 96% of the caffeine, retains more of the flavour components than the solvent methods and is currently a patented and branded product of Kraft (http://www.ineedcoffee.com; http://www.coffeeresearch.com).

2.7 CONCLUSION

Taste is the most important factor determining the purchase of food and beverages. The food and beverage industries therefore consistently are faced by the challenge of meeting consumer demands and keeping ahead of market trends. Bitterness, being one of the four primary taste sensations, is often not desirable, but, in some instances, it forms part of the characteristic flavour of a specific product. Due to the wide range of bitterness-eliciting compounds and the economic impact that the bitterness defect can have, scientists are faced with the ongoing task of providing more information on this topic. The wine industry, for example, will benefit greatly from a better understanding of wine spoilage as a result of bitterness, which could lead to solutions for preventing and/or removing excessive bitterness from wines.

The way of expressing and measuring the level of bitterness is very relative, since the ability to perceive bitter taste and the sensitivity towards it vary greatly among individuals. A number of factors, including genetic variation, age and sex, play a role in the perception of bitterness. These factors therefore should be taken into account when conducting studies on bitterness perception.

The acceptable level of bitterness in foods and beverages varies according to the product. In tonics and grapefruit juice, bitterness is a characteristic trait of the product, while in red wine; for instance, a limited degree of bitterness is needed to balance the flavour profile. In many instances, bitter tastes are masked by the consumer or producer by adding sugar or other sweeteners.

The citrus industry has had the most success in reducing or removing bitterness. Methods to debitter citrus juices use either enzymes or adsorption techniques.

Both of these methods are used commercially, and recently studies have shifted towards biotechnology. Genetic techniques have been used widely for improving and tailoring many organisms according to specific needs. The pharmaceutical industry, in particular, uses genetically modified organisms (GMOs) extensively. The use of GMOs in the prevention of bitterness in certain foods and beverages therefore is being studied extensively by food scientists. For example, the genes encoding the

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limiting enzymes in caffeine synthesis have been cloned and open up the possibility of using genetic engineering to produce transgenic tea and coffee plants that are naturally deficient in caffeine (Ashihara and Crozier, 2001). A number of enzymes from *Lactococcus lactis* have also been purified and characterised, and the construction of enzyme-overproducing strains, as well as strains containing a deletion of the genes encoding flavour-forming enzymes, are being investigated. Genetically modified strains will most probably become available for use as starter cultures in the future (Smit et al., 2000).

The wine industry would benefit enormously from information gained on the subject of bitterness. Understanding the underlying mechanisms leading to bitterness would enable scientists to design preventative or curative strategies to fight this spoilage. Wine yeasts that have been genetically engineered to express, bacteriocin-encoding genes of LAB, for example, can be used to control spoilage LAB in wine fermentations (Schoeman et al., 1999). GMOs such as these, combined with existing and developing knowledge on the issue of bitterness, will provide tools to develop improved and more effective preservation systems for the wine industry. This would be another step towards meeting consumer demands and keeping pace with a competitive market.

2.8 LITERATURE CITED

Adler, E., Hoon, M.A., Mueller, K.L., Chandrashekar, J., Ryba, N.J.P., Zuker, C.S., 2000. A novel family of mammalian taste receptors. Cell 100, 693-702.

Arnold, R.A., Noble, A.C., Singleton, V.L., 1980. Bitterness and astringency of phenolic fractions in wine. J. Agric. Food Chem. 28, 675-678.

Ashihara, H., Crozier, A., 2001. Caffeine: A well known but little mentioned compound in plant science. Trends Plant Sci. 6, 407-413.

Bakker, J., 1998. Astringency: A matter of taste. Biol. 43, 104-108.

Bartoshuk, L.M., 1994. The biological basis of food perception and food acceptance. Food Qual. Prefer. 4, 21-32.

Bartowsky, E.J., Henschke, P.A., 1995. Malolactic fermentation and wine flavour. Austr. Grapegrower & Winemaker 378a, 83-94.

Belitz, H.D., Wiester, H., 1985. Bitter compounds: Occurrence and structure activity relationships. Food Rev. Int. 1, 271-354.

Brand, J.G., 2000. Within reach of an end to unnecessary bitterness? The Lancet 356, 1371-1372.

Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56, 317-333.

Broadbent, J.R., Strickland, M., Weimer, B.C., Johnson, M.E., Steele, J.L., 1998. Peptide accumulation and bitterness in cheddar cheese made using single-strain *Lactococcus lactis* starters with distinct proteinase specificities. J. Dairy Sci. 81, 327-337.

Chandrashekar, J., Mueller, K.L., Hoon, M.A., 2000. T2Rs function as bitter taste receptors. Cell 17, 703-711.

Claisse, O., Lonvaud-Funel, A., 2000. Assimilation of glycerol by a strain of *Lactobacillus collinoides* isolated from cider. Food Microbiol. 17, 513-519.

Claisse, O., Lonvaud-Funel, A., 2001. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. J. Food Protect. 64, 833-837.

Stellenbosch University http://scholar.sun.ac.za

Cowles, J.M., Ting, P.L., Murakami, A.A., Goldstein, H., 1990. Separation and identification of hop bitter compounds in beer. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 251-263.

Daniel, R., Bobik, T.A., Gottschalk, G., 1999. Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organisation of the encoding genes. FEMS Microbiol. Rev. 22, 553-66.

Davis, C.R., Wibowo, D.J., Lee, T.H., Fleet, G.H., 1986. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. Appl. Environ. Microbiol. 51, 539-545.

Delcour, J.A., Vandenberghe, M.M., Corten, P.F., Dondeyne, P., 1984. Flavor thresholds of polyphenols in water. Am. J. Enol. Vitic. 35, 134-136.

Delfini, C., Formica, J.V., 2001. The microbiological control of wine during storage. In: Delfini, C., Formica, J.V. (Eds.), Wine Microbiology. Marcel Dekker, New York, pp. 389-408.

Drewnowski, A., Rock, C.L., 1995. The influence of genetic taste markers on food acceptance. Am. J. Clin. Nutr. 62, 506-511.

Drewnowski, A., 2001. The science and complexity of bitter taste. Nutr. Rev. 59, 163-169.

Drewnowski, A., Gomez-Carneros, C., 2000. Bitter taste, phytonutrients, and the consumer: a review. Am. J. Clin. Nutr. 72, 1424-1435.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, 74-96.

El-Ziney, M.G., Arneborg, N., Uyttendaele, M., Debevere, J., Jakobsen, M., 1998. Characterisation of growth and metabolite production of *Lactobacillus reuteri* during glucose/glycerol cofermentation in batch and continuous cultures. Biotech. Lett. 20, 913-916.

Fischer, U., Noble, A.C., 1994. The effect of ethanol, catechin concentration, and pH on sourness and bitterness of wine. Am. J. Enol. Vitic. 45, 6-10.

Glendenning, J.I., 1994. Is the bitter rejection response always adaptive? Physiol. Behav. 56, 1217-1227.

Gorga, A., Claisse, O., Lonvaud-Funel, A., 2002. Organisation of the encoding glycerol dehydratase of *Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans*. Sci. Aliments. 22, 151-160.

Greenhoff, K., Wheeler, R.E., 1981. Analysis of beer carbonyls at the part per billion level by combined liquid-chromatography and high-pressure liquid-chromatography. J. Inst. Brew. 87, 35-41.

Guldfeldt, L.U., Sorensen, K.I., Stroman, P., Behrndt, H., Williams, D., Johansen, E., 2001. Effect of starter cultures with a genetically modified peptidolytic or lytic system on cheddar cheese ripening. Int. Dairy J. 11, 373-382.

Habibi-Najafi, M.B., Lee, B.H., 1996. Bitterness in cheese: A review. Crit. Rev. Food Sci. Nutr. 36, 397-411.

Hasegawa, S., Brewster, L.C., Maier, V.P., 1973. Use of limonoate dehydrogenase of *Arthrobacter globiformis* for prevention or removal of limonin bitterness in citrus products. J. Food Sci. 38, 1153-1155.

Hasegawa, S., Pelton, V.A., 1983. Reduction of nomilin bitterness in citrus juices and juice serum with *Arthrobacter globiformis* cells immobilized in acrylamide gel. J. Agric. Food Chem. 31, 178-180.

Hasegawa, S., Maier, V.P., 1990. Biochemistry of limonoid citrus juice bitter principles and biochemical debittering processes. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 293-306.

Hayashi, K., Yamanaka, K., Toko, K., Yamafuji, K., 1990. Multichannel taste sensor with lipid membranes. Sens. Actuators B 2, 205-213.

Hoon, M.A., Adler, E., Lindemeier, J., Battey, J.F., Ryba, J.P., Zuker, C.S., 1999. Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. Cell 96, 541-551.

Ishibashi, N., Arita, Y., Kaneshisa, H., Kouge, K., Okai, H., Fukui, S., 1987. Bitterness of leucine-containing peptides. Agric. Biol. Chem. 51, 2389-2394.

Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria [Fermentation, food technology]. Antonie v. Leeuwenhoek 49, 209-224.

King, B.M., Moreau, N., 1996. A comparison of bitter perception in high-alcohol, low-alcohol and alcohol-free beer. J. Inst. Brew. 102, 419-425.

Lea, A.G.H., 1978. The analysis of cider phenolics. An. de la Nutr. Aliment. 32, 1051-1061.

Stellenbosch University http://scholar.sun.ac.za

Lea, A.G.H., Arnold, G.M., 1978. The phenolics of ciders: bitterness and astringency. J. Sci. Food Agric. 29, 478-483.

Lee, K.D., Lo, C.G., Warthesen, J.J., 1996. Removal of bitterness from the bitter peptides extracted from cheddar cheese with peptidases from *Lactococcus lactis* ssp. *cremoris* SK11[1]. J. Dairy Sci. 79, 1521-1528.

Lüthi-Peng, Q., Dileme, F.B., Puhan, Z., 2002. Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri*. Appl. Microbiol. Biotech. 59, 289-296.

Maga, J.A., 1990. Compound structure versus bitter taste. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 35-46.

Macheix, J., Fleuriet, A., Billot, J., 1990. Importance and roles of phenolic compounds in fruits. In: Macheix, J., Fleuriet, A., Billot, J. (Eds.), Fruit phenolics. CRC Press, London, pp. 265-271.

Margalith, P.Z., 1981. Flavour microbiology. CC Thomas Publishers, Springfield, Illinois.

Matsunami, H., Montmayeur, J.P., Buck, L.B., 2000. A family of candidate taste receptors in human and mouse. Nature 404, 601-604.

McCamey, D.A., Thorpe, T.M., McCarthy, J.P., 1990. Coffee bitterness. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 169-180.

Montmayeur, J., Matsunami, H., 2002. Receptors for bitter and sweet taste. Curr. Opin. Neurobiol. 12, 366-371.

Ney, K.H., 1979. Bitterness of peptides: amino acids composition and chain length. In: Boudreau, J.C. (Ed.), Bitterness of peptides: Amino acids composition and chain length. American Chemical Society, Washington D.C., pp. 149-173.

Noble, A.C., 1990. Bitterness and astringency in wine. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 145-156.

Noble, A.C., 1994. Bitterness in wine. Physiol. Behav. 56, 1251-1255.

Postel, W., Adam, L., 1983. Gas chromatographical characterisation of raspberry brandy. Deut. Lebensm.-Rundsch. 79, 117-122.

Pressman, D., Lucas, H.J., 1942. Hydration of unsaturated compounds. XI. Acrolein and acrylic acid. J. Am. Chem. Soc. 64, 1953-1957.

Puri, A., 1990. Removal of bitter compounds from citrus products by adsorption techniques. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 325-335.

Puri, M., Banerjee, U.C., 2000. Production, purification, and characterisation of the debittering enzyme naringinase. Biotechnol. Adv. 18, 207-217.

Ramalakshmi, K., Raghavan, B., 1999. Caffeine in coffee: Its removal. Why and how? Crit. Rev. Food Sci. Nutr. 39, 441-456.

Rentschler, H., Tanner, H., 1951. Das Bitterwerden der Rotweine. Mitt. Lebensm. Unters. Hygiene. 42, 463-475.

Robichaud, J.L., Noble, A.C., 1990. Astringency and bitterness of selected phenolics in wine. J. Sci. Food Agric. 53, 343-353.

Robins, R.J., Rhodes, M.J.C., Parr, A.J., Walton, N.J., 1990. Biosynthesis of bitter compounds. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 49-74.

Rouseff, R.L., 1990. Bitterness in food products: An overview. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 1-12.

Sanoner, P., Guyot, S., Marnet, N., Molle, D., Drilleau, J.P., 1999. Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.). J. Agric. Food Chem. 47, 4847-4853.

Sauvageot, N., Gouffi, K., Laplace, J.M., Auffray, Y., 2000. Glycerol metabolism in *Lactobacillus collinoides*: Production of 3-hydroxypropionaldehyde, a precursor of acrolein. Int. J. Food Microbiol. 55, 167-170.

Sauvageot, N., Muller, C., Hartke, A., Auffray, Y., Laplace, J., 2002. Characterisation of the diol dehydratase *pdu* operon of *Lactobacillus collinoides*. FEMS Microbiol. Lett. 209, 69-74.

Schiffman, S.S., Sennewald, K., Gagnon,J., 1981. Comparison of taste qualities and thresholds of D- and L-amino acids. Physiol. Behav. 27, 51-59.

Schmidt, R., 1990. Bitter compounds in dairy products. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 183-199.

Stellenbosch University http://scholar.sun.ac.za

Schoeman, H., Vivier, M.A., Du Toit, M., Dicks, L.M.T., Pretorius, I.S., 1999. The development of bactericidal yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (*ped*A) in *Saccharomyces cerevisiae.* Yeast 15, 647-656.

Schütz, H., Radler, F., 1984a. Anaerobic reduction of glycerol to propanediol-1,3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. System. Appl. Microbiol. 5, 169-178.

Schütz, H., Radler, F., 1984b. Propanediol-1,2-dehydratase and metabolism of glycerol of *Lactobacillus brevis*. Arch. Microbiol. 139, 366-370.

Shaw, P.E., Tatum, J.H., Wilson, C.W., 1984. Improved flavour of navel orange and grapefruit juices by removal of bitter components with b-cyclodextrin polymer. J. Agric. Food Chem. 32, 832-836.

Shaw, P.E., 1990. Cyclodextrin polymers in the removal of bitter compounds in citrus juices. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 309-323.

Shinoda, I., Fushimi, A., Kato, H., Okai, H., Fukui, S., 1985. Bitter taste of synthetic C-terminal tetradecapeptide of Bovine B-casein, H-Pro^{196}-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Ile-Ile-Val^{209}-OH, and its related peptides. Agric. Biol. Chem. 49, 2587-2596.

Slininger, P.J., Bothast, R.J., Smiley, K., 1983. Production of 3-hydroxypropionaldehyde from glycerol. Appl. Environ. Microbiol. 46, 62-67.

Smit, G., Verheul, A., Van Kranenberg, R., Ayad, E., Siezen, R., Engels, W., 2000. Cheese flavour development by enzymatic conversions of peptides and amino acids. Food Res. Int. 33, 153-160.

Solms, J., 1969. The taste of amino acids, peptides and proteins. J. Agric. Food Chem. 17, 689-695.

Sponholz, W.R., 1993. Wine spoilage by microorganisms. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, pp. 395-420.

Takagi, S., Toko, K., Wada, K., Ohki, T., 2001. Quantification of suppression of bitterness using an electronic tongue. J. Pharm. Sci. 90, 2042-2048.

Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. Appl. Environ. Microbiol. 56, 1195-1197.

Toko, K., 1998. A taste sensor. Meas. Sci. Technol. 9, 1919-1936.

Toko, K., 2000. Taste sensor. Sens. Actuators B 64, 205-215.

Veiga da Cunha, M., Foster, M.A., 1992. Sugar-glycerol cofermentations in lactobacilli: The fate of lactate. J. Bacteriol. 174, 1013-1019.

Wang, H., Provan, G.J., Helliwell, K., 2000. Tea flavonoids: Their functions, utilisation and analysis. Trends Food Sci. Tech. 11, 152-160.

Yamanishi, T., 1990. Bitterness/astringency in tea. In: Rouseff, R.L. (Ed.), Bitterness in Foods and Beverages. Elsevier Science Publishers, Amsterdam, pp. 159-167.

http://www.coffeeresearch.com

http://www.coffeereview.com

http://www.ineedcoffee.com

http://www.jgi.doe.gov/JGI_microbial/html

http://www.worldwidemart.com

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CHAPTER 3

RESEARCH RESULTS

Isolation, identification and characterisation of glycerol-degrading lactic acid bacteria from South African red wines

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3. RESEARCH RESULTS

Isolation, identification and characterisation of glycerol-degrading lactic acid bacteria from South African red wines

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Abstract

Two-hundred-and-forty lactic acid bacteria (LAB) were isolated from Pinotage, Merlot and Cabernet Sauvignon grapes and wine samples obtained from five wineries situated in the Western Cape, South Africa. In 2001, the LAB population on Cabernet Sauvignon grapes ranged from 10^2 to 10^4 cfu/ml. In both 2001 and 2002, the LAB population on Pinotage and Merlot grapes ranged from 10^2 to 10^3 cfu/ml. The Cabernet Sauvignon grapes carried a LAB population of between 10^3 and 10^4 cfu/ml in 2002. The number of LAB in Cabernet Sauvignon wine after alcoholic fermentation (AF) ranged from 10^3 to 10^5 cfu/ml in 2001 and from 10^2 to 10^5 cfu/ml in 2002. After AF, Pinotage and Merlot wines carried a LAB population ranging from 10^2 to 10^4 cfu/ml in 2001. In 2002, the number of LAB in Merlot and Pinotage ranged from 10^2 to 10^5 cfu/ml. The strains could be classified into the three metabolic groups based on cell shape and the ability to produce CO_2 from glucose and/or gluconate. Twenty-eight strains of the obligately heterofermentative group were identified as *Oenococcus oeni* and four as *Leuconostoc mesenteroides* by means of species-specific primers. Fifteen strains were identified as *Lactobacillus brevis* and another fifteen strains as *Lactobacillus hilgardii.* Ninety-eight strains were identified as *Lactobacillus plantarum*, three as *Lactobacillus paraplantarum* and 12 as *Lactobacillus pentosus* by means of a multiplex PCR assay using species-specific primers. Primers specific for *Lactobacillus paracasei* were used to identify 28 strains. Two strains of the obligately homofermentative group were identified as *Pediococcus acidilactici* and 35 strains were taken as *Pediococcus* spp., based on cell morphology. Twenty-six of the isolated strains were found to contain the glycerol dehydratase gene sequence. One strain was isolated from Pinotage grapes, 15 from Pinotage wine tanks, one from Cabernet Sauvignon grapes, four from Cabernet Sauvignon wine tanks and five from Merlot wine tanks. All the strains tested were able to degrade glycerol almost completely. Preliminary results suggest that the GD-possessing strains exhibit an inhibitory activity against Gram-positive and Gram-negative bacteria, and that this antimicrobial activity is similar to that of reuterin, which is produced by *Lb. reuteri.*

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3.1 INTRODUCTION

Grapes and wine are known to be part of the natural habitats of lactic acid bacteria (LAB), and several species of LAB belonging to the genera *Lactobacillus*, *Oenococcus*, *Leuconostoc* and *Pediococcus* are commonly associated with the wine environment (Lafon-Lafourcade et al., 1983; Davis et al., 1985; Wibowo et al., 1985; Ribéreau-Gayon et al., 2000; Du Toit and Pretorius, 2000). Certain LAB are able to degrade glycerol, which is one of the major products of yeast metabolism during the alcoholic fermentation of wine. These LAB possess a glycerol dehydratase enzyme that converts glycerol to 3-hydroxypropionaldehyde (3-HPA) under anaerobic conditions (Sobolov and Smiley, 1959; Schütz and Radler, 1984). This 3-HPA can then be reduced to 1,3-propanediol (1,3-PD) by a 1,3-PD oxidoreductase, or it can be dehydrated spontaneously to form acrolein. The last reaction is favoured in conditions of high temperature and low pH (Pressman and Lucas, 1942; Slininger et al., 1983; Schütz and Radler, 1984; Claisse and Lonvaud-Funel, 2000; Du Toit and Pretorius, 2000; Sauvageot et al., 2000). Acrolein can then combine with the phenolic groups of anthocyanins to form a bitter complex (**Fig. 3.1**) (Rentschler and Tanner, 1951). Voisenet (1910) first associated the acrolein taint with bitterness and, since then, the bitter off-flavour due to acrolein has been associated with apple ciders, beer, brandy, rum and whisky (Greenhoff and Wheeler, 1981; Postel and Adam, 1983; Sauvageot et al., 2000, Claisse and Lonvaud-Funel, 2001). However, very little is known about the acrolein content in wine, although it appears that approximately 10 mg/l is sufficient to cause bitterness (Margalith, 1981).

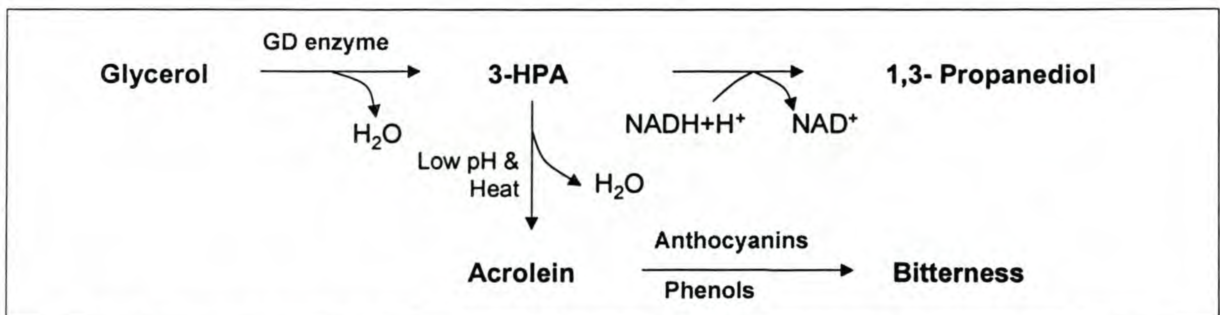

Fig. 3.1. Metabolic pathway from glycerol to 1,3-propanediol via the intermediate 3-HPA.

The coenzyme B12-dependent glycerol dehydratase has been described in *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus reuteri* and, recently, in *Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans* (Schütz and Radler, 1984; Talarico and Dobrogosz, 1990; Gorga et al., 2002). Sauvageot et al. (2002) have purified and characterised the diol dehydratase of *Lb. collinoides*. These LAB all lack the oxidative pathway of glycerol degradation and therefore cannot use glycerol as sole carbon source, but only as an external electron acceptor. The glycerol dehydratase has also been cloned and sequenced in *Clostridium pasteurianum*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter freundii* and *Salmonella enterica* serovar *typhimurium* (Daniel et al., 1999). The

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enzymes glycerol dehydratase and diol dehydratase are isofunctional (Daniel et al., 1999) and there seems to be some confusion as to the naming of these enzymes. However, comparing the results of different studies, it seems that the two enzymes exhibit the same $\alpha_2\beta_2\gamma_2$ composition, and that both have high homology to other characterised dehydratases (Gorga et al., 2002; Sauvageot et al., 2002). The two enzymes differ, however, in their substrate specificities, with the diol dehydratase having a higher affinity for 1,2-propanediol, and the glycerol dehydratase having a higher affinity for glycerol (Daniel et al., 1999). The characterised dehydratases have an $\alpha_2\beta_2\gamma_2$ composition where α, β and γ are the large, medium and small subunits, respectively. These subunits are encoded by three contiguous open reading frames (ORFs) (Daniel et al., 1999). The dehydratase of *Lb. reuteri* was found to have a similar molecular mass as the dehydratases of other genera, but it is composed of four identical subunits of 52 kDa each (Talarico and Dobrogosz, 1990). The dehydratase of *Lb. collinoides* has a heterotrimer composition, with the subunits having molecular masses of 63, 25 and 19 kDa for the α, β and γ subunits respectively (Sauvageot et al., 2002). The genomes of a few LAB, viz. *Leuconostoc mesenteroides*, *Lactobacillus gasseri*, *Oenococcus oeni* and *Pediococcus pentosaceus*, have recently been sequenced and, according to the draft sequences, none of these species (or rather, the specific strains) possesses the GD enzyme sequence (http://www.jgi.doe.gov/JGI_microbial/html).

Reuterin is a broad spectrum antimicrobial substance that is active against several Gram-positive and Gram-negative bacteria, fungi and yeasts and is produced by *Lb. reuteri* when grown on a glycerol-containing medium (Talarico et al., 1988). Talarico and Dobrogosz (1989) have shown that reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA. It is believed that this species is unique in its ability to accumulate and excrete 3-HPA (Talarico and Dobrogosz, 1990), but none or little research has been done to investigate this ability in other genera of *Lactobacilli*.

Since the GD enzyme is strain dependent, it is important to determine what percentage of strains occurring in wine fermentations possess this enzyme. Several studies have isolated one specific LAB species from cider that has the ability to degrade glycerol, namely *Lb. collinoides* (Carr and Davies, 1972; Claisse and Lonvaud-Funel, 2000; Sauvageot et al., 2000). To date, no specific studies have been conducted specifically on wine-isolated LAB to determine the presence of this GD enzyme.

The aims of this study were to enumerate and identify the naturally occurring LAB on South African Pinotage, Merlot and Cabernet Sauvignon grapes and to follow their development during the fermentation process. The isolated LAB were also screened for the presence of the GD enzyme sequence and the positive LAB strains were assessed for their ability to degrade glycerol, as well as screened for possible antimicrobial activity. This information can contribute to a better understanding of wine spoilage due to bitterness.

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3.2 MATERIALS AND METHODS

3.2.1 Collection of samples

During the 2001 and 2002 harvest seasons, grape and wine samples from three grape varieties (Pinotage, Merlot and Cabernet Sauvignon) were collected from five different commercial wineries (I to V) situated in the Western Cape region, South Africa. The grapes were harvested aseptically, crushed in sterile Ziploc bags and left at room temperature for two hours before plating out. Wine samples were collected in sterile bottles at two stages of the winemaking process, namely at the end of the alcoholic fermentation [before inoculation or onset of malolactic fermentation (MLF)] and during MLF.

3.2.2 Isolation and enumeration of lactic acid bacteria

Grape juice and wine samples were diluted 10-fold in quarter strength sterile Ringer's solution (Merck, Germany) and mixed thoroughly. Thereafter, 100 μl of each dilution were plated out, in duplicate, on the following selective media: (i) MRS agar (Biolab, Merck, South Africa), for the enumeration of lactobacilli and pediococci, and (ii) MRS agar with 20% apple juice agar (MRSA) (Bartowski and Henschke, 1999), at pH 5.5, for the enumeration of *O. oeni*. Tomato juice agar (TJA), consisting of (per litre) 10 g peptone, 10 g lactalbumin, enzymatic hydrolysate (Sigma-Aldrich, Germany), 400 ml tomato juice and 2.5% agar, at pH 6.0 to 6.2, was used for pour-plating 1 ml portions of the undiluted and diluted juice, also for the enumeration of *O. oeni*. All the plates contained 50 mg/l Actistab (Gist-Brocades, Netherlands) for the inhibition of yeast and fungi, and 25 mg/l Kanamycin sulphate (Boehringer Mannheim, Germany) for the inhibition of acetic acid bacteria. The plates were incubated in anaerobic jars containing an AnaerocultA gas-generating kit (Merck, Germany) at 30°C for 48 h (MRS plates) or up to 14 days (TJA and MRSA plates).

3.2.3 Preliminary identification of lactic acid bacteria

Approximately six colonies per plate were selected randomly on the basis of morphological differences, viz. size, form, colour, elevation and border. These colonies were then restreaked on the corresponding selective media to obtain pure cultures. Purified cultures were maintained in 30% glycerol (v/v) at –80°C. The Gram reaction and catalase activity tests were performed on the pure cultures and used as primary selection criteria (Schillinger and Lücke, 1987). Catalase-negative and Gram-positive bacteria were further divided into rods, cocci and coccobacilli on the basis of the microscopic appearance of the Gram-stained preparations. These strains were screened for the production of CO_2 from glucose and gluconate. Modified MRS broth (without beef extract and tri-ammonium citrate), containing either glucose or sodium gluconate, was used. The strains were classified into the three

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metabolic groups, namely obligately homofermentative (no CO_2 from glucose or gluconate), facultatively heterofermentative (CO_2 from gluconate) and obligately heterofermentative (CO_2 from glucose and gluconate). The method of Schillinger and Lücke (1987) was used to test for the production of ammonia from arginine. The strains were tested for the production of D(-)- and L(+)-lactic acid by means of the Roche enzymatic kit (Mannheim, Germany). Strains belonging to the genera *Leuconostoc* and *Oenococcus* were distinguished from strains belonging to the genus *Lactobacillus* by the configuration of the lactic acid produced and the production of NH_3 from arginine (Holzapfel and Schillinger, 1992).

3.2.4 Identification of the bacteria to species level

The bacteria were identified to species level by means of colony PCR with different species-specific primers. The primers used in this study are listed in **Table 3.1**. In each instance, the corresponding type strain was used as a positive control. The bacteria were all grown on either MRS or MRSA plates at 30°C to obtain single colonies. A Biometra TRIO-Thermoblock machine was used for all PCR reactions, except for the *Pediococcus* PCR, for which a Hybaid PCR Express Thermal Cycler was used. Each 50 µl PCR reaction, except the ones for *Lb. brevis* and *Lb. hilgardii*, contained one bacterial colony as template. The PCR mixture with the colony was boiled for ten minutes before adding 0.5 units of DNA Taq polymerase (Bioline, Germany). The DNA for the *Lb. brevis* and *Lb. hilgardii* PCR was obtained by suspending a single colony in 100 µl of sterile H_2O with glass beads and vortexing it for one minute, before adding 5 µl of this suspension to the PCR mixture. The PCR products were separated on a 2% (w/v) agarose gel and a 100 bp ladder, DNA molecular weight marker XIV (Roche, Germany), was used.

3.2.5 Screening of the isolates for the presence of the glycerol dehydratase enzyme

The primers GD1 and GD2 (Claisse and Lonvaud-Funel, 2001) were used to detect the presence of the glycerol dehydratase gene sequence in the isolated LAB. A single colony was used as template for the PCR reaction. A Biometra TRIO-Thermo block machine was used. The PCR reaction was performed in a total volume of 50 µl at an annealing temperature of 57°C, and the PCR products were separated on a 2% (w/v) agarose gel. A 100 bp ladder DNA molecular weight marker XIV (Roche, Germany) was used.

3.2.6 Isolation of genomic DNA

Genomic DNA was extracted from two of the LAB strains according to the procedure of Dellaglio et al. (1973) with a slight modification. The cells were incubated for 30 min at 55°C after being resuspended in solution A and lysozyme.

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Table 3.1
Species-specific primers and conditions used in this study

Target organism	Primer pair	Primer concentration	$MgCl_2$	Annealing temperature	Reference
O. oeni	On1	2.5 µM	1 mM	60°C	Zapparoli et al., 1998
	On2	2.5 µM			
Leuc. mesenteroides	Lmesf	2.5 µM	3 mM	57°C	Lee et al., 2000
	Lmesr	2.5 µM			
Lb. plantarum	planF	2 µM	1 mM	56°C	Torriani et al., 2001
Lb. paraplantarum	paraF	2.5 µM			
Lb. pentosus	pentF	2.5 µM			
	pREV	2.5 µM			
Lb. paracasei	Y2	3 µM	3 mM	48°C	Ward & Timmins, 1999
	para	3 µM			
Lb. brevis	Br1	2 µM	1.5 mM	53°C	Guarneri et al., 2001
	Br2	2 µM			
Lb. hilgardii	H2	2 µM	3 mM	36°C	Sohier et al., 1999
	8623	2 µM			
Ped. pentosaceus	PpeF	10 µM	3 mM	70°C Decreased by 1°C every second cycle until 67°C	Mora et al., 1997
Ped. acidilactici	PacF	10 µM			
	PuR	10 µM			
	ldhDF	10 µM			
	ldhDR	10 µM			

3.2.7 DNA sequence analysis

A standard freeze-squeeze procedure was used to purify amplified DNA from agarose gels. The DNA fragments were cloned into plasmid pGEM-T Easy (Plasmid pGEM-T Easy System I, Promega) and sent for sequencing.

3.2.8 Glycerol metabolism

LAB strains that tested positive with the GD1 and GD2 primers were subcultured twice in a modified MRS medium (Claisse and Lonvaud-Funel, 2000). The LAB strains were then inoculated in the assay medium at 10^5 cfu/ml. All strains were incubated at 30°C without shaking.

The base for the assay medium consisted of (per litre): 5 g casamino acids (Difco, USA), 4 g yeast extract, 0.55 g KH_2PO_4, 0.425 g KCl, 0.125 g $MgSO_4 \cdot 7H_2O$, 0.125 g $CaCl \cdot 2H_2O$, and 0.003 g $MnSO_4 \cdot H_2O$. The assay media were obtained by adding glycerol, glucose and fructose (**Table 3.2**).

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Table 3.2
Composition of assay media

Medium	Glycerol	Glucose	Fructose	pH
1	10 g/l	1 g/l	2 g/l	4.8
2	10 g/l	-	-	4.8
3	-	1 gl	2 g/l	4.8
4	10 g/l	1 g/l	2 g/l	3.5
5	10 g/l	3 g/l	-	4.8
6	10 g/l	-	3 g/l	4.8

Glycerol and glucose were measured by means of the Roche enzymatic kit and the Glucose Trinder kit (Sigma-Aldrich, USA) respectively. The assays were performed according to the instructions of the manufacturers. Samples were taken after 24 h and 48 h of incubation. Viable cells were counted on MRS plates.

3.2.7 Preliminary screening of GD-possessing strains for antimicrobial activity

LAB strains possessing the GD gene sequence were screened for antimicrobial activity against a few Gram-positive and Gram-negative bacteria. The screening method was based on the spot-on-lawn method used for detecting bacteriocin production (Schillinger and Lücke, 1989). The LAB strains were subcultured twice in a modified MRS medium (Claisse and Lonvaud-Funel, 2000). Thereafter, 10 μl of each isolate were spotted on a modified MRS plate, which was then incubated at 30°C for 24 h. The modified MRS agar had the same base composition as the assay medium in section 3.2.6, except that the KH_2PO_4 concentration was increased to 8 g/l and to it was added (per litre): 5 g glycerol, 8.7 g $K_2HPO_4{\cdot}4H_2O$ and 15 g agar. The bacteria used as indicator organisms were *Esherichia coli* DH5α, *Bacillus cereus* LMG 13569, *Acetobacter liquefaciens* DSM 5603^T, *Gluconobacter oxydans* DSM 7145^T, *Lb. fermentum* LMG 13554, *Lb. plantarum* DSM 20174, *Leuc. mesenteroides* NCDO 529, *Ped. pentosaceus* LMG 13562 and *Ped. acidilactici* PAC 1·0. *E. coli* DH5α and *B. cereus* were subcultured twice in Luria-Bertani (LB) medium (Biolab, Merck, South Africa) and yeast peptone dextrose medium (Biolab, Merck, South Africa) respectively. *E. coli* was incubated at 37°C and *B. cereus* at 30°C. The acetic acid bacteria (AAB) were cultured in mannitol medium, consisting of (per litre): 25 g mannitol, 5 g yeast extract, and 3 g peptone. All of the LAB were cultured in MRS medium.

After 24 h, the colonies were overlayed with soft agar spiked with the respective indicator organisms and the plates were incubated at 30°C for the LAB, AAB and *B. cereus* and at 37°C for *E. coli*. All the plates were examined for zone formation after 24 h.

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3.3 RESULTS

3.3.1 Enumeration of LAB

The numbers of LAB obtained from the different grape and wine samples (after alcoholic fermentation) collected during the 2001 and 2002 harvest seasons are shown in **Fig. 3.2** and **Fig. 3.3** respectively. The mean counts of the MRS and TJA or MRS and MRSA media are presented in these figures, since there were no significant differences between the results obtained from the individual media. Wine samples during MLF were collected only during the 2002 harvest season. The LAB counts obtained on MRSA medium for all three cultivars ranged from 10^4 to 10^8 cfu/ml (**Table 3.3**).

3.3.1 Preliminary identification

A total of 240 Gram-positive and catalase-negative rods, cocci and coccobacilli were isolated from Pinotage, Merlot and Cabernet Sauvignon grapes and during various stages of the fermentation process. The strains were all given a number, eg. 4, 30, 76.1, 126.1, as they were isolated. The LAB strains were divided into the three metabolic groups (facultatively heterofermentative, obligately heterofermentative and obligately homofermentative) on the basis of their ability to produce CO_2 from glucose and/or gluconate (**Fig. 3.4**). Thirty-two of the obligately heterofermentative strains had a coccobacilli morphology, produced mainly D(-)-lactic acid from glucose and produced no NH_3 from arginine. These strains were presumptively taken as belonging to either the genus *Leuconostoc* or *Oenococcus*. Thirty of the obligately heterofermentative strains were rods, most of them produced NH_3 from arginine and they were classified as belonging to the genus *Lactobacillus*. The 37 obligately homofermentative strains were cocci, and seven of these strains were able to produce NH_3 from arginine. These strains all had the typical morphology of *Pediococcus* and tetrad formation could clearly be seen microscopically. Twenty-eight of the facultatively heterofermentative strains produced mainly L(+)-lactic acid. One-hundred-and-thirteen of the facultatively heterofermentative strains produced D (-)- as well as L(+)-lactic acid. All the facultatively heterofermentative strains were presumptively taken as belonging to the genus *Lactobacillus*.

3.3.2 Identification to species level

The 32 coccobacilli strains of the obligately heterofermentative group were screened with species-specific primers for *Leuc. mesenteroides* and *O. oeni*, respectively. Twenty-eight of these strains generated a 1 025 bp fragment with the *O. oeni*-specific primers and were identified as *O. oeni* (**Fig. 3.5**). The PCR reaction with the *Leuc. mesenteroides*-specific primers generated a 1 150 bp fragment for four of the strains (**Fig. 3.6**).

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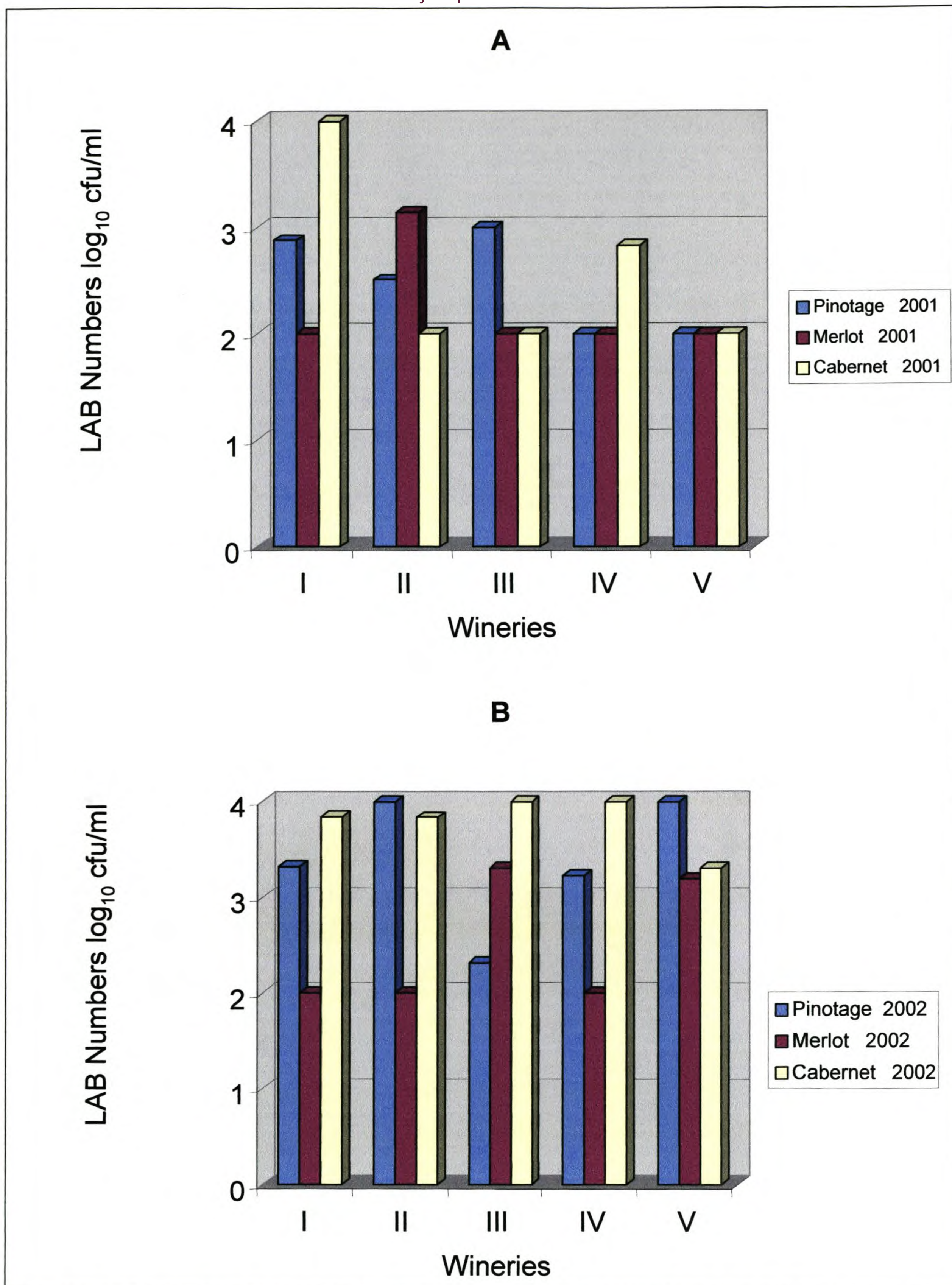

Fig. 3.2. LAB numbers found on Pinotage, Merlot and Cabernet Sauvignon grapes during the 2001 (A) and 2002 (B) harvest seasons.

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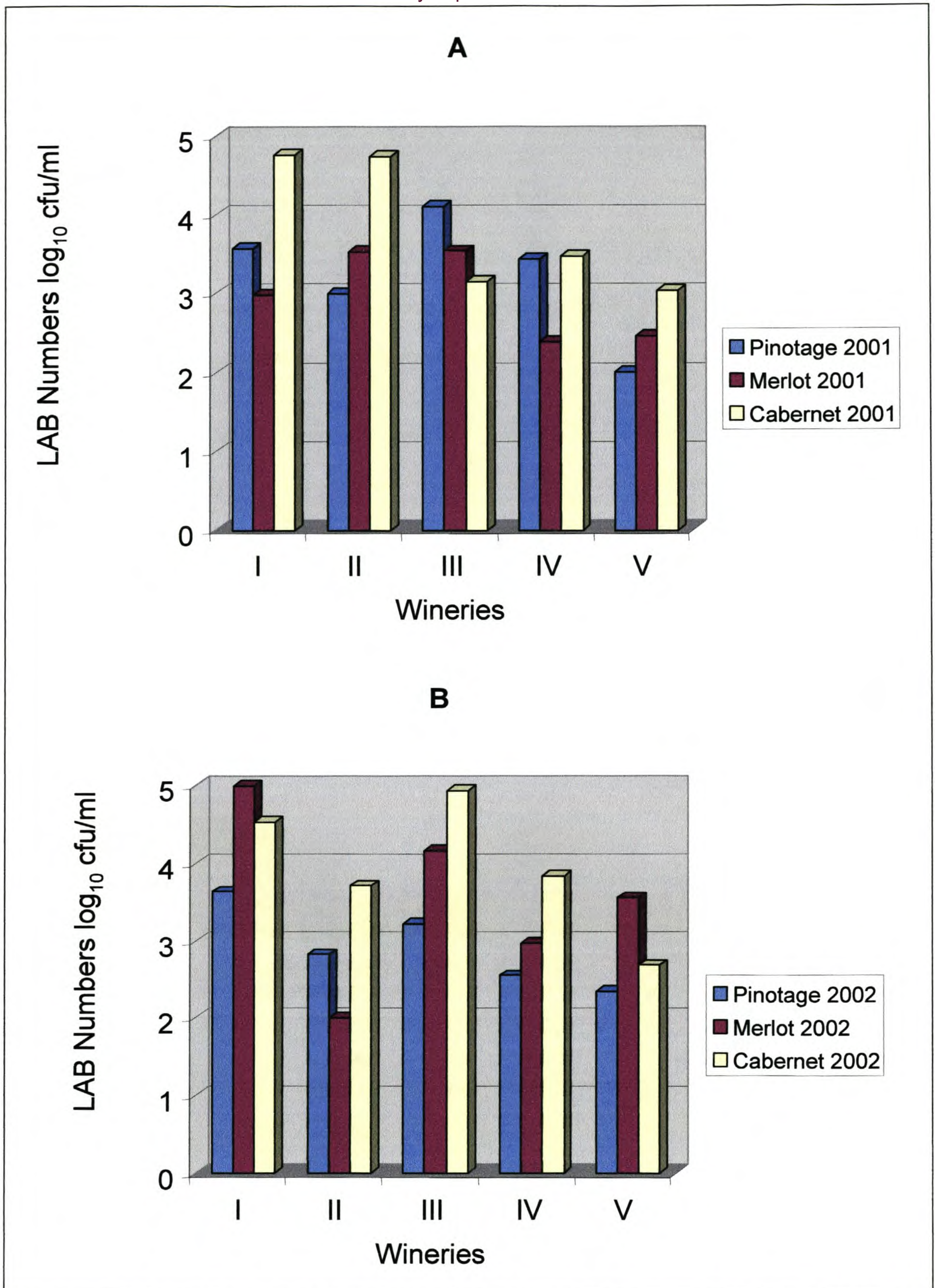

Fig.3.3. LAB numbers found in Pinotage, Merlot and Cabernet Sauvignon wine after alcoholic fermentation in 2001 (A) and 2002 (B), before inoculation or onset of MLF.

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Table 3.3
Mean LAB counts obtained from wine samples undergoing MLF during the 2002 harvest season

Winery	LAB counts (cfu/ml)		
	Pinotage	Merlot	Cabernet Sauvignon
I	9.50×10^5	3.90×10^8	ND
II	2.5×10^6	ND	ND
III	9.5×10^5	8.90×10^7	7.89×10^6
IV	8.90×10^6	ND	7.65×10^4
V	2.87×10^4	4.30×10^4	1.05×10^5

ND – counts not determined.

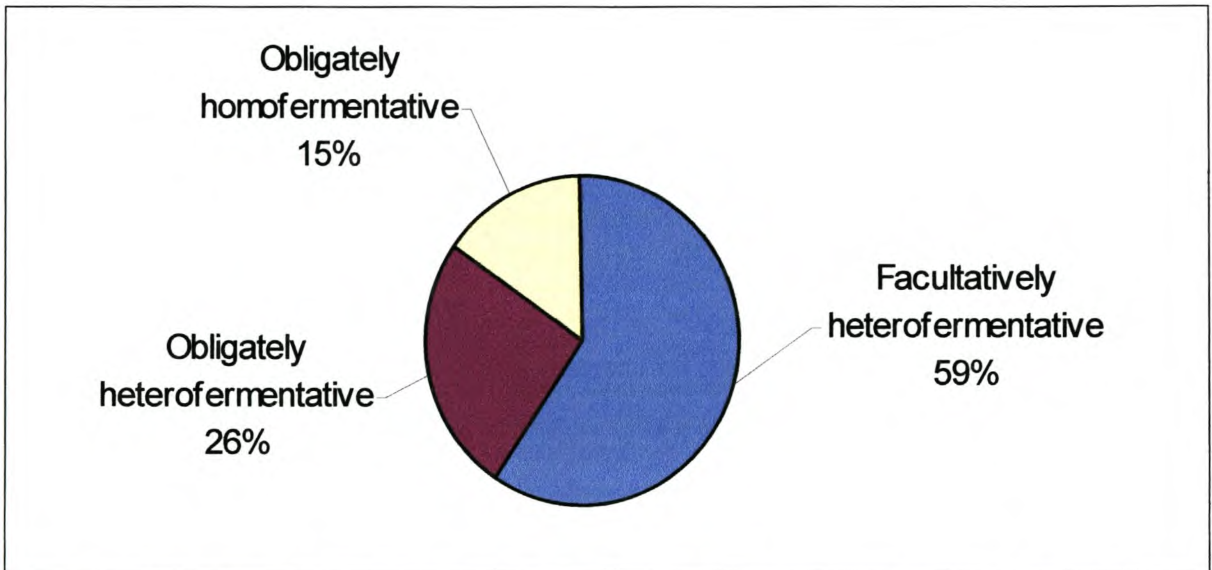

Fig. 3.4. Grouping of LAB strains on the basis of their ability to produce CO_2 from glucose and/or gluconate.

Fig. 3.5. PCR products obtained with species-specific primers for *O. oeni* (1 025 bp). M, DNA molecular weight marker XIV. Lane 1: *O. oeni* DSM 20252^T, lane 2: 76.1, lane 3: 170.2, lane 4: 164.3, lane 5: 164.2, lane 6: 126.2, lane 7: 169, lane 8: 135.1, lane 9 192.1, lane 10: 192, lane 11: 192.3, lane 12: 190.2, lane 13: 192.2, lane 14: 155.1, lane 15: 126, lane 16: 172.5, lane 17: 172.4, lane 18: 172.6, lane 19: 167, lane 20: 165.2, lane 21: 182.3, lane 22: 180, lane 23: 180.1, lane 24: 180.3, lane 25: 180.2, lane 26: 152, lane 27: 152.1, lane 28: 152.2, lane 29: 151.

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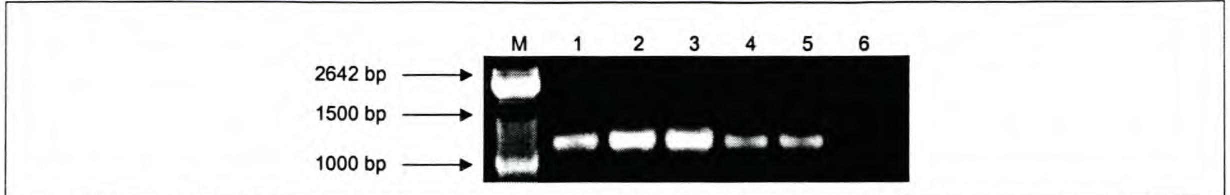

Fig. 3.6. PCR products obtained with species-specific primers for *Leuc. mesenteroides* (1 150 bp). M, DNA molecular weight marker XIV. Lane 1: *Leuc. mesenteroides* Ta33a, lane 2: 4, lane 3: 5, lane 4: 5.1, lane 5: 3.2, lane 6: *O. oeni* DSM 20252^T.

The 30 rods of the obligately heterofermentative group were screened with species-specific primers for *Lb. brevis* and *Lb. hilgardii*. Fifteen of these strains produced a 1 340 bp fragment with the primers for *Lb. brevis* (**Fig. 3.7**). A fragment of approximately 2 642 bp was generated by the remaining 15 strains with the primers for *Lb. hilgardii* (**Fig. 3.8**).

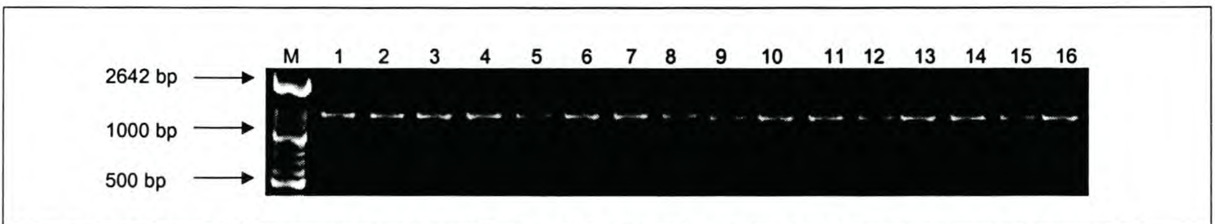

Fig. 3.7. PCR products obtained with species-specific primers for *Lb. brevis* (1 340 bp). M, DNA molecular weight marker XIV. Lane 1: *Lb. brevis* ATCC 14869^T, lane 2: 81.1, lane 3: 3.3, lane 4: 111, lane 5: 116, lane 6: 116.2, lane 7: 117.1, lane 8: 130.2, lane 9: 117, lane 10: 130.6, lane 11: 108.2, lane 12: 89, lane 13: 117.2, lane 14: 130.3, lane 15: 130.4, lane 16: 116.3.

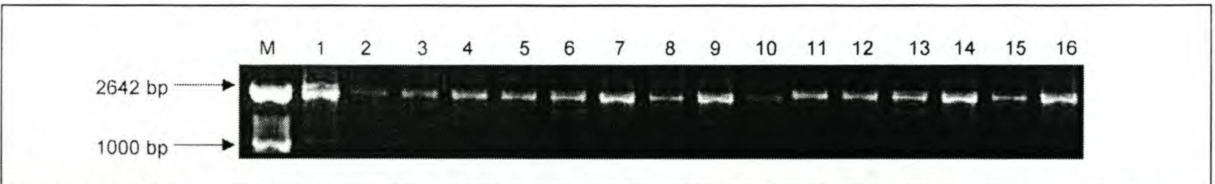

Fig. 3.8. PCR products obtained with species-specific primers for *Lb. hilgardii* (2 642 bp). M, DNA molecular weight marker XIV. Lane 1: *Lb. hilgardii* DSM 20176, lane 2: 58, lane 3: 67.1, lane 4: 87, lane 5: 81, lane 6: 89.3, lane 7: 89.1, lane 8: 89.2, lane 9: 87.2, lane 10: 87.1, lane 11: 3, lane 12: 16.1, lane 13: 17, lane 14: 51, lane 15: 51.2, lane 16: W4.

Fig. 3.9. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 1: *Lb. plantarum* DSM 20174, lane 2: *Lb. paraplantarum* DSM 10667, lane 3: *Lb. pentosus* DSM 20314, lane 4: 81.2, lane 5: 82.1, lane 6: 82.2, lane 7: 82, lane 8: 6.1, lane 9: 75, lane 10: 100, lane 11: 100.1, lane 12: 69.1, lane 13: 69, lane 14: 50, lane 15: 14, lane 16: 14.1, lane 17: 68, lane 18: 83, lane 19: 43.1, lane 20: 43, lane 21: 55, lane 22: 55.1.

A multiplex PCR assay using species-specific primers for *Lb. plantarum* (318 bp), *Lb. pentosus* (218 bp) and *Lb. paraplantarum* (107 bp) was used to screen the

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strains in the facultatively heterofermentative group. A 318 bp fragment was generated by 98 strains (**Fig. 3.8** – **Fig. 3.13**), three strains generated a 107 bp fragment (**Fig. 3.14**) and 12 strains generated a 218 bp fragment (**Fig. 3.15**).

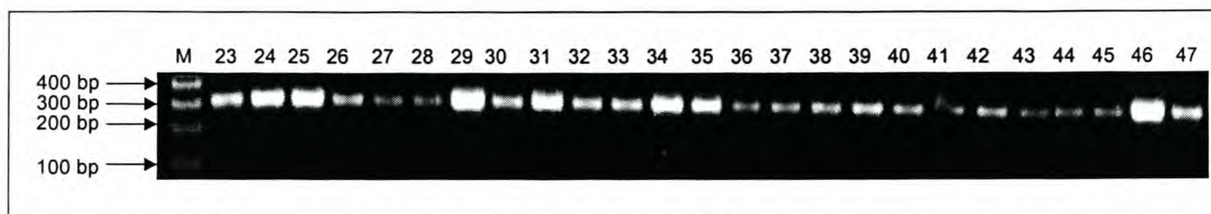

Fig. 3.10. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 23: *Lb. plantarum* DSM 20174, lane 24: 88.1, lane 25: 71, lane 26: 71.1, lane 27: 73.2, lane 28: 73.1, lane 29: 79.1, lane 30: 79.3, lane 31: 56, lane 32: 31, lane 33: 80, lane 34: 80.2, lane 35: 86, lane 36: 85.2, lane 37: 85, lane 38: 85.1, lane 39: 66, lane 40: 66.1, lane 41: 40.3, lane 42: 76.2, lane 43: 78.1, lane 44: 78, lane 45: 65, lane 46: 65.1, lane 47: 36.

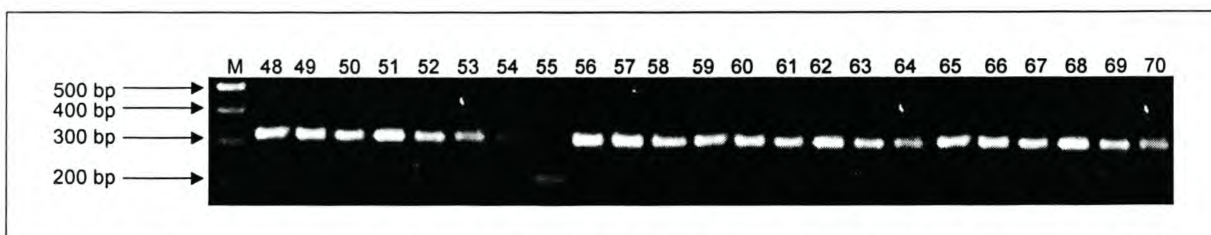

Fig. 3.11. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 48: *Lb. plantarum* DSM 20174, lane 49: 36.1, lane 50: 92, lane 51: 97, lane 52: 39.1, lane 53: 45, lane 54: 85, lane 55: 120.3, lane 56: 2.1, lane 57: 21.1, lane 58: 94.1, lane 59: 91, lane 60: 66.1, lane 61: 70, lane 62: 77.1, lane 63: 71.1, lane 64: 41.1, lane 65: 131, lane 66: 130, lane 67: 120.1, lane 68: 120, lane 69: 122.7, lane 70: 124.2.

The remaining 28 strains of the facultatively heterofermentative group generated a 290 bp fragment with the species-specific primers for *Lb. paracasei* (**Fig. 3.16** and **Fig. 3.17**).

Two strains of the obligately homofermentative group generated two fragments of 449 bp and 872 bp in the multiplex PCR assay with species-specific primers for *Ped. pentosaceus* (872 bp) and *Ped. acidilactici* (449 bp + 872 bp) (**Fig. 3.18**). The number and stage of isolation of each species are shown in **Table 3.4**.

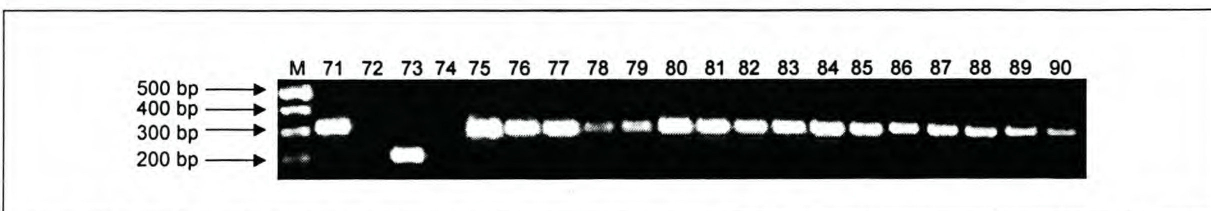

Fig. 3.12. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 71: *Lb. plantarum* DSM 20174, lane 72: *Lb. paracasei* DSM 5622, lane 73: *Lb. pentosus* DSM 20314, lane 74: *Lb. brevis* ATCC 14869^T, lane 75: 119, lane 76: 116.4, lane 77: 107.4, lane 78: 107.5, lane 79: 107.2, lane 80: 109.3, lane 81: 109, lane 82: 109.2, lane 83: 113.4, lane 84: 113.1, lane 85: 78, lane 86: 65.1, lane 87: 102.1, lane 88: 104.2, lane 89: 104, lane 90: 103.

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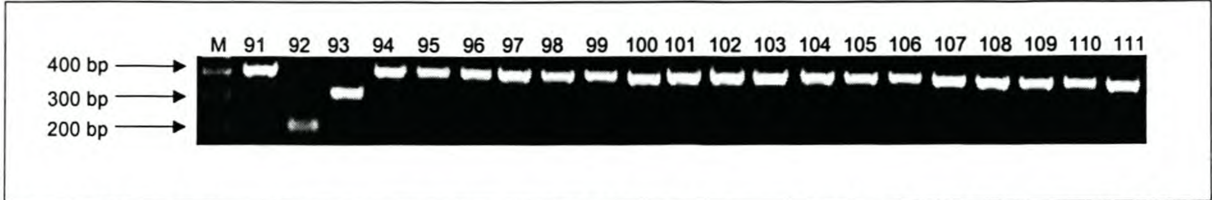

Fig. 3.13. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 91: *Lb. plantarum* DSM 20174, lane 92: *Lb. paraplantarum* DSM 10667, lane 93: *Lb. pentosus* DSM 20314, lane 94: 103.1, lane 95: 48, lane 96: 32.3, lane 97: 107, lane 98: 130.1, lane 99: 130.2, lane 100: 130.3, lane 101: 130.4, lane 102: 130.6, lane 103: 131.1, lane 104: 131.2, lane 105: 131.3, lane 106: 106.1, lane 107: 106.4, lane 108: 106.5, lane 109: 106.6, lane 110: 106.6, lane 111: 106.8.

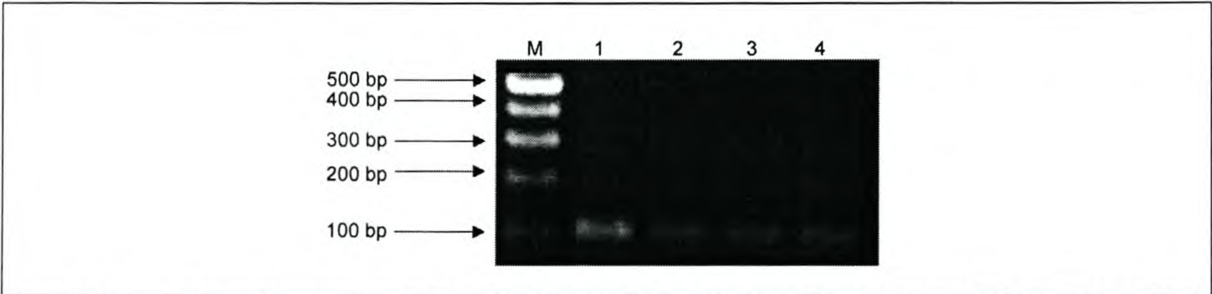

Fig. 3.14. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 1: *Lb. paraplantarum* DSM 10667, lane 2: 107.1, lane 3: 108.5, lane 4: 101.

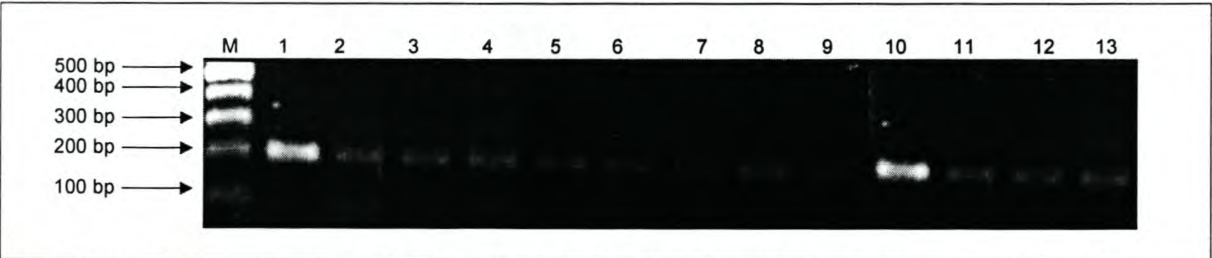

Fig. 3.15. PCR products obtained with multiplex PCR specific for *Lb. plantarum* (318 bp), *Lb. paraplantarum* (107 bp) and *Lb. pentosus* (218 bp). M, DNA molecular weight marker XIV. Lane 1: *Lb. pentosus* DSM 20314, lane 2: 120.3, lane 3: 53, lane 4: 52, lane 5: 52.1, lane 6: 46, lane 7: 42, lane 8: 42.2, lane 9: 42.1, lane 10: 79.2, lane 11: 53.1, lane 12: 65.1, lane 13: 113.

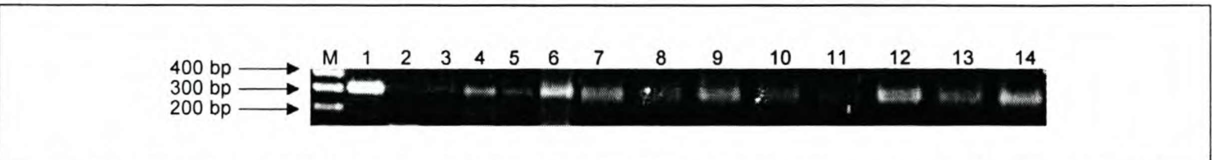

Fig. 3.16. PCR products obtained with species-specific primers for *Lb. paracasei* (290 bp). M, DNA molecular weight marker XIV. Lane 1: *Lb. paracasei* DSM 5622, lane 2: 44, lane 3: 79, lane 4: 83.1, lane 5: 84, lane 6: 54, lane 7: 39, lane 8: 77, lane 9: 50.2, lane 10: 29.1, lane 11: 90, lane 12: 101.1, lane 13: 104.1, lane 14: 105.8.

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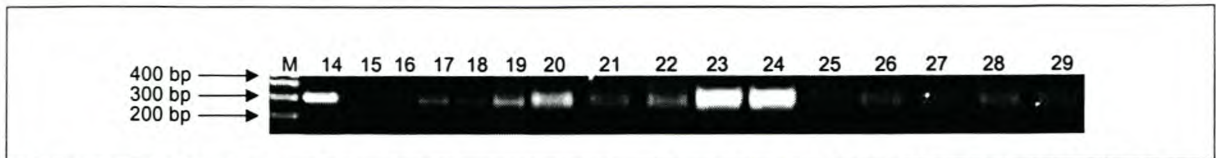

Fig. 3.17. PCR products obtained with species-specific primers for *Lb. paracasei* (290 bp). M, DNA molecular weight marker XIV. Lane 14: *Lb. paracasei* DSM 5622, lane 15: 29.2, lane 16: 30, lane 17: 36.2, lane 18: 124.1, lane 19: 124.2, lane 20: 129.7, lane 21: 129.3, lane 22: 146.1, lane 23: 152.2, lane 24: 163.1, lane 25: 165.2, lane 26: 180, lane 27: 180.2, lane 28: 181, lane 29: 10.1.

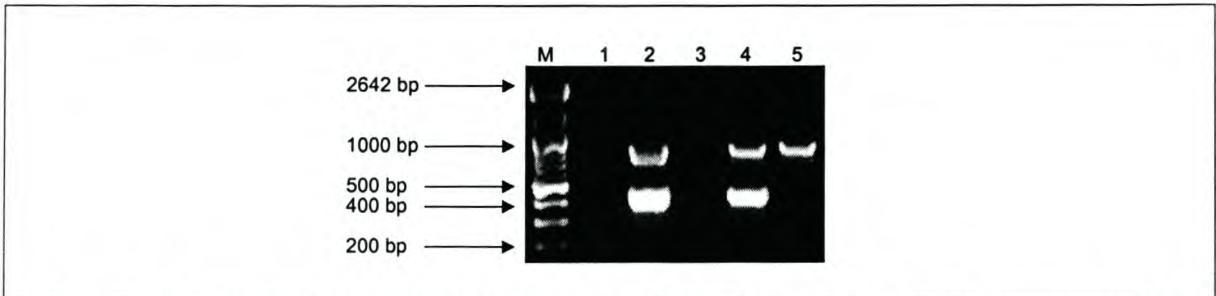

Fig. 3.18. PCR products obtained with multiplex PCR specific for *Ped. pentosaceus* (872 bp) and *Ped. acidilactici* (449 + 872 bp). M, DNA molecular weight marker XIV. Lane 1: *Ped. damnosus*, lane 2: 118, lane 3: *O. oeni* DSM 20252^T, lane 4: 118.1, lane 5: *Ped. pentosaceus* NCDO 813.

3.3.3 Screening of isolates for the presence of the glycerol dehydratase enzyme

Two-hundred-and-ten strains were screened with the GD primers. Seven strains generated a fragment of the expected size (279 bp), and 19 strains generated a 650 bp fragment (**Fig. 3.19** and **Fig. 3.20**). The 650 bp fragment was not what was expected, but it was a single band with the same intensity as the positive control (279 bp). The fragments were sequenced and it appeared that the 650 bp fragment (obtained from a wine isolate *Lb. plantarum* 76) contained stretches with some homology to the 279 bp fragments from *Lb. hilgardii* and *Lb. diolivorans* (Gorga et al., 2002) (**Fig. 3.21**). The primers GD1 and GD2 were designed from a partially sequenced GD gene sequence of *Lb. collinoides* LMG 18850 (Claisse and Lonvaud-Funel, 2001) and the 650 bp fragment showed homology to the α-subunit of the diol dehydratase of *Lb. collinoides* (Sauvageot et al., 2002).

One of the GD-possessing strains was isolated from Pinotage grapes, 15 from Pinotage wine tanks, five from Merlot wine tanks, one from Cabernet grapes and four from Cabernet wine tanks. Fifteen of these strains were identified as *Lb. plantarum*, one as *Lb. pentosus*, five as *Lb. hilgardii*, two as *Lb. paracasei*, two as *Lb. brevis* and one as a *Pediococcus* spp.

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Table 3.4
Identification of LAB isolated from Pinotage, Merlot and Cabernet Sauvignon grapes, after completion of the AF and during MLF

Species identified	Number of strains	Stage of isolation		
		Grapes	AF	MLF
Pinotage				
Leuconostoc mesenteroides	4	0	4	0
Oenococcus oeni	14	0	0	14
Pediococcus acidilactici	2	0	2	0
Pediococcus spp.	22	4	10	8
Lactobacillus plantarum	45	10	35	0
Lactobacillus paraplantarum	3	2	1	0
Lactobacillus pentosus	2	0	2	0
Lactobacillus paracasei	8	3	5	0
Lactobacillus brevis	8	6	1	1
Lactobacillus hilgardii	5	0	5	0
Merlot				
Leuconostoc mesenteroides	0	0	0	0
Oenococcus oeni	11	0	2	9
Pediococcus acidilactici	0	0	0	0
Pediococcus spp.	6	0	4	2
Lactobacillus plantarum	20	17	3	0
Lactobacillus paraplantarum	0	0	0	0
Lactobacillus pentosus	2	1	1	0
Lactobacillus paracasei	9	4	5	0
Lactobacillus brevis	4	1	3	0
Lactobacillus hilgardii	6	0	5	1
Cabernet Sauvignon				
Leuconostoc mesenteroides	0	0	0	0
Oenococcus oeni	3	0	1	2
Pediococcus acidilactici	0	0	0	0
Pediococcus spp.	7	1	3	3
Lactobacillus plantarum	33	11	22	0
Lactobacillus paraplantarum	0	0	0	0
Lactobacillus pentosus	8	7	1	0
Lactobacillus paracasei	11	4	7	0
Lactobacillus brevis	3	2	1	0
Lactobacillus hilgardii	4	0	4	0

AF: completion of alcoholic fermentation; MLF: during malolactic fermentation.

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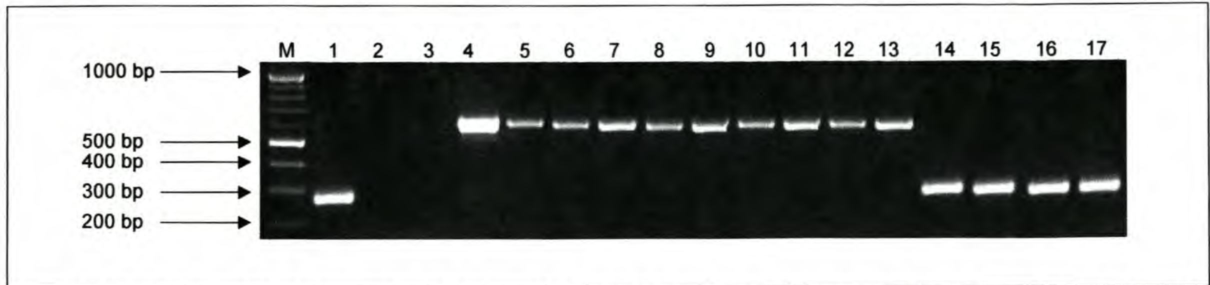

Fig. 3.19. PCR products obtained with GD primers. M, DNA molecular weight marker XIV. Lane 1: *Lb. collinoides* LMG 18850, lane 2: *O. oeni*, lane 3: 88.1, lane 4: 76, lane 5: 14, lane 6: 14.1, lane 7: 36.1, lane 8: 36.2, lane 9: 46, lane 10: 70, lane 11: 83, lane 12: 89, lane 13: 84.1, lane 14: 87.2, lane 15: 67.1, lane 16: 55, lane 17: 31.2.

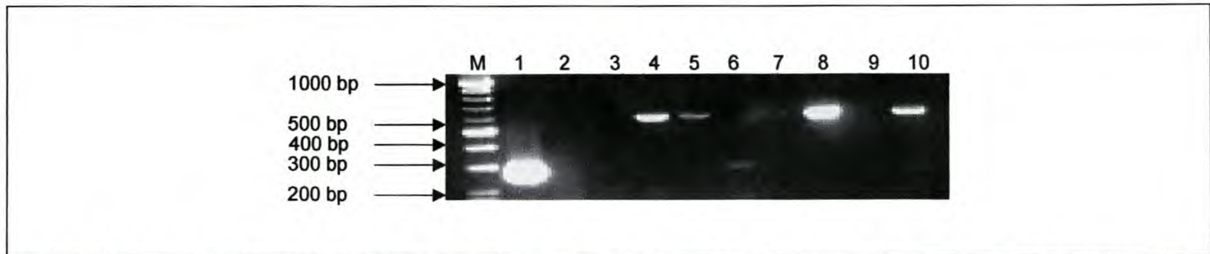

Fig. 3.20. PCR products obtained with GD primers. M, DNA molecular weight marker XIV. Lane 1: *Lb. collinoides* LMG 18850, lane 2: Lalvin 31, lane 3: 118, lane 4: 81, lane 5: 100, lane 6: 58, lane 7: 85.1, lane 9: 5, lane 10: 84.

```
Lb. hilgardii     1            KD----N----P---VQIAAD-AAEA---ALRGFP--EQ----- 22
Lb. collinoides   1            KD----N----P---VQIAAD-AAEA---ALRVFP--EQ----- 22
Lb. diolivorans   1            KD----K----P---VQIAAD-AAEA---SLRGFP--EQ----- 22
Lb. plantarum 76  1   ASTRWLSHMVDLQAANSLVIPGGVVAVGEDPVSPTNNFSIGSKPTHPQTQFVS 53

Lb. hilgardii     23  --ETT---TAVARYAP-MNISIMVGSQT-GRPGVITQ---CSVEESEE----- 60
Lb. collinoides   23  --ETT---TAVARYAP-MNISIMVASAT-GRPGVITQ---CSVEEADE----- 60
Lb. diolivorans   23  --ETT---TAVARYAP-LSISIMVGSQT-GRPGVITQ---CSVEESEE----- 60
Lb. plantarum 76  54  FFNTLGDNTRTACLGPNHDNSVHWRIPSNGSGGFFFRNPTCSFGSISRDLHRV 106

Lb. hilgardii     61  -LSLGMRGFTAYAETIS---VYGTDRVFTD---G------------DDTPW   92
Lb. collinoides   61  -LSLGMRGFTAYAETIP---VYGTDRVFTD---G------------DDTPW   92
Lb. diolivorans   61  -LSLGMRGFTAYAETIS---VYGTDRVFTD---G------------DDTP    91
Lb.plantarum 76   107 ILTLGYLIYSHYPESVKQPHHWDICLIAMKLLMGQFQPHHHRQQPPLSAPVCP 159
```

Fig. 3.21. Amino acid sequence alignment of the GD fragments from *Lb. hilgardii*, *Lb. collinoides*, *Lb. diolivorans* (Gorga et al., 2002) and *Lb. plantarum* 76. Identical amino acid residues are shown on a pink background, while similar residues are shown on a green background.

3.3.4 Glycerol metabolism

Sixteen of the strains possessing the GD gene sequence, as well as a positive control, *Lb. collinoides* LMG 18850, were tested for their ability to metabolise glycerol. These strains were *Lb. plantarum* (8), *Lb. paracasei* (2), *Lb. pentosus* (1), *Lb. brevis* (1), *Lb. hilgardii* (3) and *Pediococcus* spp. (1). The strains were inoculated into the growth media at 10^5 cfu/ml. After 48 h, twelve of the strains increased to 10^9 cfu/ml, while four strains increased to 10^8 cfu/ml. Initially (after 24 h), the strains grew very slowly in Medium 4 (**Table 3. 2**), but they increased to the same level as in the other growth media after 48 h.

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All strains were able to degrade glycerol by more than 90% in all the growth media (**Table 3. 5**). Medium 4 and 5 seemed to be more favourable. In medium 1 (pH 4.8), glycerol and glucose were degraded to a large extent. In comparison, glycerol was also degraded in medium 4 (pH 3.5), but less glucose was used. The same applied to medium 5 (glycerol: glucose 3:1). The strains were also able to use glycerol as a sole carbon source in medium 2, but this may be because the base medium is a rich medium. The strains need to be tested in minimal growth medium to investigate this finding. There seemed to be no significant difference between the ability of the different species to metabolise glycerol.

3.3.5 Preliminary antimicrobial activity

The strains tested all showed a degree of inhibition towards the indicator organisms. Inhibition was seen as a zone of no growth forming around the colony. There was a definitive discrepancy between the levels of inhibition against the different organisms, as indicated by the diameter of the zone. Surprisingly, the strains seemed to be more inhibitory against the AAB than the LAB, which rules out the possibility that the inhibitory action might be due to a bacteriocin.

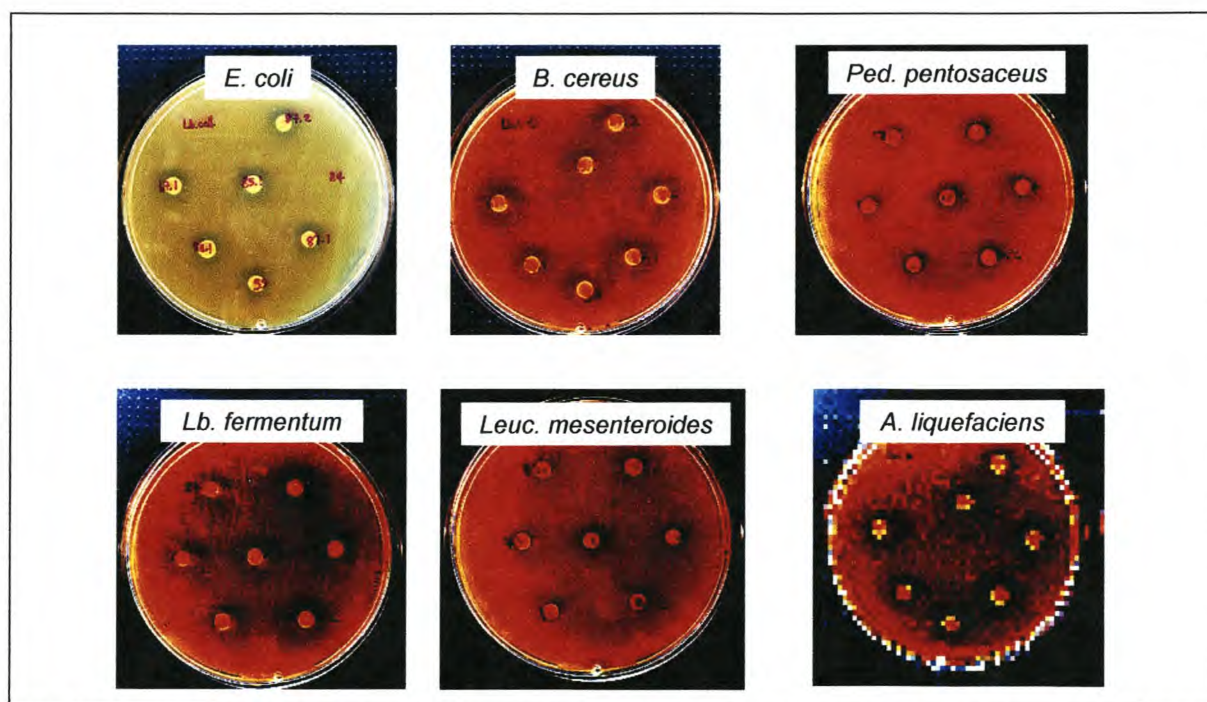

Fig. 3.22. Preliminary results obained with the antimicrobial activity assay. A clear zone around the colony indicates inhibition of growth.

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Table 3.5
Glycerol metabolism of GD possessing strains in media with different compositions[a] grown under anaerobic conditions for 48h

Isolate	Medium 1		Medium 2		Medium 3		Medium 4		Medium 5		Medium 6	
	[Glycerol]	[Glucose]	[Glycerol]	[Glucose]	[Glycerol]	[Glucose]	[Glycerol]	[Glucose]	[Glycerol]	[Glucose]	[Glycerol]	[Glucose]
Lb. collinoides	0.398	0.101	0.204	0	0	0.444	0.085	0.56	0.104	0.694	0.096	0
14	0.24	0.05	0.3	0	0	0.048	0.074	0.453	0.096	0.051	0.636	0
36.2	0.355	0.071	0.381	0	0	0.05	0.095	0.0461	0.076	0.187	0.103	0
46	0.298	0.048	0.524	0	0	0.076	0.087	0.0424	0.054	0.066	0.655	0
70	0.377	0.047	0.478	0	0	0.05	0.094	0.679	0.059	0.05	0.104	0
83	0.299	0.049	0.536	0	0	0.145	0.112	0.43	0.031	0.049	0.674	0
89	0.327	0.048	0.535	0	0	0.05	0.079	0.15	0.073	0.049	0.105	0
87.1	0.399	0.048	0.379	0	0	0.049	0.075	0.421	0.064	0.05	0.684	0
67.1	0.415	0.074	0.515	0	0	0.049	0.095	0.456	0.096	0.23	0.104	0
81	0.515	0.048	0.435	0	0	0.076	0.072	0.46	0.083	0.15	0.695	0
100	0.576	0.049	0.275	0	0	0.048	0.094	0.588	ND*	0.05	0.092	0
85.1	0.57	0.058	0.501	0	0	0.121	0.002	0.464	0.11	0.051	0.693	0
84	0.455	0.047	0.385	0	0	0.06	0.075	0.341	0.102	0.05	0.179	0
91	0.382	0.051	0.027	0	0	0.048	0.083	0.368	0.104	0.049	0.67	0
55	0.219	0.074	0.0465	0	0	0.049	0.108	0.344	0.098	0.058	0.19	0
35.1	0.164	0.05	0.52	0	0	0.0.79	0.118	0.449	0.089	0.62	0.667	0
76	0.388	0.048	0.572	0	0	0.047	0.09	0.408	ND	0.051	ND	0

[a]Medium 1: 10g/l glycerol, 1g/l glucose, 2g/l fructose; Medium 2: 10g/l glycerol; Medium 3: 1g/l glucose, 2g/l fructose;

Medium 4: 10g/l glycerol, 1g/l glucose, 2g/l fructose (pH 3.5); Medium 5: 10g/l glycerol, 3g/l glucose; Medium 6: 10g/l glycerol, 3g/l fructose

*ND – not determined

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3.4 DISCUSSION

The LAB population on Pinotage, Merlot and Cabernet Sauvignon grapes ranged from 10^2 to 10^4 cfu/ml during the 2001 and 2002 harvest seasons. The number of LAB occurring on Cabernet grapes was slightly higher than that on Pinotage and Merlot grapes. Generally, the number of LAB decreases to about 10^2 cfu/ml at the end of the alcoholic fermentation (Lonvaud-Funel, 1999), and this phase is known as the lag phase. The lag phase may last several days, but it also might not occur at all (Ribéreau-Gayon et al., 2000). Results from several studies support this absence of a lag phase after AF, and it has been found that the LAB population after AF is often in the range of 10^4 to 10^7 cfu/ml, depending on the pH, alcohol and SO_2 levels of the wine (Lafon-Lafourcade et al., 1983; Fleet et al., 1984; Edwards et al., 1993). In this study, the LAB population in the Cabernet Sauvignon, Pinotage and Merlot wines after alcoholic fermentation ranged from 10^2 to 10^5 cfu/ml.

The range of LAB species found on the grapes and in the wine has been shown to vary between different cultivars and regions (Costello et al., 1983; Lafon-Lafourcade et al., 1983; Fleet et al., 1984; Lonvaud-Funel, 1999). *Lb. hilgardii*, *Lb. plantarum*, *Lactobacillus casei*, *Lb. brevis*, *Lb. fermentum*, *Lb. buchneri*, *Lactobacillus cellobiosus*, *Lactobacillus fructivorans*, *Ped. damnosus*, *Ped. pentosaceus* and *Leuc. mesenteroides* have been isolated from red grapes, must and wine after AF (Lafon-Lafourcade et al., 1983; Fleet et al., 1984; Wibowo et al, 1985, Dicks and Van Vuuren, 1988). However, predominately *O. oeni* was isolated from wine during or after MLF (Lafon-Lafourcade et al., 1983; Wibowo et al., 1985; Lonvaud-Funel, 1995). Ten different species have been isolated and identified from Pinotage, Merlot and Cabernet Sauvignon grapes, from the wine at the end of alcoholic fermentation and from the wine during MLF, viz. *O. oeni*, *Leuc. mesenteroides*, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus*, *Lb. paracasei*, *Lb. brevis*, *Lb. hilgardii*, *Ped. acidilactici* and *Pediococcus* spp. Of all the species identified in this study, only two, *O.oeni* and *Lb. hilgardii*, were isolated only from the wine and not from the grapes as well. Fourteen of the strains identified as *Lb. hilgardii* were isolated from the wine at the end of alcoholic fermentation before the onset or inoculation of MLF. Most of the *O. oeni* strains were isolated from wine undergoing MLF. The most predominant species isolated from the grapes and the wine of all three cultivars was *Lb. plantarum*, followed by *Pediococcus* spp., which were mostly isolated at the end of alcoholic fermentation and during MLF. These results indicate that Pinotage carries a more diverse LAB population in comparison to Merlot and Cabernet Sauvignon, and also that the percentage of *Pediococcus* spp., which is often considered a spoilage bacterium, is higher in Pinotage.

The presence of the GD gene was detected in 26 strains. Fifteen of these strains were identified as *Lb. plantarum*, one as *Lb. pentosus*, five as *Lb. hilgardii*, two as *Lb. paracasei*, two as *Lb. brevis* and one as a *Pediococcus* spp. To our knowledge, this is the first report of the presence of the GD gene in *Lb. plantarum*, *Lb. pentosus*

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and *Lb. paracasei*. One of the GD-possessing strains was isolated from Pinotage grapes, 15 from Pinotage wine tanks, five from Merlot wine tanks, one from Cabernet Sauvignon grapes and four from Cabernet Sauvignon wine tanks. The primers used to detect the presence of the GD gene, GD1 and GD2, are supposed to amplify a fragment of 279 bp (Claisse and Lonvaud-Funel, 2001). However, when used in this study, the primers amplified a fragment of approximately 650 bp for 14 of the strains tested. This fragment was of the same intensity as the fragment for the positive control, and was subsequently sequenced together with the fragment of the correct size (279 bp). Sequence analysis showed that the 650 bp fragment had some homology to the 279 bp fragments of *Lb. collinoides*, *Lb. hilgardii* and *Lb. diolivorans* (Gorga et al., 2002). These fragments all show homology to the α-subunit of the diol dehydratase of *Lb. collinoides* (Sauvageot et al., 2002), as well as of other characterised diol/glycerol dehydratases. The strains generating the 650 bp fragment were therefore also taken as possessing the GD gene sequence, since negative strains, which could not degrade glycerol, did not generate this fragment.

Sixteen of the strains possessing the GD gene were tested for their ability to degrade glycerol in different growth media. The strains tested represented most of the different species identified. All the strains were able to degrade glycerol by more than 90%, and there was no significant difference between the ability of the different species to metabolise glycerol. In medium 4 and medium 5, slightly more glycerol was degraded than in the other media. It seems that pH might have an influence on glycerol metabolism, seeing that the only difference between medium 1 and medium 4 was a pH of 3.5 in medium 4. In medium 1, glycerol, together with glucose, were degraded to a large extent, whereas in medium 4 and medium 5, more glycerol and less glucose was used. As reported in the literature, glycerol metabolism was favoured when the molar ratio of glycerol:glucose was 3:1, as in medium 5 (Sauvageot et al., 2000). Seeing that the base for the assay media contains a fair amount of nutrients, all the strains were also able to grow in the medium containing only glycerol as a carbon source. However, the ability of these strains to use glycerol as a sole carbon source needs to be tested in a minimal growth medium.

The GD-possessing strains all showed varying degrees of inhibition towards LAB, AAB, *E. coli* and *B. cereus*. The plates used for the antimicrobial assay were buffered to eliminate the possibility of inhibition due to acid formation. Seeing that the LAB also effectively inhibited the AAB, the possibility of bacteriocin activity was ruled out. No inhibition activity was evident when the GD-possessing strains were grown on a medium without glycerol, which strongly suggests that this antimicrobial activity might be similar to that of reuterin – an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA – produced by *Lb. reuteri*. However, the assay to detect reuterin activity is done in a liquid medium, because it was shown that the presence of the sensitive organisms stimulates the production of reuterin (Chung et al., 1989). The results obtained with the plate assay therefore need to be confirmed by the liquid assay. Keeping in mind that there is a world-wide

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shift towards more environmentally friendly and less heavily preserved wines, this antimicrobial activity of the LAB against AAB in particular might be exploited for use as a biopreservative in wine.

3.5 CONCLUSION

This study forms part of a research project being done by the South African wine industry to unravel the causes of bitterness in wines. It was found that there are definite differences between the LAB ecology of Pinotage, Merlot and Cabernet Sauvignon. Cabernet Sauvignon had a higher LAB population, while Pinotage, a unique South African variety, had the most diverse population. The two most dominant species isolated were *Lb. plantarum* and *Pediococcus* spp., with *Pediococcus* spp. being isolated mostly from Pinotage. To our knowledge, this is the first time that *Lb. plantarum*, *Lb. paracasei* and *Lb. pentosus* are reported to possess the ability to degrade glycerol. Interestingly, 62% of the GD-possessing strains were isolated from Pinotage. This study also reports the finding of possible antimicrobial activity of LAB against a range of Gram-positive and Gram-negative bacteria. The results suggest that this activity might be similar to that of reuterin, which is produced by *Lb. reuteri*. Further research needs to be done on the characterisation of the glycerol-degrading ability of the GD-possessing strains, and the specific products formed from glycerol have to be determined. In order to determine the ability of LAB to form acrolein, and thus assess their potential to cause bitterness, an analytical method for the detection of acrolein in wine has to be optimised.

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3.7 LITERATURE CITED

Bartowsky, E.J., Henschke, P.A., 1999. Use of a polymerase chain reaction for specific detection of the malolactic fermentation bacterium *Oenococcus oeni* (formerly *Leuconostoc oenos*) in grape juice and wine samples. Austr. J. Grape Wine Res. 5, 39-44.

Carr, J.G., Davies, P.A., 1972. The ecology and classification of strains of *Lactobacillus collinoides* nov. spec.: A bacterium commonly found in fermenting apple juice. J. Appl. Bacteriol. 35, 463-471.

Chung, T.C., Axelsson, L., Lindgren, S.E., Dobrogosz, W.J., 1989. In vitro studies on reuterin synthesis by *Lactobacillus reuteri*. Microb. Ecol. Health Dis. 2, 137-144.

Claisse, O., Lonvaud-Funel, A., 2000. Assimilation of glycerol by a strain of *Lactobacillus collinoides* isolated from cider. Food Microbiol. 17, 513-519.

Stellenbosch University http://scholar.sun.ac.za

Claisse, O., Lonvaud-Funel, A., 2001. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. J. Food Protec. 64, 833-837.

Costello, P.J., Morrison, G.J., Lee, T.H., Fleet, G.H., 1983. Numbers and species of lactic acid bacteria in wines during vinification. Food Tech. Austr. 35, 14-18.

Daniel, R., Bobik, T.A., Gottschalk, G., 1999. Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organisation of the encoding genes. FEMS Microbiol. Rev. 22, 553-66.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H., Fleet, G.H., 1985. Practical implications of malolactic fermentation: A review. Am. J. Enol. Vitic. 36, 290-301.

Dellaglio, F., Bottazzi, V., Trovatelli, L.D., 1973. Deoxyribonucleic acid homology and base composition in some thermophilic lactobacilli. J. Gen. Microbiol. 74, 289-297.

Dicks, L.M.T., Van Vuuren, H.J.J., 1988. Identification and physiological characteristics of heterofermentative strains of *Lactobacillus* from South African red wines. J. Appl. Bacteriol. 64, 505-513.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, 74-96.

Edwards, C.G., Powers, J.R., Jensen, K.A., Weller, K.M., Peterson, J.C., 1993. *Lactobacillus* spp. from Washington State wines: Isolation and characterisation. J. Food Sci. 58, 453-458.

Fleet, G.H., Lafon-Lafourcade, S., Ribéreau-Gayon, P., 1984. Evolution of yeasts and lactic acid bacteria during the fermentation and storage of Bordeaux wines. Appl. Environ. Microbiol. 48, 1034-1038.

Gorga, A., Claisse, O., Lonvaud-Funel, A., 2002. Organisation of the encoding glycerol dehydratase of *Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans*. Sci. Aliments. 22, 151-160.

Greenhoff, K., Wheeler, R.E., 1981. Analysis of beer carbonyls at the part per billion level by combined liquid-chromatography and high-pressure liquid-chromatography. J. Inst. Brew. 87, 35-41.

Guarneri, T., Rossetti, L., Giraffa, G., 2001. Rapid identification of *Lactobacillus brevis* using the polymerase chain reaction. Lett. Appl. Microbiol. 33, 377-381.

Holzapfel, W.H., Schillinger, U., 1992. The Genus *Leuconostoc*. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K. (Eds.), The Prokaryotes (2nd ed.). A handbook on the biology of bacteria: Ecophysiology, isolation, identification, applications. Springer-Verlag, New York, pp. 1508-1534.

Lafon-Lafourcade, S., Carre, E., Ribereau-Gayon, P., 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. 46, 874-880.

Lee, H., Park, S., Kim, J., 2000. Multiplex PCR-based detection and identification of *Leuconostoc* species. FEMS Microbiol. Lett. 193, 243-247.

Lonvaud-Funel, A., 1995. Microbiology of the malolactic fermentation: Molecular aspects. FEMS Microbiol. Lett. 126, 209-214.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76, 317-331.

Margalith, P.Z., 1981. Flavour microbiology. CC Thomas Publishers, Springfield, Illinois.

Mora, D., Parini, C., Fortina, M.G., Manachini, P.L., 1997. Development of molecular RAPD marker for the identification of *Pediococcus acidilactici* strains. System. Appl. Microbiol. 23, 400-408.

Postel, W., Adam, L., 1983. Gas chromatographical characterisation of raspberry brandy. Deut. Lebensm.-Rundsch. 79, 117-122.

Pressman, D., Lucas, H.J., 1942. Hydration of unsaturated compounds. XI. Acrolein and acrylic acid. J. Am. Chem. Soc. 64, 1953-1957.

Rentschler, H., Tanner, H., 1951. Das Bitterwerden der Rotweine. Mitt. Lebensm. Unters. Hygiene. 42, 463-475.

Ribéreau-Gayon, P., Dubourdieu, D., Donénche, B., Lonvaud-Funel, A., 2000. Handbook of Enology. The Microbiology of Wine and Vinifications (Vol. 1). John Wiley & Sons Ltd., England.

Sauvageot, N., Gouffi, K., Laplace, J.M., Auffray, Y., 2000. Glycerol metabolism in *Lactobacillus collinoides*: Production of 3-hydroxypropionaldehyde, a precursor of acrolein. Int. J. Food Microbiol. 55, 167-170.

Sauvageot, N., Muller, C., Hartke, A., Auffray, Y., Laplace, J., 2002. Characterisation of the diol dehydratase *pdu* operon of *Lactobacillus collinoides*. FEMS Microbiol. Lett. 209, 69-74.

Stellenbosch University http://scholar.sun.ac.za

Schillinger, U., Lücke, F., 1987. Identification of lactobacilli from meat products. Food Microbiol. 4, 199-208.

Schillinger, U., Lücke, F., 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55, 1901-1906.

Schütz, H., Radler, F., 1984. Anaerobic reduction of glycerol to propanediol-1,3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. System. Appl. Microbiol. 5, 169-178.

Slininger, P.J., Bothast, R., Smiley, K., 1983. Production of 3-hydroxypropionaldehyde from glycerol. Appl. Environ. Microbiol. 46, 62-67.

Sobolov, M., Smiley, K.L., 1959. Metabolism of glycerol by an acrolein-forming lactobacillus. J. Bacteriol. 79, 261-266.

Sohier, D., Coulon, J., Lonvaud-Funel, A., 1999. Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. Int. J. Syst. Bacteriol. 49, 1075-1081.

Talarico, T.L., Casas, I.A., Chung, T.C., Dobrogosz, W.J., 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob. Agent. Chemother. 32, 1854-1858.

Talarico, T.L., Dobrogosz, W.J., 1989. Chemical characterisation of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob. Agent. Chemother. 33, 674-679.

Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterisation of glycerol dehydratase from *Lactobacillus reuteri*. Appl. Environ. Microbiol. 56, 1195-1197.

Torriani, S., Felis, G., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Appl. Environ. Microbiol. 67, 3450-3454.

Voisenet, E., 1910. Formation d'acroleine dans la maladie de l'amertume des vins. C.R. Acad. Sci. 150, 1614-1616.

Ward, L.J.H., Timmins, M.J., 1999. Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. Lett. Appl. Microbiol. 29, 90-92.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.L., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.

Zapparoli, G., Torriani, S., Pesente, P., Dellaglio, F., 1998. Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. Lett. Appl. Microbiol. 27, 243-246.

http://www.jgi.doe.gov/JGI_microbial/html

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

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4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION AND CONCLUDING REMARKS

Spoilage, be it due to microbial actions, chemical reactions or both, poses a serious threat to the food and beverage industries. Not only can spoilage lead to great economic losses, but it can also cause industries to lose their competitive edge in the economic market. The challenge to ensure constant consumer satisfaction thus relies on the simultaneous production of high quality, as well as healthy and environmentally friendly products. Quality in this sense includes the flavour and aroma profile and also relies on consumer preference (Bisson et al., 2002; Pretorius and Bauer, 2002). Considering all the modern technology and the range of preservation techniques that are available, it is surprising that spoilage is still an economic problem (Huis in't Veld, 1996; Loureiro, 2000). Over the years, various types of spoilage mechanisms found during winemaking have been well documented and characterised. There are, however, certain types of spoilage for which the underlying mechanisms still have to be elucidated. Unpalatable bitterness in wine and the role of lactic acid bacteria (LAB) in causing this bitterness are amongst these.

Grapes and wine are known to part of the natural habitats of LAB and several species of LAB belonging to the genera *Lactobacillus*, *Oenococcus*, *Leuconostoc*, and *Pediococcus* are commonly associated with the wine environment (Lafon-Lafourcade et al., 1983; Davis et al, 1985; Wibowo et al., 1985; Du Toit and Pretorius, 2000; Ribéreau-Gayon et al., 2000). The role of LAB, like that of yeast, depends on the species (and sometimes a specific strain) that are present, and the stage of the fermentation process during which they grow (Lonvaud-Funel, 1999; Du Toit and Pretorius, 2000). It therefore is important to know the typical microbial composition of specific grapes/wine and to determine their effect on the spoilage characteristics. The presence of high numbers of LAB at the wrong time eventually will lead to the deterioration of the product, but the time between reaching these high numbers and the actual spoilage can be controlled better if the underlying mechanisms of the spoilage are well defined and understood.

Wine spoilage due to unpalatable bitterness and the role of LAB in causing this bitterness have received much attention over the years, but no definite understanding has yet emerged (Rentschler and Tanner, 1951; Sobolov and Smiley, 1959; Slininger et al., 1983; Claisse and Lonvaud-Funel, 2000; Sauvageot et al., 2002). In all these studies, the occurrence of bitterness in wine and cider has been linked to the degradation of glycerol to 3-HPA. The spontaneous dehydration of 3-HPA to form acrolein is favoured in conditions of low pH and high temperature (Pressman and Lucas, 1942; Claisse and Lonvaud-Funel, 2000). The acrolein can combine with the phenolic groups of anthocyanins to form a bitter complex (Rentschler and Tanner, 1951) and the bitter off-flavour due to acrolein has been associated with apple ciders,

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beer, brandy, rum and whisky (Greenhoff and Wheeler, 1981; Postel and Adam, 1983; Sauvageot et al., 2000; Claisse and Lonvaud-Funel, 2001). Very little, however, is known about the acrolein content of wine, but it appears that approximately 10 mg/l is sufficient to cause bitterness (Margalith, 1981).

The enzyme responsible for the degradation of glycerol to 3-HPA, glycerol dehydratase (GD), has been characterised in a few *Lactobacillus* species, namely *Lactobacillus reuteri*, *Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans* (Talarico and Dobrogosz, 1990; Gorga et al., 2002; Sauvageot et al., 2002). This enzyme has also been studied in *Lactobacillus brevis* and *Lactobacillus buchneri* (Veiga da Cunha and Foster, 1992).

Reuterin, which is produced by *Lactobacillus reuteri* when grown on a glycerol-containing medium, is a broad spectrum antimicrobial substance that is active against several Gram-positive and Gram-negative bacteria, yeasts and fungi (Talarico et al., 1988). Talarico and Dobrogosz (1989) have shown that reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA. Several studies have shown that the accumulation of 3-HPA was favoured at a molar ratio of glucose to glycerol of 0.33 (Veiga da Cunha and Foster, 1992; El-Ziney et al., 1998; Lüthi-Peng et al., 2002).

To date, no specific studies have been conducted on wine-isolated LAB to determine their glycerol-degrading abilities. In order to design adequate strategies to prevent bitterness in wine, it is important to first identify the typical indigenous LAB populations associated with grapes and wine and to assess their role in causing bitterness.

This study therefore attempted to identify the LAB populations associated with three red grape varieties, viz., Pinotage, Merlot and Cabernet Sauvignon, and to assess their ability to degrade glycerol. The ability of the isolated LAB to inhibit Gram-positive and Gram-negative bacteria was also tested.

The LAB population differed between the three varieties, with the Cabernet Sauvignon carrying higher numbers of LAB, while Pinotage, a unique South African variety, carried a more diverse LAB population. Two-hundred-and-forty strains were isolated in this study and were identified as *Lb. hilgardii*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lb. brevis*, *Pediococcus. acidilactici*, *Pediococcus* spp., *Leuconostoc mesenteroides* and *Oenococcus oeni* by using species-specific primers. All of these species were isolated from the grapes, as well as from the wine, except for *Lb. hilgardii* and *O. oeni*, which was isolated only from wine. Most of the *O. oeni* strains were isolated from wine undergoing malolactic fermentation (MLF). The most predominant species isolated from the grapes and the wine of all three cultivars was *Lb. plantarum*, followed by *Pediococcus* spp., which was isolated mostly at the end of alcoholic fermentation and during MLF. These results also suggest that the percentage of *Pediococcus* spp., which is often considered a spoilage organism, is higher in Pinotage.

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The GD gene sequence was detected in 26 strains by using the GD1 and GD2 primers (Claisse and Lonvaud-Funel, 2001). These strains were *Lb. plantarum* (15), *Lb. pentosus* (1), *Lb. hilgardii* (5), *Lb. paracasei* (2), *Lb. brevis* (2) and *Pediococcus* spp. (1). To our knowledge, this is the first report of the presence of the GD gene in *Lb. plantarum*, *Lb. paracasei* and *Lb. pentosus*. Interestingly, 62% of the GD-possessing strains were isolated from Pinotage. The strains were all able to degrade glycerol by more than 90% and there was no significant difference between the glycerol-degrading ability of the different species.

The results of this study also showed that the GD-possessing LAB exhibit an antimicrobial effect against a range of Gram-positive and Gram-negative bacteria. The possibility of a bacteriocin being involved is eliminated by the fact that the LAB also inhibited *Eschericia coli* and AAB. This inhibition by the LAB was only evident when the strains were grown on a glycerol-containing medium, which strongly suggest that the antimicrobial activity might be similar to that of reuterin. In the current era, during which there is a world-wide shift towards more environmentally friendly and less heavily preserved wines, this antimicrobial activity of the LAB may well prove to be a very effective biopreservative, since LAB are naturally occurring organisms in the grape and wine environment.

Further work is required to characterise the glycerol-degrading ability of the GD-possessing LAB strains. The specific products obtained from glycerol degradation and the influence of wine conditions on the levels of these products must be determined. The antimicrobial activity should be confirmed in a liquid assay against a wider range of microorganisms.

In conclusion, modern technology cannot prevent wine spoilage without a clear understanding about the underlying mechanisms of all types of spoilage. In order to gain knowledge relating to certain types of spoilages, classical microbiology needs to be combined with molecular biology to obtain data on specific spoilage bacteria. From the results, it is clear that the ecology of grapes and wine differ between varieties and during different stages of the winemaking process. This study can form the foundation for unravelling the causes of bitterness in red wines. Combining the results of this study with analytical, sensory and molecular data may very well provide the industry with valuable tools with which to combat the occurrence of bitterness.

4.3 LITERATURE CITED

Bisson, L.F., Waterhouse, A.L., Ebeler, S.E., Walker, M.A., Lapsley, J.T., 2002. The present and future of the international wine industry. Nature 418, 696-699.

Claisse, O., Lonvaud-Funel, A., 2000. Assimilation of glycerol by a strain of *Lactobacillus collinoides* isolated from cider. Food Microbiol. 17, 513-519.

Claisse, O., Lonvaud-Funel, A., 2001. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. J. Food Protect. 64, 833-837.

Stellenbosch University http://scholar.sun.ac.za

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H., Fleet, G.H., 1985. Practical implications of malolactic fermentation: A review. Am. J. Enol. Vitic. 36, 290-301.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, 74-96.

El-Ziney, M.G., Arneborg, N., Uyttendaele, M., Debevere, J., Jakobsen, M., 1998. Characterisation of growth and metabolite production of *Lactobacillus reuteri* during glucose/glycerol cofermentation in batch and continuous cultures. Biotech. Lett. 20, 913-916.

Gorga, A., Claisse, O., Lonvaud-Funel, A., 2002. Organisation of the encoding glycerol dehydratase of *Lactobacillus collinoides*, *Lactobacillus hilgardii* and *Lactobacillus diolivorans*. Sci. Aliments. 22, 151-160.

Greenhoff, K., Wheeler, R.E., 1981. Analysis of beer carbonyls at the part per billion level by combined liquid-chromatography and high-pressure liquid-chromatography. J. Inst. Brew. 87, 35-41.

Huis in't Veld, J.H.J., 1996. Microbial and biochemical spoilage of foods: An overview. Int. J. Food Microbiol. 33, 1-18.

Lafon-Lafourcade, S., Carre, E., Ribereau-Gayon, P., 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. 46, 874-880.

Loureiro, V., 2000. Spoilage yeasts in foods and beverages: characterisation and ecology for improved diagnosis and control. Food Res. Int. 33, 247-256.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie van Leeuwenhoek 76, 317-331.

Lüthi-Peng, Q., Dileme, F.B., Puhan, Z., 2002. Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri*. Appl. Microbiol. Biotech. 59, 289-296.

Margalith, P.Z. 1981. Flavour microbiology. CC Thomas Publishers, Springfield, Illinois. pp. 173-224.

Postel, W., Adam, L., 1983. Gas chromatographical characterisation of raspberry brandy. Deut. Lebensm.-Rundsch. 79, 117-122.

Pressman, D., Lucas, H.J., 1942. Hydration of unsaturated compounds. XI. Acrolein and acrylic acid. J. Am. Chem. Soc. 64, 1953-1957.

Pretorius, I.S., Bauer, F.F., 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotech. 20, 426-432.

Rentschler, H., Tanner, H., 1951. Das Bitterwerden der Rotweine. Mitt. Lebensm. Unters. Hygiene 42, 463-475.

Ribéreau-Gayon, P., Dubourdieu, D., Donénche, B., Lonvaud-Funel, A., 2000. Handbook of Enology. The Microbiology of Wine and Vinifications. John Wiley & Sons Ltd., England.

Sauvageot, N., Gouffi, K., Laplace, J.M., Auffray, Y., 2000. Glycerol metabolism in *Lactobacillus collinoides*: Production of 3-hydroxypropionaldehyde, a precursor of acrolein. Int. J. Food Microbiol. 55, 167-170.

Sauvageot, N., Muller, C., Hartke, A., Auffray, Y., Laplace, J., 2002. Characterisation of the diol dehydratase pdu operon of *Lactobacillus collinoides*. FEMS Microbiol Lett. 209, 69-74.

Slininger, P.J., Bothast, R., Smiley, K., 1983. Production of 3-hydroxypropionaldehyde from glycerol. Appl. Environ. Microbiol. 46, 62-67.

Sobolov, M., Smiley, K.L., 1959. Metabolism of glycerol by an acrolein-forming lactobacillus. J. Bacteriol. 79, 261-266.

Talarico, T.L., Casas, I.A., Chung, T.C., Dobrogosz, W.J., 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob. Agent. Chemother. 32, 1854-1858.

Talarico, T.L., Dobrogosz, W.J., 1989. Chemical characterisation of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. 33, 674-679.

Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterisation of glycerol dehydratase from *Lactobacillus reuteri*. Appl. Environ. Microbiol. 56, 1195-1197.

Veiga da Cunha, M., Foster, M.A., 1992. Sugar-glycerol cofermentations in lactobacilli: The fate of lactate. J. Bacteriol. 174, 1013-1019.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.L., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.