Characterisation of biogenic amine genes in lactic acid bacteria isolated from 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.

Lynn Downing

SUMMARY

The winemaking process involves a complex microbial flora where the interaction of yeasts, lactic acid bacteria and acetic acid bacteria play an important role in the quality and wholesomeness of the final product. Yeasts are primarily responsible for alcoholic fermentation. Malolactic fermentation follows alcoholic fermentation and is conducted by lactic acid bacteria. These bacteria are important in winemaking and can have a positive or negative effect on the wine quality. Biogenic amines are one of the compounds produced by lactic acid bacteria, which affect the hygienic quality and wholesomeness of the wine negatively and directly pose a health risk to the consumer. The demand of consumers for higher quality and healthier foods has led to renewed interest in studies on biogenic amines. Biogenic amines occur in a wide variety of food products, such as cheese, dried sausage, sauerkraut, fishery products, chocolates, wine and beer. This thesis focussed on the presence of biogenic amines in wine.

The first objective of the study was to determine the ability of lactic acid bacteria isolated from South African wine to produce biogenic amines, using a decarboxylase screening plate method. The potential to produce the biogenic amines histamine, tyramine, putrescine and cadaverine was investigated. The results obtained showed that *Lactobacillus* species (*Lactobacillus brevis* and *Lactobacillus hilgardii*) might be the lactic acid bacteria responsible for tyramine and putrescine production and that it can contribute significantly to the overall biogenic amine content in wines. The results also suggest that amine production is strain dependent and not species specific. None of the lactic acid bacteria tested had the ability to produce histamine or cadaverine. It is important to remember that the ability of the lactic acid bacteria to produce biogenic amines has only been investigated in synthetic media and that it does not necessarily imply similar behaviour in wine. Wine represents a complex environment with a wide number of factors influencing microbial growth and decarboxylase activity and, thus, further investigation is necessary to determine if these amine-producing bacteria behave similarly in wine conditions.

In addition, the polymerase chain reaction (PCR) amplification method was used for the identification of the tyrosine decarboxylase (TDC) gene in some of the tyramine-producing lactic acid bacteria. This was followed by the sequencing of the amplified products, which are partial TDC gene sequences, of two *L. brevis* strains and of a *L. hilgardii* strain. Only one *tdc* gene sequence has been described for bacteria (*Enterococcus faecalis*), while a partial TDC gene sequence from *L. brevis* IOEB 9809 was described. An amino acid sequence alignment of the three TDC gene fragments, obtained in this study, with the known TDC gene fragment of *L. brevis* IOEB 9809 and the *tdc* gene of *E. faecalis* showed a high degree of relatedness and conserved regions.

To meet consumer demands, procedures are necessary to prevent the formation of amines in food products. One way of preventing the formation of

biogenic amines is to relate amine production with certain lactic acid bacteria species involved in the winemaking process. Another possible way would be to develop a rapid detection method for bacteria carrying amino acid decarboxylase genes. The results of this study provide knowledge about which lactic acid bacteria in the winemaking process could contribute to the production of biogenic amines and the sequencing of additional partial TDC genes could possibly assist in the development of a rapid detection method for tyramine-producing lactic acid bacteria in food products.

OPSOMMING

Die wynmaakproses behels 'n komplekse mikrobiese flora waar die interaksie van giste, melksuurbakterieë en asynsuurbakterieë 'n belangrike rol speel in die kwaliteit en heilsaamheid van die finale produk. Giste is primêr verantwoordelik vir alkoholiese fermentasie. Appelmelksuurgisting volg op alkoholiese fermentasie en word deur melksuurbakterieë uitgevoer. Hierdie bakterieë is belangrik in die maak van wyn en kan 'n positiewe of negatiewe uitwerking op die kwaliteit van wyn hê. Biogeniese amiene is een van die komponente wat deur melksuurbakterieë geproduseer kan word en wat die higiëniese kwaliteit en heilsaamheid van die wyn benadeel. Dit hou ook 'n gesondheidsrisiko vir die verbruiker in. Die vereiste van verbruikers vir hoër kwaliteit en gesonder voedselprodukte het nuwe belangstelling in studies op biogeniese amiene ontlok. Biogeniese amiene kom in 'n wye verskeidenheid voedselprodukte voor, soos kaas, droëwors, suurkool, vis, sjokolade, wyn en bier. Hierdie tesis fokus op die teenwoordigheid van biogeniese amiene in wyn.

Die eerste doelwit van die studie was om melksuurbakterieë, wat uit Suid-Afrikaanse wyn geïsoleer is, se vermoë te bepaal om biogeniese amiene op dekarboksilase-agarplate te produseer. Die potensiaal om die biogeniese amiene histamien, tiramien, putresien en kadawerien te produseer, is bestudeer. Die resultate wat verkry is, toon dat *Lactobacillus*-spesies (*Lactobacillus brevis* en *Lactobacillus hilgardii*) vir tiramien- en putresienproduksie verantwoordelik is en dat hulle 'n belangrike bydrae kan lewer tot die totale biogeniese amienkonsentrasie in wyn. Die resultate dui ook daarop dat die produksie van amiene afhanklik is van die ras, en nié 'n spesifieke spesie nie. Geen melksuurbakterieë wat getoets is, het die vermoë getoon om histamien of kadawerien te produseer nie. Dit is belangrik om in ag te neem dat die vermoë van die melksuurbakterieë om amiene te produseer slegs in sintetiese media bestudeer is en dat dit nie noodwendig dieselfde gedrag in wyn sal toon nie. Wyn is 'n komplekse omgewing met 'n wye verskeidenheid faktore wat die mikrobiese groei en dekarboksilase-aktiwiteit kan beïnvloed, daarom is verdere studie nodig om vas te stel of hierdie amien-produserende bakterieë dieselfde gedrag in wyn sal toon.

Die polimerase-kettingreaksie (PKR) amplifikasie-metode is vir die identifikasie van die tirosiendekarboksilase-geen (TDK) in sommige van die tiramien-produserende melksuurbakterieë gebruik. Dit is gevolg deur die volgordebepaling van die geamplifiseerde produkte, wat gedeeltelike TDK-geenvolgordes is, van twee *L. brevis*- en van een *L. hilgardii*-ras. Slegs een *tdk*-geenvolgorde is al voorheen vir bakterieë beskryf, nl. *Enterococcus faecalis*, asook 'n gedeeltelike TDK-geenvolgorde vir *L. brevis* IOEB 9809. 'n Vergelyking van die aminosuurvolgordes van die drie TDK-geenfragmente wat in die studie verkry is, het 'n hoë graad van ooreenkoms en gekonserveerde areas met die bekende TDK-geenfragment van *L. brevis* IOEB 9809 en die *tdk*-geen van *E. faecalis* getoon.

Om verbruikers se behoeftes te bevredig, is dit noodsaaklik dat die vorming van amiene in voedselprodukte voorkom word. Een manier van voorkoming is om amienproduksie aan sekere melksuurbakterieë wat in die wynmaakproses betrokke is, te koppel. 'n Ander manier sal wees om 'n vinnige metode te ontwikkel vir die opsporing van bakterieë wat aminosuurdekarboksilase-gene dra. Die resultate van die studie verskaf kennis van watter melksuurbakterieë in die wynmaakproses tot die produksie van biogeniese amiene kan bydra. Die volgordebepaling van addisionele gedeeltelike TDK-gene kan moontlik tot die ontwikkeling van 'n vinnige opsporingsmetode van tiramien-produserende melksuurbakterieë in voedselprodukte bydra.

What lies behind us and what lies before us
are tiny matters compared to
what lies within us.

OLIVER WENDELL HOLMES

BIOGRAPHICAL SKETCH

Lynn Downing was born in Bellville, Cape Town, South Africa on the 5th day of June 1978. She attended Brackenfell Primary School and matriculated at Stellenberg High School, Bellville in 1996.

Lynn entered Stellenbosch University and obtained a BSc degree in Microbiology and Biochemistry in 1999. In 2000, she completed a BScHons degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University. Lynn enrolled for the MSc degree in Wine Biotechnology in 2001.

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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**
Biogenic amines in foods and beverages

Chapter 3 **RESEARCH RESULTS**
Characterisation of biogenic amine-encoding genes in lactic acid bacteria isolated from South African wine

Chapter 4 **GENERAL DISCUSSION AND CONCLUSIONS**

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Since the beginning of civilisation, wine seems to have been part of the daily life of people. Evidence of wine production dates back as far as 6000 BC and is woven into the tapestry of human history like few other products. Throughout time, wine has played a role as part of religious ceremonies, as a medicine and antiseptic, as a social lubricant, a water purifier, a transformer of meals into feasts, and as a comforting friend and a courageous partner. In general, it has been regarded as a source of pleasure for mankind.

The association of microorganisms with the winemaking process was confirmed in the 1800s, when Louis Pasteur showed that yeasts were responsible for the conversion of grape sugar into ethanol. Apart from yeasts, lactic acid bacteria (LAB) and acetic acid bacteria are part of the natural flora of grapes and the subsequent fermentation. *Saccharomyces cerevisiae* is the dominant yeast during alcoholic fermentation and *Oenococcus oeni* is usually the dominant LAB occurring during malolactic fermentation. These microorganisms can either be beneficial to the quality of wine, or can be detrimental, by causing defects. LAB can influence the wine quality positively by reducing wine acidity, contributing to the flavour profile of wine and playing a part in microbiological stability (Davis et al., 1985). In contrast, undesired effects include bitterness, off-flavours and cosmetic problems, such as viscosity (Du Toit and Pretorius, 2000). Moreover, LAB also produce precursors of ethyl carbamate, a carcinogen, and compounds such as biogenic amines, which affect the wholesomeness of wine and directly pose a health risk to the consumer (Du Toit and Pretorius, 2000).

A flood of scientific evidence is coming to the fore to support the contention that drinking two glasses of wine a day has beneficial health results, from the prevention of neurodegenerative diseases, such as Alzheimer's and Parkinson's, to the prevention of cardiovascular diseases and cancer.

In the early 1990s, epidemiological studies showed a correlation between red wine consumption and the reduced incidence of heart disease in France (Renaud and De Lorgeril, 1992). The French, who eat higher amounts of saturated fats and smoke more, have one of the lowest heart attack rates in the world. This contradiction is popularly known as the French Paradox. Several possible explanations have been offered, but the best explanation for resolving this paradox has been the effect of red wine phenolics. Polyphenols in red wine act as radical scavengers and antioxidants. They prevent the oxidation of low-density lipoprotein (LDL), also known as bad cholesterol, to a harmful chemical state (Frankel et al., 1993). LDL oxidation accounts for the build-up of fat cells in the arteries, leading to fatal heart attacks. Wine also has been shown to reduce thrombosis by inhibiting

platelet aggregation (Demrow et al., 1995). Thus, wine helps to reduce the risk of cardiac diseases.

Studies showed that certain polyphenols in wine have anticancer activities (Soleas et al., 1997, 2002; Yang et al., 2001). These polyphenols include the flavanols, quercetin and catechin, and a stilbene resveratrol. It has also been found that resveratrol increases the effectiveness of the neural enzyme MAP kinase (mitogen-activated protein) by up to seven times (Miloso et al., 1999; http://www.winevault.co.uk/Health/wineandhealth.html). This enzyme stimulates nerve cells to regenerate. In neurodegenerative diseases, such as Alzheimer's and Parkinson's, these cells are broken down. Neurodegeneration therefore can be reduced and prevented by moderate wine consumption.

Wine can also be used as a digestive aid. As a result of its alcohol and acid content, wine has shown to inhibit the growth of microorganisms that cause diseases in man. It has an antibacterial property against *Salmonella*, *Shigella* and pathogenic *Escherichia coli*, which are responsible for food-related ailments (Weisse et al., 1995). It also has been shown to kill cholera bacteria, combat typhoid (http://www.winehorizon.com/healtheffects.htm) and prevent ulcer infections (http://wine.about.com/library/bl_health.htm). Some polyphenols in red wine are also effective against viruses, such as those that cause cold sores (http://www.winehorizon.com/healtheffects.htm). Wine is also known to have a calming and relaxing influence. This is something that may seem incidental, but should not be forgotten. It acts as a mild tranquilliser that can help reduce stress.

Indeed, wine is truly beneficial to health and, above all, it is enjoyable at the same time. Unfortunately, a hazard of wine drinking can be headaches (Littlewood et al., 1988; Daeschel, 1996; Jarisch and Wantke, 1996). In tasting rooms across the world, visitors often turn down samples of red wine for fear of getting a headache. There are several types of headaches, including vascular, tension, pressure or traction and inflammatory headaches. Wine headaches are generally vascular and include the category of migraine headaches. Vascular headaches result from the expansion or contraction of blood vessels within the head, which causes pain. It is not yet clear from the research which compounds in wine cause these reactions, but the biogenic amines, histamine and tyramine, are considered to be among the major culprits.

Biogenic amines occur not only in wine, but also in a wide variety of foods, such as cheese, dried sausage, sauerkraut, fishery products and chocolate (Shalaby, 1996; Silla Santos, 1996). The ingestion of food containing large amounts of biogenic amines, particularly histamine and tyramine, can cause health problems, such as hypertension, respiratory distress and migraine, and psychiatric disorders, such as schizophrenia (Ten Brink et al., 1990; Buckland et al., 1997). Moreover, certain amines, like putrescine and cadaverine, are potential precursors of carcinogenic nitrosamines and, as ethanol, potentiate the toxicity of other amines (Huis in't Veld et al., 1990; Ten Brink et al., 1990). In foods and beverages, these

amines are usually formed by decarboxylation of the corresponding amino acids through specific amino acid decarboxylases of the microorganisms present in the food.

Histamine, tyramine and putrescine are the major biogenic amines found in wine (Lonvaud-Funel, 2001). They can be produced by the action of yeasts during alcoholic fermentation, by the action of LAB during malolactic fermentation and/or by the action of contaminant microorganisms associated with the winemaking process. Amines are believed to be produced mainly by LAB. As consumers are demanding better and healthier foods, there is increasing interest in identifying these amine-producing bacteria. A better understanding of the range of microorganisms capable of producing biogenic amines should help to overcome this problem. One of the most important developments in microbiology today is the use of molecular tools for the early and rapid detection of undesirable bacteria. Le Jeune et al. (1995) developed a polymerase chain reaction (PCR) detection system for the identification of histidine-decarboxylating LAB by studying the amino acid and nucleotide sequences of the histidine decarboxylase genes of *Lactobacillus* 30a and *Clostridium perfringens*. Recently, a rapid PCR-based assay was used to detect tyrosine decarboxylase genes in a few *Lactobacillus brevis* strains (Lucas and Lonvaud-Funel, 2002). When more lactic acid bacterial tyrosine decarboxylase genes are sequenced, it should be possible to design primers in conserved regions and to develop a rapid detection method for tyramine-producing LAB. In this way, it may be possible to prevent the formation of tyramine in food and beverages. This concept could also be applied to other important amino acid decarboxylase genes.

At present there are no regulations regarding the level of biogenic amines in wines. However, the US Food and Drug Administration established regulations for histamine levels (50 ppm) in fish products (FDA, 1996). According to Lehtonen (1996), upper limits for histamine in wine have been recommended in Germany (2 mg l^{-1}), Belgium (5 to 6 mg l^{-1}), France (8 mg l^{-1}) and Switzerland (10 mg l^{-1}). Amine levels in wines may also be regulated in the future and, to ensure that winemakers are one step ahead when these regulations are implemented, research on biogenic amines in wine is necessary.

1.2 PROJECT AIMS

As biogenic amines are becoming a concern to human health, there has been renewed interest in studies on biogenic amines in wine (Coton et al., 1998; Lasekan and Lasekan, 2000; Moreno-Arribas et al., 2000; Lonvaud-Funel, 2001). This study deals with the ability of lactic acid bacteria isolated from South African wines to produce biogenic amines.

The specific aims of this study were the following:

(i) to determine the ability of lactic acid bacteria strains isolated from South African wine to produce histamine, tyramine, putrescine and cadaverine;

(ii) to clone and sequence fragments of TDC genes of different tyramine-producing lactic acid bacterial species; and

(iii) to compare these amino acid sequences to all known prokaryotic TDC sequences to detect conserved regions.

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CHAPTER 2

LITERATURE REVIEW

Biogenic amines in foods and beverages

2. LITERATURE REVIEW

BIOGENIC AMINES IN FOODS AND BEVERAGES

Biogenic amines are a group of biologically active compounds that are widespread in nature. They are formed during normal metabolic processes in all living organisms and therefore are present in everyday food products. The characteristics and biological functions of amines are very diverse. They may have beneficial or harmful effects on humans. On the one hand, biogenic amines are necessary for several critical functions in humans. On the other hand, the ingestion of food containing relatively high levels of biogenic amines can result in several toxicological problems. Moreover, biogenic amines are of concern in relation to food spoilage. They can be the result of the proteolytic and amino acid decarboxylase activity of undesired contaminating microbial flora. Therefore, biogenic amines can be used as indicators of food quality. Recent trends in food security are promoting an increasing search for trace compounds that can affect human health. Biogenic amines belong to this group of substances. Although they are present in low quantities in fermented and non-fermented foods and beverages, they exhibit interactions with normal human metabolism. The possible harmful effects of these amines on human health and their impact on food quality justify the research based on their presence in foods and beverages.

2.1 BIOGENIC AMINES: A DEFINITION

The term amine is used for basic nitrogenous compounds of low molecular weight that are produced within the normal metabolism of humans, animals, plants or microorganisms (Ten Brink et al., 1990). When these amines are formed by the action of living organisms, they are designated biogenic amines (Shalaby, 1996). In foods and beverages, biogenic amines are formed mainly by the decarboxylation of the corresponding precursor amino acids (**Table 2.1**). This reaction is catalysed by substrate-specific enzymes, decarboxylases, of the microbial flora of the food products. They are generated either as the result of endogenous decarboxylase-positive microorganisms in raw food materials or by the growth of contaminating decarboxylase-positive microorganisms (Ten Brink et al., 1990). Biogenic amines are usually formed by microbial decarboxylation of amino acids. However, in plants, a common mode of formation is amination from corresponding aldehydes (Smith, 1981; Maijala et al., 1993). Amines are considered to be naturally present in food originating from plant matter, such as fruit and vegetables (Lovenberg, 1973).

The chemical structure of biogenic amines can either be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, β-phenylethylamine) or heterocyclic (histamine, tryptamine) (**Fig. 2.1**) (Silla Santos, 1996).

Table 2.1
Important biogenic amines and their amino acid precursors

Biogenic amine	Precursor
Histamine	Histidine
Tyramine	Tyrosine
Putrescine	Ornithine
Cadaverine	Lysine
β-phenylethylamine	Phenylalanine
Tryptamine	Tryptophane
Spermine	Arginine
Spermidine	Arginine

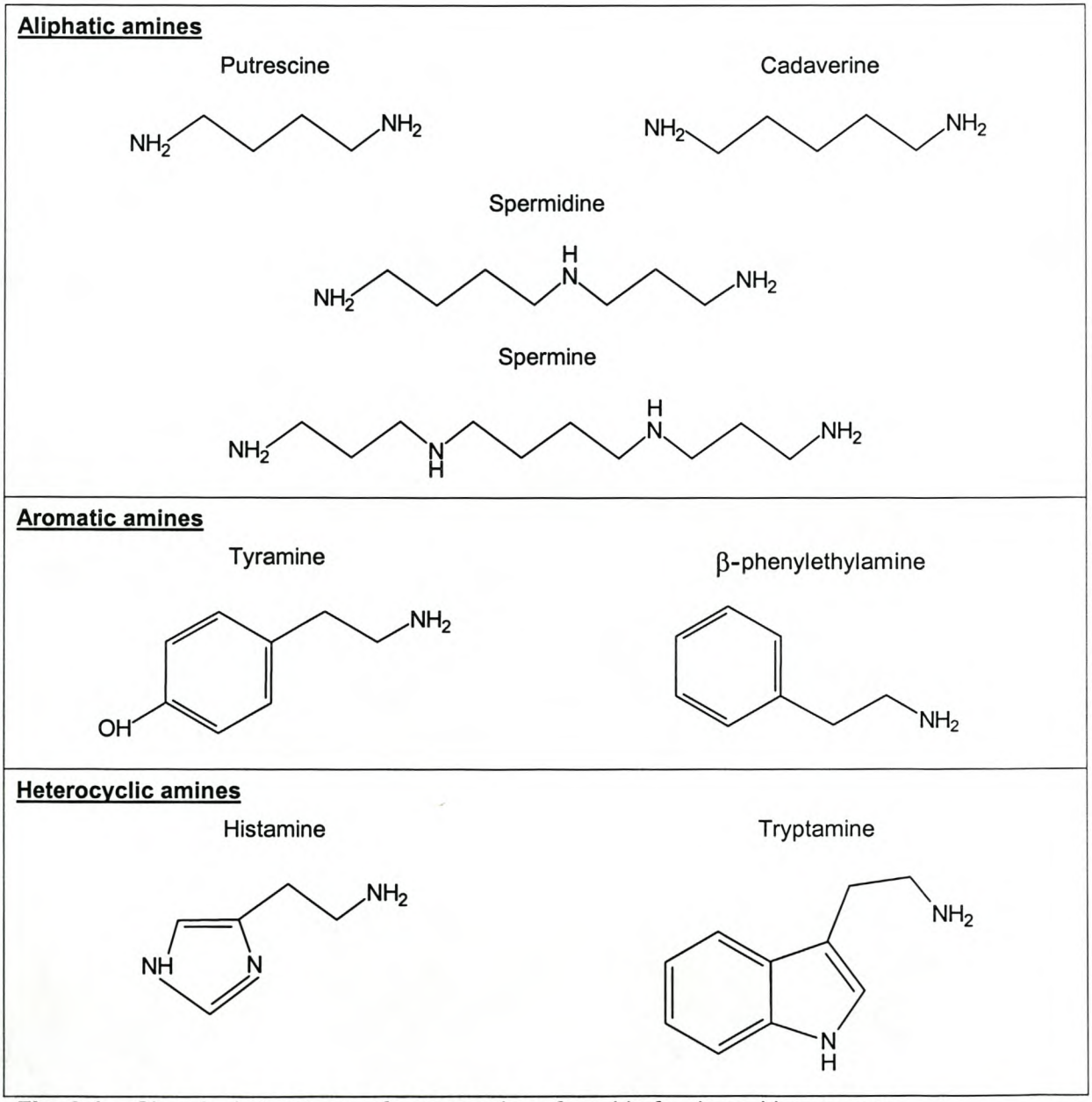

Fig. 2.1. Chemical structures of some amines found in foods and beverages.

Foods likely to contain biogenic amines include fish and fish products, meat products, cheese, vegetables, dried sausage, wine, beer and other fermented products. Histamine, putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, spermine and spermidine generally are considered to be the most important biogenic amines occurring in foods and beverages (Shalaby, 1996). The factors that govern the formation of amines in foods include (a) the availability of free amino acids, (b) the presence of microorganisms that can decarboxylate the amino acids and (c) favourable conditions for the growth of the microorganisms that produce the decarboxylases (Ten Brink et al., 1990).

Two possible reasons exist for the decarboxylation of amino acids by microorganisms. It has been reported that microorganisms could be encouraged to produce decarboxylase enzymes as a protective mechanism against acidic environments (Eitenmiller et al., 1978). The decarboxylation reaction induces an increase in the pH due to the production of more alkaline biogenic amines from the amino acids. Thus, this reaction is thought to favour the growth and survival of microorganisms in acidic media. Moreover, several decarboxylation pathways have been shown to provide energy to some microorganisms (Leuschner et al., 1998a). Therefore, amino acid-decarboxylating microorganisms might survive longer than those that do not decarboxylate.

2.2 PHARMACOLOGICAL AND TOXICOLOGICAL ASPECTS

Histamine, tyramine, putrescine, cadaverine, β-phenylethylamine and tryptamine are a group of biologically active compounds that have important physiological effects (**Table 2.2**) in humans. They may exert either psychoactive or vasoactive effects. Psychoactive amines affect the nervous system by acting on neural transmitters, while vasoactive amines act on the vascular system (Lovenberg, 1973). Tyramine, tryptamine and β-phenylethylamine are included in the presser amine group, which consists of vasoactive amines. They cause a rise in blood pressure by constricting the vascular system, increasing the heart rate and forcing the heart to contract. In contrast, histamine reduces the blood pressure by causing vasodilatation (Smith, 1981).

It is important to emphasise that harmful effects resulting from the consumption of food rich in biogenic amines can be expected only when these amines gain access to the blood stream. This is normally prevented by the action of a defence mechanism of the body that is situated in the digestive tract (Taylor, 1985).

2.2.1 HISTAMINE

2.2.1.1 Pharmacological actions of histamine

Histamine is a normal constituent of the body, where it mediates several important functions. Firstly, histamine is a mediator of allergic reactions (Taylor, 1985).

Special granules of the mast cells and blood basophils contain large amounts of histamine, which is released into the bloodstream in response to an allergic reaction. The release of histamine by mast cell degranulation and the intake of food containing histamine can exert the same effects (Ten Brink et al., 1990).

Histamine exerts its effects by binding to receptors on cellular membranes. Two types of histamine receptors, known as the H_1 and H_2 receptors, exist in humans and other related species (Taylor, 1986). Both these receptors are found in the cardiovascular system. The interaction of histamine with these receptors results in the dilatation of peripheral blood vessels, capillaries and arteries (Stratton et al., 1991). Furthermore, capillary permeability is increased, which leads to the leakage of plasma into the tissues and to haemoconcentration (Joosten, 1988a). Histamine also causes an increase in the rate and strength of the heartbeat (Joosten, 1988a).

Histamine receptors are also found in various secretory glands. Gastric acid secretion is regulated by histamine through H_2 receptors located on the parietal cells (Soll and Wollin, 1977). Secretion by the pancreas, the intestine and the bronchii is stimulated by histamine (Joosten, 1988a). Histamine can also liberate adrenaline and noradrenaline from the suprarenal gland and, as a result, directly stimulate the heart (Joosten, 1988a; Shalaby, 1996). Mediated by H_1 receptors, histamine also excites the smooth muscles of the uterus, the intestine and the respiratory tract (Taylor, 1986).

In addition, histamine is probably a neurotransmitter in the central nervous system. It is responsible for sensory and motor neuron stimulation, which is mediated by H_1 receptors (Taylor, 1986).

2.2.1.2 Toxicological actions of histamine

The most frequent food-borne intoxications caused by biogenic amines are related to histamine. Histamine poisoning is a chemical intoxication with a short incubation period (Taylor, 1986). The onset of the symptoms usually begins several minutes to a few hours after the ingestion of histamine-containing food. Not many incidences of histamine poisoning have been recorded. The symptoms of food allergy and those of histamine poisoning are very similar and physicians occasionally make a faulty diagnosis (Ten Brink et al., 1990). Moreover, both allergic symptoms and histamine poisoning are responsive to antihistamine treatment (Becker et al., 2001).

Histamine can directly stimulate the heart, cause the smooth muscles of the uterus, intestine and respiratory tract to contract or relax, stimulate both sensory and motor neurons and control gastric acid secretion. Thus, it is not surprising that histamine poisoning is characterised by a wide variety of symptoms. Characteristic symptoms affecting the cutaneous (i.e. skin) system include rash, urticaria, edema and localised inflammation (Taylor, 1986). Gastrointestinal involvement is characterised by nausea, vomiting, diarrhoea and abdominal cramps (Taylor, 1985). Other symptoms include hypotension, headache, heart palpitations, tingling, flushing and burning sensations in the mouth (Gilbert et al., 1980; Taylor, 1985).

Shock, bronchospasm, suffocation and severe respiratory distress have been reported in severe cases (Franzen and Eysell, 1969). However, the illness usually has a mild character and the symptoms do not last long (Joosten, 1988a).

2.2.2 TYRAMINE

2.2.2.1 Pharmacological actions of tyramine

Unlike histamine, tyramine is not an important metabolite in the body and it is generally present at a very low concentration.

Tyramine mainly acts indirectly by releasing noradrenaline from the sympathetic nervous system. The most prominent effect of this action is an increase in blood pressure as a result of the vascular system being constricted and the cardiac output being increased (Stockley, 1973; Joosten, 1988a). Tyramine also dilates the pupils and palpebral tissue, causes lacrimation and salivation and increases respiration. If present in high concentrations, the blood sugar level is also increased (Franzen and Eysell, 1969; Joosten, 1988a).

2.2.2.2 Toxicological actions of tyramine

The importance of tyramine in foods is principally due to its toxicological implication. Tyramine is toxic in itself, but, apart from that, it reacts with monoamine oxidase inhibitor (MAOI) drugs to give rise to a hypertensive crisis (Shalaby, 1996). One of the functions of MAO (monoamine oxidase) in the intestine and liver is to destroy potentially harmful amines, specifically tyramine, before they reach the blood. The use of MAOI drugs for the treatment of depression eliminates this detoxification mechanism. Thus, high concentrations of the presser amine derived from food accumulate in the blood. The affected patients then become very susceptible to the toxic actions of tyramine, resulting in a hypertensive crisis (Blackwell, 1963). Such attacks may take place as long as three weeks after drug withdrawal. This is the time needed to restore the MAO enzyme (Smith, 1981).

Cheese was the food initially associated with the hypertensive attacks noted in patients receiving treatment with MAOI (Blackwell and Mabbitt, 1965). Therefore, the increase in blood pressure is now known as the cheese reaction (**Fig. 2.2**). The cheese reaction can cause severe headache and may induce a brain haemorrhage or heart failure (Smith, 1981). Other foods, such as yeast extract (Blackwell et al., 1965a, b), pickled herring (Nueslle et al., 1965), beef liver (Boulton et al., 1970), chicken liver (Hedberg et al., 1966) and beer (Murray et al., 1988; Tailor et al., 1994; Shulman et al., 1997), were shown to cause similar hypertensive attacks due to tyramine.

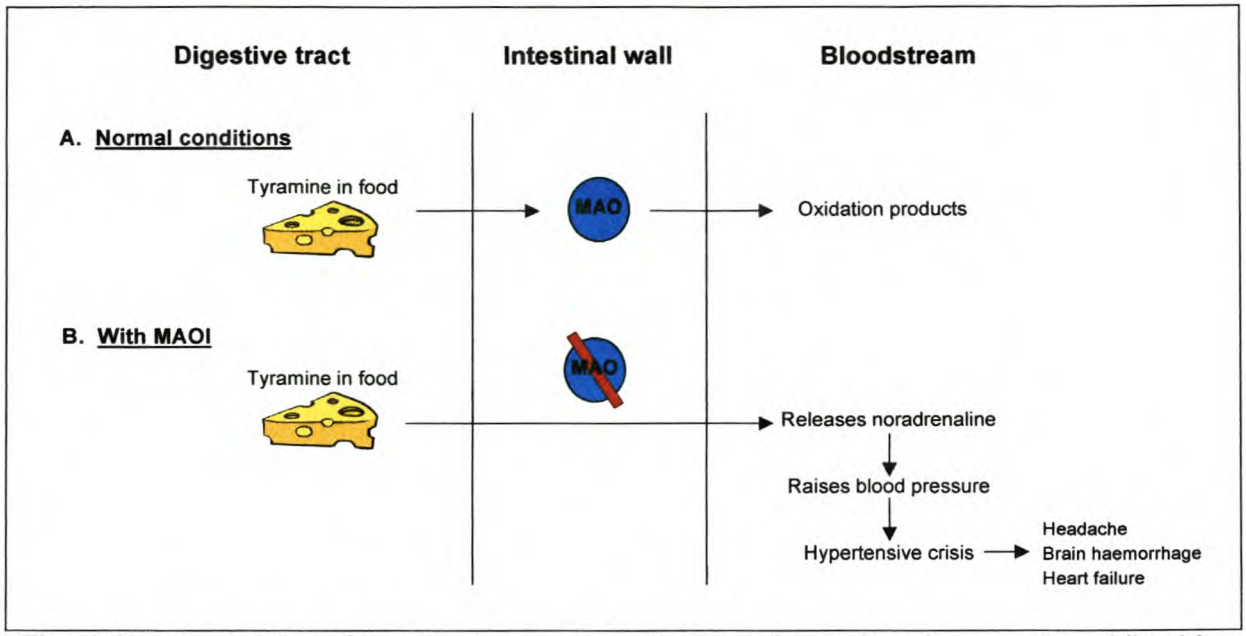

Fig. 2.2. Mechanism of the cheese reaction. A: Tyramine in food is normally oxidised by monoamine oxidase to non-toxic products before it reaches the blood. B: In patients taking monoamine oxidase inhibitors, the detoxification enzyme is suppressed and tyramine enters the bloodstream, causing a hypertensive crisis (cheese reaction) (adapted from Smith, 1981).

2.2.3 PUTRESCINE AND CADAVERINE

Both putrescine and cadaverine seem to have a much lower pharmacological activity than histamine and tyramine. Putrescine and cadaverine and the polyamines spermidine and spermine are indispensable components of living cells. These amines are important in the regulation of nucleic acid function and protein synthesis, and probably also in the stabilisation of membranes (Bardócz et al.,1993; Halász et al., 1994).

Toxic effects are observed only after ingestion of very large amounts of putrescine and cadaverine. Intoxication symptoms that have been reported are hypotension, bradycardia, dyspnoea, lockjaw and paresis of the extremities (Franzen and Eysell, 1969).

The most important effect of the presence of these compounds in food is their ability to potentiate the toxicity of other amines (Taylor, 1986). Furthermore, it should be mentioned that secondary amines, such as putrescine and cadaverine, can react with nitrite to form the heterocyclic carcinogenic nitrosamines, nitrosopyrrolidine and nitrosopiperidine (Huis in't Veld et al., 1990).

2.2.4 PHENYLETHYLAMINE

Pharmacologically, phenylethylamine resembles tyramine, as it also is a neurosympathomimetic amine. In some instances, hypertensive crisis incidents were related to the ingestion of foods containing phenylethylamine (Joosten, 1988a). In addition to its sympathomimetic action, phenylethylamine has also been associated

with migraine (Sandler et al., 1974) and psychiatric disorders such as schizophrenia (O'Reilly et al., 1991; Buckland et al., 1997). The presence of phenylethylamine in food is less frequent than that of tyramine.

2.2.5 TRYPTAMINE

Tryptamine is also a neurosympathomimetic amine. However, it can also exert its effect directly by stimulating smooth muscles and thereby increasing the blood pressure (Joosten, 1988a).

Table 2.2
The physiological effects of biogenic amines (adapted from Shalaby, 1996)

Amine	Physiological effects
Histamine	Liberates adrenaline and noradrenaline Excites the smooth muscles of the uterus, the intestine and the respiratory tract Stimulates both sensory and motor neurons Controls gastric acid secretion
Tyramine	Peripheral vasoconstriction Increases cardiac output Causes lacrimation and salivation Increases respiration Increases blood sugar level Releases noradrenaline from the sympathetic nervous system Causes migraine
Putrescine and cadaverine	Hypotension Bradycardia Dyspnoea Lockjaw Paresis of the extremities Potentiate the toxicity of other amines
β-phenylethylamine	Releases noradrenaline from the sympathetic nervous system Increases the blood pressure Causes migraine Schizophrenia
Tryptamine	Increases the blood pressure

2.2.6 DETOXIFICATION OF BIOGENIC AMINES

Biogenic amines do not usually represent any health hazard to individuals. Many foods contain small amounts of amines, which can easily be tolerated. A fairly efficient detoxification system exists in the intestinal tract of mammals (Taylor, 1985). The detoxification system is composed of two distinct enzymes, MAO and diamine

oxidase (DAO) (Ten Brink et al., 1990). Mono- and diamine oxidases are present in eukaryotes and were also described for bacteria (Voigt and Eitenmiller, 1978; Murooka et al., 1979; Ishizuka et al., 1993; Yamashita et al., 1993). These enzymes convert amines into non-toxic products, which are then excreted. For example, histamine can be metabolised by one of two enzymatic pathways (**Fig. 2.3**). In the first pathway, the ring structure of histamine is methylated by histamine N-methyltransferase (HMT) to form N-methylhistamine. This product can be further oxidised by MAO to form N-methyl imidazole acetic acid. In the second pathway, histamine is oxidised by DAO to form imidazole acetic acid (Stratton et al., 1991). Tyramine is excreted as *p*-hydroxyphenylacetic acid, after oxidation by MAO (Smith, 1981). Putrescine and cadaverine can be degraded by DAO and possibly also by MAO (Franzen and Eysell, 1969). Phenylethylamine and tryptamine are destroyed by MAO (Sandler et al., 1974; Joosten, 1988a).

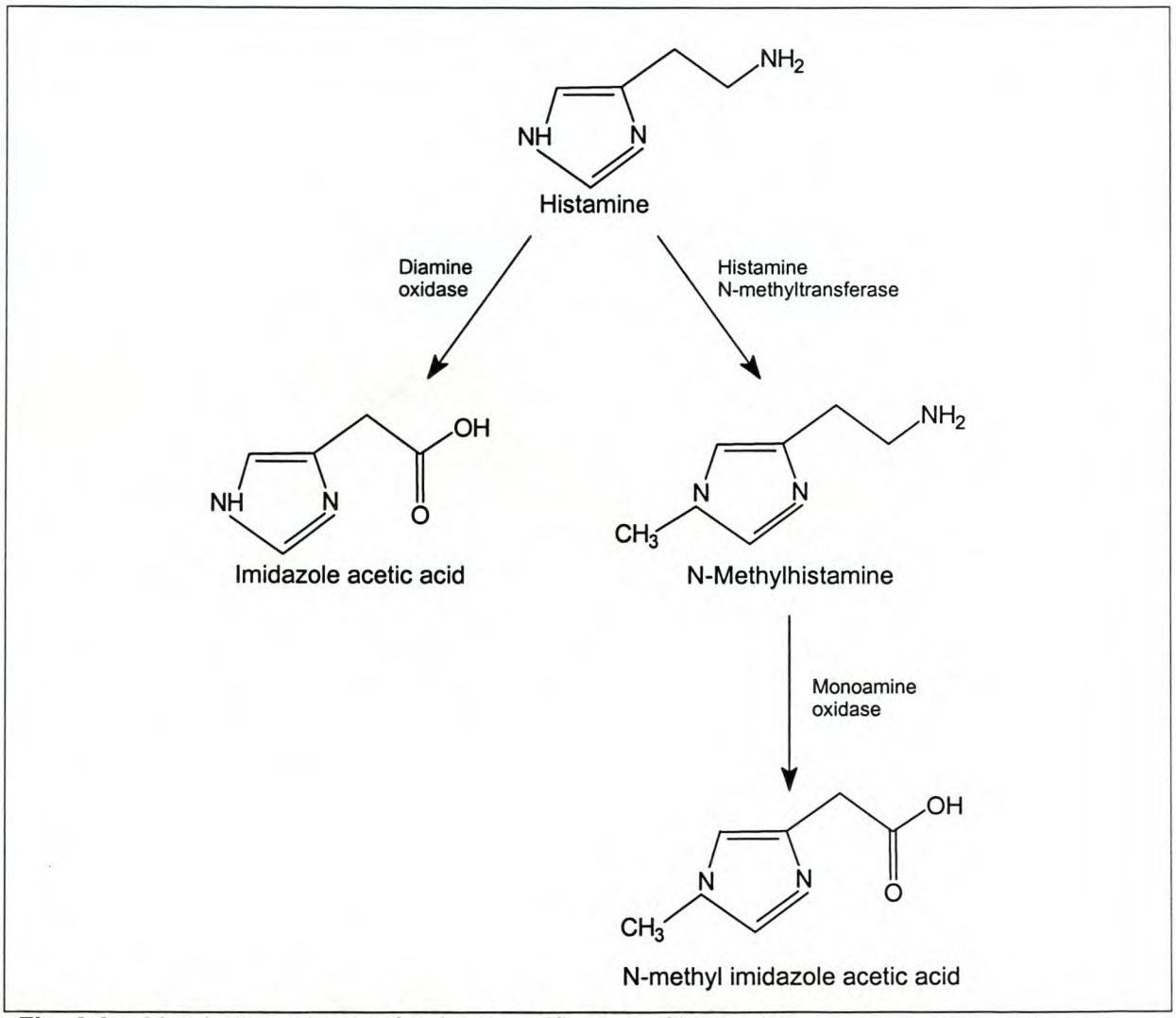

Fig. 2.3. Metabolic pathways for the detoxification of histamine.

This detoxification system is capable of metabolising normal dietary intakes of biogenic amines. However, it apparently fails to eliminate the large amounts of biogenic amines ingested with spoiled foods. The consumption of a high dose of

amines saturates the amine-metabolising capacity of the human body (Ten Brink et al., 1990). One or more substances known as potentiators can also suppress the activity of the detoxifying enzymes and thereby lower the efficiency of detoxification. Potentiators can either be classified as food-borne putrefactive amines or as pharmacological agents (Stratton et al., 1991). Drugs known to suppress these enzymes are antihistamines (Barth and Lorenz, 1978), antimalarials (Cohn, 1965) and other medications, such as monoamine oxidase inhibitors used as antidepressants (Horwitz et al., 1964), and isoniazid, an antituberculosis agent (Kahana and Todd, 1981). Some amines, specifically putrescine and cadaverine, inhibit the detoxifying enzymes, DAO and HMT (Hui and Taylor, 1985). Other biogenic amines that may act as potentiators include tyramine, tryptamine and phenylethylamine. Tyramine can inhibit MAO (Voigt and Eitenmiller, 1978), while tryptamine inhibits DAO (Stratton et al., 1991). Phenylethylamine is a DAO and HMT inhibitor (Hui and Taylor, 1985). In addition, alcohol also increases sensitivity to biogenic amines (Ten Brink et al., 1990). It is also possible that the detoxification system may be genetically deficient (Halász et al., 1994).

2.3 MECHANISMS OF AMINE FORMATION

Amine build-up usually results from the decarboxylation of amino acids by enzymes of bacterial origin. Amino acid decarboxylation takes place by removal of the α-carboxyl group of the amino acid, giving rise to the corresponding amine. By means of decarboxylation reactions, tyrosine produces tyramine, histidine yields histamine and cadaverine is derived from lysine. Tryptamine and β-phenylethylamine are derived in the same manner from tryptophane and phenylalanine respectively. Arginine gives rise to putrescine by means of an intermediate state represented by ornithine. Furthermore, putrescine is also an intermediate of a metabolic pathway that leads to the formation of spermidine and spermine (**Table 2.1**) (**Fig. 2.3.**) (Shalaby, 1996).

Two mechanisms of amino acid decarboxylation have been identified: (a) a pyridoxal phosphate-dependent reaction and (b) a non-pyridoxal phosphate-dependent reaction (Eitenmiller and De Souza, 1984). In the pyridoxal phosphate-dependent reaction, pyridoxal phosphate join in a Schiff base linkage to the amino group of a lysyl residue forms the active site of the enzyme. Pyridoxal phosphate itself is considered to be the portion of the enzyme that actually takes part in the reaction. The carbonyl group of pyridoxal phosphate reacts with amino acids to form Schiff base intermediates. These intermediates are then decarboxylated, water is eliminated and the corresponding amines and the original pyridoxal phosphate moiety are generated (Eitenmiller and De Souza, 1984). Non-pyridoxal phosphate decarboxylation reactions involve a pyruvoyl residue, instead of pyridoxal phosphate (Snell et al., 1975). The pyruvoyl group, which is derived from a serine residue, is covalently bound to the amino group of a phenylalanine residue on the enzyme

(Recsei and Snell, 1982). The pyruvoyl residue acts in a similar manner to pyridoxal phosphate in the decarboxylation reaction (Eitenmiller and De Souza, 1984).

Most amino acid decarboxylases are pyridoxal phosphate dependent (Snell, 1990). With regard to histidine decarboxylase (HDC), mammalian HDCs are stimulated by pyridoxal phosphate, while bacterial HDC enzymes from *Clostridium perfringens*, *Lactobacillus buchneri* (Recsei et al., 1983), *Lactobacillus* 30A (Chang and Snell, 1968), *Micrococcus* sp. (Prozorouski and Jörnvall, 1975) and *Oenococcus oeni* (Rollan et al., 1995) are pyridoxal phosphate independent. Two bacterial tyrosine decarboxylases from *Enterococcus faecalis* (Børressen et al., 1989) and *Lactobacillus brevis* IOEB 9809 (Morena-Arribas and Lonvaud-Funel, 2001) also require pyridoxal phosphate for activity.

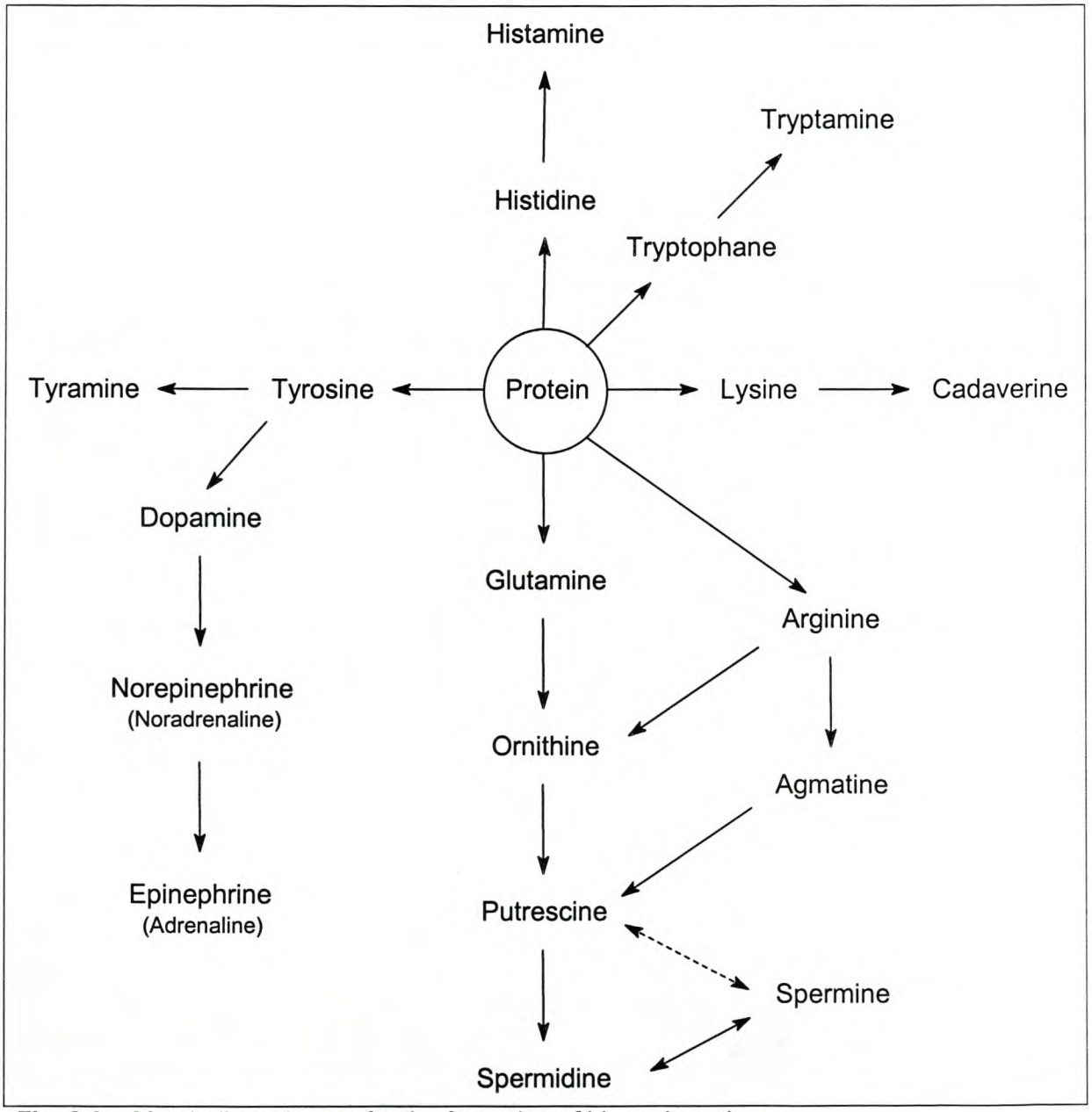

Fig. 2.3. Metabolic pathways for the formation of biogenic amines.

2.4 ANALYTICAL METHODS

The importance of estimating the levels of biogenic amines in food and beverages is related to their impact on human health and food quality. Studies of the occurrence of biogenic amines, as well as of controlling their limits in foods and food products, would not be possible without the supporting analytical methods.

Several methods have been reported for the analysis of amines, including selective plate assays, enzymatic, fluorometric and chromatographic techniques. Among these, only the chromatographic techniques have the ability to separate the different biogenic amines.

2.4.1 SELECTIVE MEDIA

Many of the detection methods for amine-producing bacteria are based on a specific medium that is selective and/or differential for such organisms. MRS broth, when supplemented with histidine, can be used in the differentiation of histamine-producing lactobacilli (Sumner et al., 1985). The detection of histamine-producing bacteria has also been done by applying diamine oxidase and leucocrystal violet directly to the growth media (Sumner and Taylor, 1989). Recently, an improved screening plate method was developed for the detection of amino acid decarboxylase-positive microorganisms, especially lactic acid bacteria (Bover-Cid and Holzapfel, 1999). The suitability and detection level of the designed medium was quantitatively evaluated by an HPLC procedure. The screening plate method showed a good correlation with the chromatographic analysis. Therefore, due to its simplicity, it is presented as a suitable and sensitive method to investigate biogenic amine production by lactic acid bacteria.

One of the limitations of amine production by microorganisms in laboratory media is that the organisms may not show a similar behaviour in a food product. It should be considered that food products are complex systems with a wide number of factors influencing microbial growth and activity.

2.4.2 ENZYMATIC METHODS

Many enzymatic methods exist for detecting histamine in blood and biological tissues (Snyder, 1971; Beavan and Horakova, 1978; Dyer et al., 1982; Verburg et al., 1988). These methods utilise histamine N-methyltransferase (HMT) and radioactive S-adenosylmethionine, a substance required by HMT as a methyl donor. Radio-enzymatic methods for histamine are quite sensitive and are able to detect μg amounts (Verburg et al., 1988). These methods have not been applied to foods (Stratton et al., 1991). The use of an enzyme-linked immunosorbent assay (ELISA) for the routine quantitation of amines would be advantageous, because of the rapidity, simplicity and convenience offered by the test (Guesdon et al., 1986). Expensive instrumentation such as an HPLC or fluorometer would also not be necessary (Stratton et al., 1991). Guesdon et al. (1986) developed an ELISA assay

to quantify histamine in biological fluids. An ELISA method for the quantitative determination of histamine in foods has been described (www.researchd.com/rdikits/re59211.htm). More recently, the application of an enzyme sensor array was described for the simultaneous determination of three biogenic amines (histamine, tyramine and putrescine) in different food samples, such as fish, meat, sauerkraut, diary products, beer and wine (Lange and Wittmann, 2002).

2.4.3 FLUOROMETRIC METHODS

Fluorometric methods have been used for the determination of amines individually. The AOAC procedure is the official method for analysing histamine in foods in the U.S. (Williams, 1984). Staruszkiewicz et al. (1977) originally developed this method for determining histamine in fish products. The method involves the extraction of the sample with methanol, the separation of histamine from interfering substances using an anion exchange column, derivation with *o*-phthalaldehyde (OPA), followed by fluorometric measurement. Lerke and Bell (1976) developed a very similar procedure, in which trichloroacetic acid (TCA) and a cation exchange resin were used instead of methanol and the anionic exchange resin of the AOAC method. A similar method is used for the determination of tyramine in meat and meat derivative products (Santos-Buelga et al., 1981). After an appropiate extraction procedure, tyramine is cleaned up by anion exchange and detected fluorometrically prior to its reaction with α-nitroso-β-naphtol.

2.4.4 CHROMATOGRAHIC METHODS

Since several biogenic amines often occur simultaneously in the same extract, analytical procedures that enable the separation and estimation of these compounds in the same aliquot of an extract are desirable (Shalaby, 1994). In this regard, numerous chromatographic methods utilising paper chromatography, gas chromatography (GC), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been developed.

The extraction of the amines from the food samples is an important step prior to the separation of biogenic amines. In a solid matrix, the extraction of amines can be carried out with water, at different temperatures, so that only free amines are extracted (Moret and Conte, 1996). Acid solvents, such as perchloric acid ($HClO_4$) (Koehler and Eitenmiller, 1978; Yen, 1986), TCA (Mietz and Karmas, 1977; Zee et al., 1983a) or hydrochloric acid (HCl) (Rice et al., 1975; Chang et al., 1985), are used more frequently so that amines linked to other matrix components can also be extracted. Several organic solvents, such as methanol (Carlucci and Karmas, 1988), dichloromethane-$HClO_4$ (Takeba et al., 1990), acetone or acetonitrile-$HClO_4$ (Moret and Conte, 1996), are also used. The reagents $HClO_4$, TCA, HCl and methanol are commonly used for the extraction of amines from foods (Shalaby, 1996). The relative

extraction efficiencies of these solvents depend on the type and nature of the amines and the foods from which they are being extracted.

Since most amines show neither natural UV absorption nor fluorescence, most methods require that amines should be derivatised before detection. Different derivatisation reagents have been used for amine analysis, e.g. ninhydrin in amino acid analysers as a post-column derivatisation reagent (Simon-Sarkadi and Holzapfel, 1994) and dansyl chloride (Dns-Cl) (Mietz and Karmas, 1978; Rosier and Petegham, 1988; Eerola et al., 1993; Moret and Conte, 1996), dabsyl chloride (Bockhardt et al., 1996; Romero et al., 2000), OPA (Busto et al., 1995, 1997; Oguri et al., 1997), fluorescein isothiocyanate (Nouadje et al., 1995) and 9-fluorenylmethyl chloroformate (FMOC-Cl) with pre-column derivatisation (Kirschbaum et al., 1994). Dansyl derivatives can easily be detected at a very low concentration under UV light due to their fluorescent characteristics. However, dansyl chloride is a non-specific reagent and was found to react with all amino compounds, such as amines, ammonia and free amino acids (Fleischer, 1979). Dabsyl chloride is an excellent reagent for the spectrophotometric detection of amines, as it forms coloured compounds that can be detected in the visible zone. The dabsyl derivatives of primary and secondary amines are also stable at room temperature (Bockhardt et al., 1996). OPA seems to be the generally preferred fluorescence-labelling reagent. The most important advantage that OPA has over other derivatisation reagents is that it reacts with amines quickly, it gives rise to highly fluorescent derivatives and enables the biogenic amines to be detected at femtomol levels. However, the use of OPA has some drawbacks. It only reacts with primary amines, which prevents the determination of polyamines such as spermine and spermidine. Some of the derivatives formed with OPA also have limited stability (Busto et al., 1997; Fernandes and Ferreira, 2000). Primary and secondary amines can react with FMOC-Cl to give rise to the corresponding fluorescent 9-fluorenylmethyl carbamates. These fluorescent FMOC derivatives of biogenic amines are reasonably stable (Kirschbaum et al., 1994).

A few reports have been published on the simultaneous detection of multiple amines, but only in a few food products. An HPLC method has been developed for the simultaneous analysis of histamine, cadaverine, putrescine, spermidine and spermine in fish after reaction with dansyl chloride (Mietz and Karmas, 1978). Pre-column derivatisation was also used for the simultaneous determination of histamine, tyramine, cadaverine, putrescine, tryptamine and phenylethylamine by HPLC in both cheese and fish (Hui and Taylor, 1985). After the extraction of amines from sausages, nine dansylated biogenic amines were separated and determined by HPLC (Eerola et al., 1993). A reverse-phase HPLC method for the detection of eight biogenic amines as dabsyl derivates in wines has been reported (Romero et al., 2000). Fernandes and Ferreira (2000) presented an accurate and sensitive GC-mass spectrometry method to permit the simultaneous quantitative determination of the most relevant diamines, polyamines and aromatic amines found in wines and

grape juice. HPLC seems to be the most widely used analytical approach to assay biogenic amines in foods and beverages.

There is a need for a single quantitative analytical method that is able to determine biogenic amines in all kinds of food products. Kovács et al. (1999) developed a high-performance capillary electrophoresis (HPCE) method for separating histamine, tyramine, putrescine, cadaverine, tryptamine, spermine and spermidine in foods. Derivatisations of amines were carried out using AccQ-Fluor reagent. AccQ-Fluor (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) derivatising reagent was first used for amino acid analysis. It reacts rapidly with primary and secondary amines and converts them to stable UV and fluorescent derivatives. One of the greatest advantages of this reaction is its tolerance towards salts in the samples (Kovács et al., 1999). The method was shown to be rapid, simple and reproducible. Complete separation of seven amines was achieved within 30 minutes. The amine profiles of three different food samples (wine, salami and chive) were determined and quantitated. The examples in the analysis show that this method can be applied to the determination of biogenic amines in different foodstuffs.

Methods such as HPCE and HPLC require expensive and sophisticated instrumentation, technical skill, and are costly and time-consuming to operate. TLC was found to be rapid and, more importantly, less expensive. However, TLC methods are not as efficient, sensitive and repeatable as HPLC methods (Shakila et al., 2001). A TLC procedure for the semiquantitative screening of food samples for eight biogenic amines (histamine, cadaverine, putrescine, phenylethylamine, tyramine, tryptamine, spermine and spermidine) has been described (Shalaby, 1995). About two hours are needed for the semiquantitation of the amine content of 14 samples simultaneously. Fish, meat products and cheese samples have been screened successfully. The advantage of this method over earlier reported methods is its simplicity, rapidity, versatility, applicability to a large number of samples in minimal time, cheapness in terms of reagents and equipment, as well as its applicability to food in general. Therefore, it seems that the above semiquantitative TLC method may be used as a routine control procedure and can be put to use by regulatory agencies and food industries for determining the quality of food with respect to biogenic amines.

2.5 OCCURRENCE OF BIOGENIC AMINES IN FOODS AND BEVERAGES

Biogenic amines in foods are a concern with regard to both food spoilage and food safety. They are present in a wide range of food products, including fish, meat, cheese, vegetables, fruit, wine and beer. The total amount and type of biogenic amines formed depend on the nature of the food and on the kind of microorganisms present. For instance, the tissue of scombroid fish contains high levels of free histidine, which can be converted into histamine by the presence of decarboxylase-positive microorganisms (Ten Brink et al., 1990).

Amino acid decarboxylation is the most common mode of synthesis of amines in foodstuffs. Decarboxylase-positive microorganisms may be part of the associated flora of the food or may be introduced by contamination before, during or after the processing of the food (Halász et al., 1994). In non-fermented foods, the presence of biogenic amines above a certain level is indicative of undesired microbial activity (Silla Santos, 1996). Therefore, the amine level could be used as an indicator of the degree of spoilage of food. However, the presence of biogenic amines in food does not necessarily correlate with the growth of spoilage organisms, as they are not all decarboxylase positive. During the preparation of fermented foods, the product is incubated for days, weeks or even months to reach the desired degree of fermentation and maturation. All kinds of microorganisms can proliferate, especially during the early stages of fermentation (Ten Brink et al., 1990). The microorganisms naturally present in raw materials, introduced throughout the processing of the food or added as starter cultures can critically influence biogenic amine production during the manufacturing of fermented products.

2.5.1 NON-FERMENTED FOODS

2.5.1.1 Fish and fish products

In terms of the biogenic amine content of food, fish and fish products have received the greatest attention. Fish of the families *Scombridae* and *Scomberesocidae* are commonly implicated in incidents of histamine poisoning, hence the term scombroid fish poisoning (Taylor et al., 1989). Fish that have been implicated include mackerel, tuna, saury, bonita, seerfish and butterfly kingfish. Tuna and mackerel are the fish generally associated with the poisoning (Flick et al., 2001). Non-scombroid fish, such as sardines, pilchards, anchovies, herring, mahimahi and marline, have also been implicated in cases of histamine poisoning (Taylor, 1985). As this condition is not specific to scombroid fish, histamine poisoning is a more appropriate term to describe this food-related illness. *Morganella morganii* (*Proteus morganii*) (Arnold and Brown, 1978; Eitenmiller et al., 1981), *Klebsiella pneumonia* (Lerke et al., 1978; Taylor et al., 1979) and *Hafnia alvei* (Havelka, 1967) are histamine-producing bacteria that have been implicated in scombroid poisoning.

The muscle tissues of fish contain high levels of free histidine. Two mechanisms of histidine catabolism exist in fish: (a) amino acid deamination to obtain urocanic acid or (b) histidine decarboxylation to form histamine (Mackie and Fernández, 1977). The deamination activity is the principal mechanism under normal physiological conditions. The decarboxylation activity is the result of microorganisms that possess the histidine decarboxylase enzyme. Microorganisms proliferate on the surface of fish that have been handled incorrectly (e.g. improperly refrigerated) (Becker et al., 2001). These histamine-forming microbes usually belong to the bacterial family, *Enterobacteriaceae* (Taylor, 1986, 1988). It appears to be generally accepted that the production of histamine is bacterial in origin and therefore the

amine levels act as indicators of the spoilage or deterioration of fish products (Shalaby, 1996). The bacteria *Proteus vulgaris*, *Proteus mirabilis*, *Clostridium perfringens*, *Enterobacter aerogens*, *Vibrio alginolyticus* (Middlebrooks et al., 1988), *Lactobacillus curvatus*, *Lactobacillus buchneri* (Leuschner and Hammes, 1999), *Alteromonas putrefaciens* (Frank et al., 1985), *Photobacterium phosphoreum* (Morii et al., 1986) and *Plesiomonas shigelloids* (López-Sabater et al., 1994a) have all been isolated from fish and found to produce histamine.

The biogenic amines putrescine, cadaverine, tyramine, spermine, spermidine and agmatine have also been detected in fish and fish products (Shalaby, 1996). Recently, it was observed that an important pathogenic bacteria, *Stenotrophomonas maltophilia* (previously known as *Pseudomonas* and *Xanthomonas maltophilia*), produces significantly high levels of cadaverine in albacore tuna (Ben-Gigirey et al., 1999). Other amines, such as trimethylamine and dimethylamine, are also present and give fish its characteristic smell. Trimethylamine-*N*-oxide (TMAO) is formed in salt water fish and crustacea as a harmless excretion compound for excess nitrogen. Trimethylamine is produced in dead fish by bacteria from TMAO. Dimethylamine is not formed by bacteria, but from the activity of an endogenous enzyme on TMAO (Smith, 1981).

The formation of biogenic amines in fish depends on several factors. Some researchers have concluded that more amines are produced in red muscle fish, such as tuna and mackerel, than in white muscle species, such as rockfish (Takagi et al., 1969). The amine concentrations in freshly caught fish are generally very low (Flick et al., 2001). After capture, the most important factor that contributes to the production of amines is the storage time at specific temperatures. It seems that rapid chilling of fish on the fishing vessel and maintaining fish at temperatures lower than 0°C throughout storage and distribution are the best ways to prevent amine formation (Becker et al., 2001). Tuna can be especially vulnerable to temperature fluctuations. When caught, their average body temperature tends to be several degrees warmer than that of other types of fish (López-Sabater et al., 1994b). The effect of temperature on amine formation has been studied frequently. Although there is great variability in these results, it is clear that longer storage times and higher temperatures induce amine production. The control of biogenic amine production by low temperatures is a constant observation.

Processing steps appear to influence amine production. Vacuum packaging was not shown to have any significant effect on controlling amine production and bacterial growth (Wei et al., 1990). Low temperature storage was more effective than vacuum packaging. Amines are not easily formed during salting due to the inhibitory effect of salt on bacterial growth. However, halotolerant bacteria, identified as *Staphylococcus* spp., *Vibrio* spp. and *Pseudomonas* spp., produced biogenic amines in sardine meat containing 12% NaCl (Yatsunami and Echigo, 1993). Smoking the fish can also influence the levels of biogenic amines. The temperatures used for a hot smoking process may inhibit amine producers, but cold smoking does not expose

the fish to temperatures high enough to inhibit amine-forming bacteria. Zotos et al. (1995) reported the effect of hot-smoking on histamine formation in previously frozen mackerel. Whole mackerel (*Scomber scombrus*) were stored at -20°C. At suitable intervals (11, 22 and 33 weeks), the fish were thawed and subsequently hot smoked. An increase in histamine formation was observed as a result of frozen storage alone. After smoking, a significant increase (at 95% level) in histamine formation was observed in the 11 and 33 week mackerel samples. This increase in amine formation, with the exception of the 22 week sample, was solely due to the smoking process and independent of the frozen storage time of the mackerel samples prior to smoking. Before smoking, fish often will be eviscerated. Belly meat might be more susceptible to bacterial contamination during the evisceration process, as it is very close to the fish gut cavity where amine-forming bacteria reside (Becker et al., 2001). In general, ungutted fish spoils more rapidly than gutted fish (Fernández-Salguero and Mackie, 1987; Haaland et al., 1990). However, Dawood et al. (1988) reported that eviscerated fish contained lower concentrations of amines than whole samples of rainbow trout. It is possible that evisceration may have different effects on biogenic amine levels, depending on the sanitary conditions of the process. Furthermore, fish exposed to multiple thawing and refreezing cycles is more likely to contain biogenic amines (Becker et al., 2001).

2.5.1.2 Meat and meat products

The levels of biogenic amines in fresh and processed pork have been studied (Zee et al., 1983a). Both products contained high levels of adrenaline, spermidine and spermine, but low levels of noradrenaline, putrescine, histamine, cadaverine and tyramine. Chen et al. (1994) described the effect of storage at different temperatures on the amine levels in fresh pork. Increases in the biogenic amine content seemed to be temperature dependent. Pork stored at 30°C had higher amine levels than pork stored at 4°C. The amine levels of pork stored at -18°C did not alter over a nine-month period.

In cooked and uncooked ground beef, the concentrations of putrescine, 1,3-diaminopropane, spermine, spermidine, cadaverine and tyramine correlated positively with both the time and temperature (4 to 10°C) of storage (Halász et al., 1994). Biogenic amines were also formed in fresh vacuum-packaged beef during storage at 1°C for 120 days (Smith et al., 1993). Significant levels were detected after 20 days of storage.

2.5.1.3 Fresh fruit and vegetables

It has been observed that the biogenic amines putrescine, spermidine and spermine are present in very low levels in developing olive tissues (Tattini et al., 1993). Phenylethylamine is the amine that occurs in chocolate and chocolate products and derives from roasted cocoa beans (Schweitzer et al., 1975). Different biogenic

amines have been found in variable concentrations in several juices, nectars and lemonades made from oranges, raspberries, lemons, grapefruit, mandarins, strawberries, currants and grapes. Putrescine is the predominant amine in most fruit juice samples (Silla Santos, 1996). Halász et al. (1994) have reported high amine levels in orange juice (noradrenaline, tryptamine), tomato fruit (tyramine, tryptamine, histamine), banana fruit (tyramine, noradrenaline, serotonin) and plum fruit (tyramine, noradrenaline). This is contradictory to amine concentrations published for the same types of fruit by Maga (1978). The reason for the considerable variation may lie in the changes in the fruit associated with the different degrees of maturity (Halász et al., 1994).

Vegetables represent another plant food from which amines have been identified. Spinach leaves contain histamine in high concentrations (Halász et al., 1994). High levels of pyrrolidine have been detected in white and black pepper (Pfundstein et al., 1991). Spermidine concentrations are relatively high in green peas (Kalač et al., 2002). Some species of mushrooms were found to contain high levels of phenylethylamine (Pfundstein et al., 1991). In a reported case, a very high concentration of phenylethylamine was observed in mushrooms and resulted in adverse reactions with typical tachycardia, an extremely rapid heart rate (Beck et al., 1998).

2.5.1.4 Yeast extract

Yeast extracts are ingredients of foods such as canned soups, sauces, relishes, brawns or moulded meat products and also sandwich spreads and beverages (Lyall, 1963; Blackwell et al., 1969). Yeast extracts are prepared by a process that involves plasmolysis and autolysis (Acraman, 1966). Waste yeast from the brewing industry is plasmolysed to disrupt the yeast cells in order to liberate protein and enzymes from the cells. Plasmolysis is followed by autolysis, during which the liberated yeast protein is converted to amino acids by proteases, polypeptidases and dipeptidases.

It has been estimated that yeast extracts contain tyramine and histamine in large quantities (Blackwell et al., 1965a). One of the most commonly eaten yeast products is a sandwich spread, "Marmite". The amount of histamine in "Marmite" is about ten times more than the amounts found in other foods (Tabor, 1954). Fortunately, the high salt content and savoury flavour of yeast extracts limit the amount consumed.

There is little knowledge concerning the organisms responsible for amine production during the manufacturing of yeast extracts. However, it is known that brewer's yeast is often contaminated with lactobacilli (Blackwell et al., 1969).

2.5.2 NON-FERMENTED BEVERAGES

2.5.2.1 Milk

The amine concentration in fresh (human and cow) milk is very small (Smith, 1981). The amines present in milk include propylamine, hexylamine (Cole et al., 1961), cadaverine, putrescine, spermidine and spermine (Sanguansermsri et al., 1974).

2.5.2.2 Tea

Methylamine and ethylamine have been found in tea leaves (Smith, 1981). It has been reported that tea is probably the main source of the ethylamine found in human urine (Asatoor, 1966).

2.5.3 FERMENTED FOODS

2.5.3.1 Fermented meat products

The manufacturing of fermented meat products, such as dried sausage, involves a fermentation and a drying process. These processes give rise to a safe and stable product and are responsible for the typical flavour of the product (Bover-Cid et al., 2000). The production of fermented meat offers favourable conditions for biogenic amine formation, since the main factors that are required are present. There is growth of microorganisms over several days, a certain degree of proteolysis takes place, giving rise to the presence of free amino acids as precursors of biogenic amines, and, also there is an acidic environment that favours the amino acid decarboxylase activity of microorganisms.

The microbial flora naturally present in the raw meat materials seem to have a strong influence on biogenic amine formation. The use of good quality hygienic raw materials helps to minimise the number of amine-producing microorganisms and thus the final concentration of biogenic amines in the fermented product (Maijala and Eerola, 1993; Bover-Cid et al., 2000). Besides the initial biogenic amine contents and the decarboxylase activity present in the raw materials, the microbial flora required for fermentation may also contribute to the final biogenic amine pool. Meat can be spontaneously fermented by indigenous microbial flora or it can be inoculated with a starter culture. Bauer et al. (1994) reported that the addition of a starter culture did not affect biogenic amine formation. In contrast, it has been observed that the addition of an amine-negative starter culture (*Pediococcus pentosaceus*) decreased the biogenic amine levels during the production of dried sausage (Maijala et al., 1995). It is important to ensure that the selected starter cultures have beneficial effects on biogenic amine formation.

Furthermore, amine formation may be influenced by the thawing time of the raw materials. The microbial counts seem to be higher in raw materials with longer thawing times (Maijala et al., 1995). For spontaneous fermentations, manufacturers

sometimes thaw raw materials at refrigeration temperatures for several days to enhance the growth of indigenous microbial flora. These long thawing times can also facilitate proteolysis, thus increasing the amount of free amino acids available to be decarboxylated (Bover-Cid et al., 2000).

The length of fermentation can also contribute to the biogenic amine concentration. Semi-dried sausage is fermented for short periods, often by adding lactic acid bacterial cultures, while dried sausage is fermented for a longer period by the natural microflora (Genigeorgis, 1976). Bover-Cid et al. (1999) provided data about the presence of biogenic amines in Spanish dried fermented sausage according to their diameter. Generally, amine levels in the sausages with the biggest diameter were higher than in the thinnest sausages. Sausages with a big diameter need a longer drying period than thinner sausages. The drying process leads to a decrease in water activity as well as an increase in salt concentration. Both phenomena are less marked in sausages with a bigger diameter. Consequently, microbial growth in fermented products with a big diameter would be higher and amine production may be more intense. In addition, the difference in amine levels among different types of meat products may be due to the different processing technologies used (Bover-Cid et al., 1999).

Since the potential sources of biogenic amines in fermented meat products are multiple and difficult to identify, these products show a wide variation in the composition and levels of biogenic amines (Vidal-Carou et al., 1990b; Hernández-Jover et al., 1997). The production of amines in meat and meat products has been related to pseudomonads, enterobacteria, enterococci and lactobacilli. Amine-producing lactic acid bacteria, such as *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus divergens* (*Carnobacterium divergens*), *Lactobacillus hilgardii*, *Lactobacillus carnis* (*Carnobacterium piscicola*) and *Lactobacillus curvatus*, have been isolated from meat and meat products (Maijala et al., 1993). Edwards et al. (1983) found that cadaverine concentrations increased when high numbers of *Enterobacteriaceae* were present. In a study by Bover-Cid et al. (2001), *Enterobacteriaceae* was shown to yield cadaverine, but also putrescine. Enterococci are mainly associated with tyramine formation. Some *Enterococcus faecalis* strains were shown to produce tyramine, while related strains produced tyramine, putrescine and cadaverine (Maijala et al., 1993). Masson et al. (1996) tested *Carnobacterium* strains for amine production. All strains of *Carnobacterium divergens*, *Carnobacterium piscicola* and *Carnobacterium gallinarum* produced high concentrations of tyramine. These authors suggested that tyramine production is a characteristic of the genus *Carnobacterium*.

2.5.3.2 Fermented fish products

Although little research has been conducted to determine the biogenic amine content of fermented fish products, it appears that the levels of amines vary extensively. Yankah et al. (1993) have studied changes in the chemical characteristics during the

processing and storage of 'momoni', a Ghanian fermented fish product. 'Momoni' is produced by a combination of salting, fermenting and drying processes, usually using African jack mackerel (*Caranx hippos*). In this investigation, Japanese jack mackerel, which are very similar to African jack mackerel, were used. After fermentation, spermidine had the highest concentration. Trace quantities of putrescine, tyramine, tryptamine and agmatine were also detected. Histamine was not detected. According to a survey of fishery products in 1982, sugar-salted herring in barrels contained high levels of histamine (Taylor, 1985). However, no incidents of histamine poisoning associated with this type of fermented herring have been reported. In a survey of fermented Asian and Pacific foods, Mower et al. (1989) reported that salted Ziganid fish contained very little tyramine. Ziganid fish is a traditional fermented fish product of the Philippines. Recently, the occurrence of biogenic amines in the traditional Korean salted and fermented fish products, Jeotkals, has been investigated (Mah et al., 2002). Low levels of putrescine, cadaverine, histamine, tyramine, spermine and spermidine were found in most of the samples analysed. One sample had higher amine levels than the other samples tested, with the cadaverine concentration specifically being very high.

Fermented fish paste is traditionally made from small fish such as anchovies, prawns, oysters or shrimps. The preparation of the paste involves the addition of salt, sun drying, kneading to form a paste and fermentation at the surrounding temperature for several weeks. The microorganisms responsible for the fermentation originate from the fish, the salt and the environment. Histamine and β-phenylethylamine were the major amines found at high levels in fermented fish paste made in Malaysia, Thailand and the Philippines. Moderate to low levels of cadaverine, tyramine and tryptamine have also been found (Fardiaz and Markakis, 1979).

2.5.3.3 Cheese

After fish, cheese is the next most commonly implicated food item associated with histamine poisoning. The first reported case occurred in the Netherlands in 1967 and involved Gouda cheese (Doeglas et al., 1967). The other reported incidents of cheese-related outbreaks of histamine poisoning mostly involved Swiss cheese (**Table 2.3**).

Besides histamine, a variety of biogenic amines such as tyramine, cadaverine, putrescine, tryptamine and phenylethylamine are present in a wide range of cheese varieties (**Table 2.4**).

Proteolysis is probably the most important phenomenon in the development of cheese texture and flavour. This occurs through the action of native milk proteases, milk coagulants and proteolytic enzymes from microorganisms that naturally are present in milk or are added as starters. During cheese ripening, the principal protein found in cheese, casein, is slowly degraded by proteolytic enzymes to peptides and free amino acids (Fox, 1989). These peptides and free amino acids can be subjected

to breakdown reactions by bacterial decarboxylases to give rise to the formation of biogenic amines.

The final flavour in cheese is achieved during a prolonged ripening process. As this maturation process is a long and costly industrial process, attempts have been undertaken to accelerate maturation. El Soda and Pandian (1991) reviewed some of these strategies. Generally, accelerated ripening is accompanied by an intensified proteolysis. Thus, it favours an increase of substrate availability and renders the cheese more susceptible to amine formation (Joosten and Northolt, 1989; Leuschner et al., 1998b).

Table 2.3
Reported incidents of cheese-related outbreaks of histamine poisoning (Stratton et al., 1991)

Year	Location	Type of cheese	Cases	References
1967	Netherlands	Gouda	1	Doeglas et al., 1967
1976	Washington	Swiss	38	Taylor, 1985
1976	California	Swiss	1	Taylor, 1985
1977	France	Cheshire	1	Uragoda and Lodha, 1979
1980	Canada	Cheddar	1	Kahana and Todd, 1981
1980	New Hampshire	Swiss	6	Taylor et al., 1982
1980-83	France	Gruyere	4	Taylor, 1985

It is not clear which factors are most important in the formation of biogenic amines in cheeses. Factors such as available substrate, ripening temperature, pH and salt concentration all appear to affect the build-up of biogenic amines in cheese (Joosten and Van Boekel, 1988). These factors not only influence the proteolytic activity, which liberates the peptides and amino acids in cheese, but also influence the growth and possibly the decarboxylase activity of contaminant and starter bacteria. Joosten (1988b) reported that higher ripening temperatures (21°C), higher pH values (pH 5.39) and a lower salt concentration resulted in higher levels of histamine in a Gouda cheese made from milk containing a histamine-producing *Lactobacillus buchneri* strain.

The general experience is that the potential of amine formation in cheese from pasteurised milk is smaller than in cheese from raw milk (Ordóñez et al., 1997; Schneller et al., 1997). Pasteurisation reduces the number of viable bacteria in milk. Therefore, decarboxylase-positive bacteria are more abundant in raw milk cheese than in pasteurised milk cheese.

Many organisms may be responsible for the production of amines in cheese. The bacteria *Streptococcus lactis* and *Lactobacillus helveticus*, which are used as starter cultures in the dairy industry, have been identified as histamine producers (Stratton et al., 1991). Edwards and Sandine (1981) isolated histamine-producing organisms from Swiss cheese. These strains included *Streptococcus faecium*, *Streptococcus mitis*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum* and propionibacteria. A histamine-producing strain of *Lactobacillus buchneri* was isolated

from a sample of the Swiss cheese that had been implicated in an outbreak of histamine poisoning in New Hampshire in 1980 (Sumner et al., 1985). Another five *Lactobacillus buchneri* histidine-decarboxylating strains were isolated from Gouda cheese, as was a tyrosine-decarboxylating *Lactobacillus brevis* strain (Joosten and Northolt, 1989).

Table 2.4
Amines in various cheeses (Stratton et al., 1991)

Cheese	Amines
Brie	Cadaverine Putrescine
Brick	Tryptamine Tyramine
Camembert	Cadaverine Putrescine Tyramine
Cheddar	Cadaverine β-Phenylethylamine Putrescine Tyramine
Colby	Cadaverine Putrescine Tyramine
Edam	β-Phenylethylamine Putrescine Tryptamine Tyramine
Gouda	Cadaverine Putrescine Tryptamine Tyramine
Gruyere	Putrescine
Mozzarella	Cadaverine
Parmesan	Tyramine
Provolone	Cadaverine Putrescine
Romano	Tyramine
Roquefort	Cadaverine Putrescine Tyramine
Swiss	Cadaverine Putrescine Tyramine

2.5.3.4 Fermented vegetables

Biologically active amines are also present in many fermented vegetables. The main biogenic amines in sauerkraut are histamine, tyramine, putrescine and cadaverine (Shalaby, 1996). Although sauerkraut routinely contains levels of histamine not considered to be toxic to consumers, it has been implicated in an incident of histamine poisoning in Europe (Stratton et al., 1991).

In a study of biogenic amines occurring in mixed fermented vegetables, only very low levels were detected (Andersson, 1988). Similar results were obtained in a study of Asian foods. Low levels of tyramine were found in commercial samples of Japanese pickled vegetables (urume-zuke) and in kim chee, a traditional Korean fermented cabbage (Mower et al., 1989). However, homemade urume-zuke and kim chee were found to contain higher levels of tyramine than their commercial counterparts (Mower et al., 1989), although the levels were not high enough to cause illness. Very little histamine, putrescine, cadaverine, spermidine, spermine and tyramine were observed in fermented carrots (Choudhury et al., 1990).

Miso and soy sauce are fermented soybean products. Since several varieties of moulds, yeasts and lactic acid bacteria are involved in the fermentation process of these products and the raw materials contain considerable amounts of protein, the formation of various amines could be expected (Chin and Koehler, 1983). Tyramine and histamine have been found at different levels in both these products (Stratton et al., 1991). As a matter of interest, the first food from which histamine was isolated was 'tamari shoyu', a traditionally fermented soy sauce (Ienistea, 1971). Yen (1986) reported that the biogenic amine content of soy sauce made from black soybean is generally much higher than that of regular soy sauce. In another study, tyramine was found at moderately high levels in fermented salted black beans prepared in Hong Kong (Mower et al., 1989).

2.5.4 FERMENTED BEVERAGES

2.5.4.1 Wine

The presence of biogenic amines in wines was first described in 1954 by Tarantola (Fernandes and Ferreira, 2000). Tarontola observed histamine in a Fracia wine while investigating the amino acids of wines with the then modern method of paper chromatography (Radler and Fäth, 1991).

In wines, amines occur as salts. They are odourless at the pH of wine, but with the pH prevailing in the mouth, amines are released and their flavour can be tasted (Lehtonen, 1996). To date, about 30 different amines have been observed in wines (**Table 2.5**). Their concentrations vary greatly, with histamine, tyramine and putrescine being the major biogenic amines in wine. Many authors have determined the amine content of wines. On the basis of the literature studied, the average concentrations of the three major amines in wines (red and white) from different countries are shown in **Table 2.6**. The highest histamine and tyramine contents

measured in South African red wines were 49.1 mg l^{-1} and 6.4 mg l^{-1} respectively (Cilliers and Van Wyk, 1985). It seems that there is no general rule for the evolution and presence of biogenic amines in wines. Obviously, the degree of maturation of the grapes, the elaboration method used for the wine and the oenological treatment of the wine are some factors that determine the final amount of biogenic amines in wine (Torrea and Ancín, 2001).

Table 2.5
Amines found in wine

Agmatine	Histamine	Iso-Propylamine
Butylamine	Indole	Putrescine
Iso-butylamine	Methylamine	Pyrrolidine
Cadaverine	2-Methylbutylamine	2-Pyrrolidone
1,3-Diaminopropane	Morpholine	Serotonin
Diethylamine	β-Phenylethylamine	Spermidine
Dimethylamine	Pentylamine	Spermine
Ethanolamine	Iso-Pentylamine	Tryptamine
Ethylamine	Piperidine	Tyramine
Hexylamine	Propylamine	

Table 2.6
Average amine concentrations (mg l^{-1}) in red and white wines from different countries

Amine	Canada[1]	France[1]	USA[1]	Switzerland[2]	Spain[3]	South Africa[4]
Red wines						
Histamine	3.7	8.1	7.3	2.0	4.1	4.8
Tyramine	4.3	7.3	8.6	2.8	3.0	0.5
Putrescine	2.2	7.6	5.5	21.4		
White wines						
Histamine	1.9	4.4	3.6	1.5	0.8	0.1
Tyramine		6.5	3.2	7.5	1.5	< 0.1
Putrescine	1.3	2.3	1.7	11.1		

[1] Zee et al., 1983b

[2] Gafner, 2002

[3] Vidal-Carou et al., 1990a

[4] Cilliers and Van Wyk, 1985

The abundance of amines is related to the microflora, but also to the amino acid composition of the wine. On the one hand, the latter result from the amino acid composition of the grape must. Amino acids are naturally present in grapes. Their concentration is affected by the geographic location, climatic conditions, vineyard fertilisation and grape variety (Soufleros et al., 1998). On the other hand, yeast

metabolism influences the variation in amino acid content. Amino acids act as a source of nitrogen for yeast during alcoholic fermentation and can be partially or totally metabolised. Some are excreted by live yeasts at the end of the fermentation or released by proteolysis during the autolysis of dead yeast. Other amino acids can be produced by the enzymatic degradation of grape proteins (Lehtonen, 1996).

With respect to the microflora present in wines, biogenic amines are formed by the action of the yeasts in alcoholic fermentation (Buteau et al., 1984a; Torrea and Ancín, 2001, 2002), by the action of lactic acid bacteria during malolactic fermentation and/or by the presence of contaminant microorganisms that are responsible for the spoilage of wine. In winemaking, the species of *Oenococcus oeni* usually dominate during malolactic fermentation. *Oenococcus oeni* strains are able to produce histamine (Lonvaud-Funel and Joyeux, 1994), putrescine and cadaverine (Guerrini et al., 2002). Other lactic acid bacteria in wine, specifically *Lactobacillus brevis* and *Lactobacillus hilgardii*, have been shown to produce tyramine (Morena-Arribas et al., 2000). High levels of histamine have been related to wine spoilage by *Pediococcus* sp. (Delfini, 1989). Buteau et al. (1984b) also indicated that some enteric bacteria, such as *Klebsiella* and *Proteus*, are active producers of amines. These microorganisms are introduced into the wine through contamination and unsanitary conditions.

There are numerous reports presenting evidence that biogenic amines increase in wines during or after malolactic fermentation (Cilliers and Van Wyk, 1985; Vidal-Carou et al., 1990a; Soufleros et al., 1998; Ribéreau-Gayon et al., 2000). This may explain in part why red wines contain significantly more amines than white wines. Many red wines undergo malolactic fermentation spontaneously, especially during wood maturation. Wood maturation offers favourable conditions for the growth of some bacteria, which could produce amines (Cilliers and Van Wyk, 1985). Bentonite, which is more often used during white wine than during red wine vinification, partially absorbs amines and therefore contributes to the lower amine levels of white wines (Cilliers and Van Wyk, 1985). Red wine vinification is usually carried out in the presence of grape skins. This causes a higher amount of amino acids in the must (Zee et al., 1983b). However, some authors observed no differences in the amine contents of red and white wines (Ough, 1971) and also that there was no close relationship between malolactic fermentation and amine content (Ough et al., 1987).

Other factors, such as the addition of sulphur dioxide and the pH of the medium, also affect the biogenic amine concentration. After malolactic fermentation, wine is sulphited in order to eliminate yeasts and bacteria, which are no longer desirable and could possibly influence the amine contents. However, sulphur dioxide does not completely stop the growth of all microorganisms (Lonvaud-Funel, 2001). It has been found that the amine concentration is higher in wines with a pH above 3.77 (Mayer, 1976; Cilliers and Van Wyk, 1985). This is a consequence of a higher total growth and of a greater bacterial diversity at a higher pH.

Although amines are frequently found in wine, they are present at low or even minute concentrations. At these concentrations, they are not considered to be dangerous to consumers. Nevertheless, it is advisable to prevent any avoidable formation of biologically active amines in wine.

2.5.4.2 Beer

Barley variety, malting technology, wort processing and fermentation conditions seem to affect the total biogenic amine content of beer (Halász et al., 1994; Izquierdo-Pulido et al., 1994). Putrescine and the polyamines agmatine, spermine and spermidine could be considered as natural constituents of beer because of their presence in the raw materials (malt and hops) (Izquierdo-Pulido et al., 1994). The presence of tyramine, histamine and cadaverine is considered to be an indicator of microbial contamination during brewing (Donhauser et al., 1993; Halász et al., 1994). In general, tyramine appears to occur in beer at higher levels than histamine and other amines (Zee and Simard, 1981; Vidaud et al., 1989).

It is not clear whether amine production results from yeast activity during fermentation or from contaminant microorganisms, such as lactic acid bacteria. A few studies have shown that brewer's yeast lacks the ability to form biogenic amines. *Saccharomyces uvarum*, a top or ale yeast (Chen and Van Gheluwe, 1979; Zee et al., 1981), and *Saccharomyces cerevisiae* var. *uvarum*, a bottom fermentative yeast (Izquierdo-Pulido et al., 1995), were unable to form amines. *Lactobacillus* spp. and *Pediococcus* spp. are the lactic acid bacteria most often related to amine build-up in beers (Zee et al., 1981; Donhauser et al., 1993; Izquierdo-Pulido et al., 1996). Contamination could occur during wort elaboration or during fermentation through a brewer's yeast spoiled by microorganisms. Yeast recycling is a normal brewing practice. It has been pointed out that, as yeast becomes 'older', the number of microorganisms increase considerably due to contamination of the recycled yeast (Haikara, 1986). Phosphoric acid treatment for recycling seems to be a proper control method to keep bacterial contamination down (Hough, 1991). However, the time, pH and temperature of these washing steps are critical. It could be that the bacteria are not killed or, at the other extreme, that the yeast is damaged by the treatment.

2.6 CONCLUSION

Biogenic amines are found in many foods and beverages. They are produced mainly by lactic acid bacteria through the decarboxylation of amino acids. Their presence in food at high levels is of great significance to health. When considering the toxic levels of biogenic amines, one should not exclusively focus on the concentration of one particular amine in an incriminated food product. The amount of food consumed and the presence of other amines in the food are very important. The use of alcohol and medicine (for instance MAOI treatments) must also be taken into account.

Furthermore, the toxic dose depends on the efficiency of the detoxification systems, which may vary considerably between individuals (Ten Brink et al., 1990). It is also understandable that the simultaneous consumption of foods and beverages could result in biogenic amine poisoning, whereas the consumption of each of these products alone might not give rise to symptoms (Lonvaud-Funel, 2001). Hence, it is difficult to determine the degree of toxicity of biogenic amines in foods and beverages and, thus, to establish toxic threshold levels for these food microcomponents.

Upper limits of 100 mg of histamine kg^{-1} in foods, 2 mg of histamine l^{-1} in alcoholic beverages and 100 to 800 mg kg^{-1} tyramine and 30 mg kg^{-1} phenylethylamine in foods have been suggested (Ten Brink et al., 1990). The only legal limit has been established by the US Food and Drug Administration for histamine levels in fish products (FDA, 1996). This maximum level is 50 ppm. In the future, the amine levels in other food products may also be regulated. Thus, it is necessary to prevent the accumulation of high levels of amines in food and beverage products. Better knowledge and understanding of the microorganisms capable of producing biogenic amines and of the corresponding decarboxylase genes should help to overcome this problem. The use of molecular tools for the early and rapid detection of undesirable bacteria is one of the most important developments in microbiology today. It is now possible to detect the histidine decarboxylase gene in lactic acid bacterial strains by a PCR-based method (Le Jeune et al., 1995). Fortunately, this shows that the development of rapid detection methods is possible and can be expected from the results of molecular studies on other amino acid decarboxylase genes.

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CHAPTER 3

RESEARCH RESULTS

Characterisation of biogenic amine-encoding genes in lactic acid bacteria isolated from South African wine

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3. RESEARCH RESULTS

Characterisation of biogenic amine-encoding genes in lactic acid bacteria isolated from South African wine

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The biogenic amine-producing ability of 400 lactic acid bacteria (LAB) isolated from South African wine was determined, using a decarboxylase screening plate method. The potential to produce the biogenic amines histamine, tyramine, putrescine and cadaverine was investigated. Tyramine was the main amine formed by the LAB strains investigated. Putrescine was also produced. *Lactobacillus hilgardii* strains were shown to be mainly responsible for biogenic amine formation. Two *Lactobacillus brevis* strains also produced biogenic amines. None of the LAB produced histamine or cadaverine. In addition, polymerase chain reaction amplification was used to determine the partial tyrosine decarboxylase (TDC) gene sequences of two *L. brevis* strains (M58 and W78) and of a *L. hilgardii* strain (M2). Only one *tdc* gene sequence was described for bacteria (*Enterococcus faecalis*), while a partial TDC gene sequence from *L. brevis* IOEB 9809 was described. Since the presence of biogenic amines in foods and beverages holds health implications for the consumer, their formation should be prevented. The sequencing of three additional partial TDC genes could possibly assist in the development of a rapid detection method for tyramine-producing LAB in food products.

Keywords: Biogenic amines, lactic acid bacteria, wine, tyrosine decarboxylase

3.1 INTRODUCTION

Biogenic amines occur in a wide variety of foods, such as fishery products, cheese, wine, beer, dried sausage and other fermented foods (Shalaby, 1996; Silla Santos, 1996). They result mainly from the decarboxylation of amino acids through substrate-specific decarboxylases of the microorganisms present in the food. The occurrence and hazard levels of biogenic amines in food are becoming an economic problem. The intake of large amounts of these compounds can cause health problems. Histamine and tyramine are the most studied biogenic amines due to the toxicological effects derived from their vasoactive and psychoactive properties (Ten Brink et al., 1990). Histamine has been recognised as the causative agent of scombroid poisoning, whereas tyramine has been related to food-induced migraines and hypertensive crisis. Biogenic amines can also be responsible for symptoms such as nausea, respiratory distress, hot flushes and heart palpitations (Ten Brink et al., 1990) and are involved in psychiatric disorders such as shizophrenia (Buckland et al., 1997). Secondary amines, such as putrescine and cadaverine, can react with nitrite to form carcinogenic nitrosamines (Huis in't Veld et al., 1990). These two amines and alcohol also potentiate the toxicity of other amines (Ten Brink et al., 1990).

The formation of amines in food depends on (a) the availability of free amino acids, (b) the presence of microorganisms with decarboxylase activity and (c) conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (Ten Brink et al., 1990). As it has often been reported that the concentration of biogenic amines in wine increases after malolactic fermentation (Cilliers and Van Wyk, 1985; Vidal-Carou et al., 1990; Soufleros et al., 1998; Ribéreau-Gayon et al., 2000), it is believed that amines are produced mainly by lactic acid bacteria (LAB). Four genera of LAB are associated with grape juice and wine: *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Amerine and Kunkee, 1968; Fleet, 1993; Lonvaud-Funel, 1999). They enter the wine through the grapes and winery equipment such as crushers, presses, storage tanks, pipes, pumps, filtration units, bottling machines, etc (Wibowo et al., 1985). During alcoholic fermentation, the LAB population does not multiply and generally declines. At the end of alcoholic fermentation, only one species, *Oenococcus oeni*, is generally present, and it then multiplies to conduct malolactic fermentation. Species of the genera *Lactobacillus* and *Pediococcus* may survive in high pH wines. They are potent spoilage agents and can affect the quality of the wine (Wibowo et al., 1985; Lonvaud-Funel, 1999).

The biogenic amines histamine, tyramine and putrescine are found most frequently in wine. Their formation has been associated with a lack of hygiene during the winemaking process. Spoilage bacteria, mainly *Pediococcus* spp., have been related to high levels of histamine in wine (Delfini, 1989). In 1994, a strain of *Oenococcus oeni* (*O. oeni* IOEB 9204), the main LAB species responsible for malolactic fermentation, was isolated from wine and showed to produce histamine (Lonvaud-Funel and Joyeux, 1994). In a study by Coton et al. (1998), all the LAB responsible for histamine production in the wines tested belonged to *O. oeni*.

Guerrini et al. (2002) also found that 60% of the investigated *O. oeni* strains were able to produce histamine and, among these, seven strains showed the additional property of producing both putrescine and cadaverine. In recent years, *Lactobacillus* spp. isolated from wine were found to produce tyramine. These lactobacilli include *Lactobacillus brevis* IOEB 9809, IOEB 9901, IOEB 8511 and IOEB 8907 and *Lactobacillus hilgardii* IOEB 9649 (Moreno-Arribas and Lonvaud-Funel, 1999; Moreno-Arribas et al., 2000). *L. brevis* IOEB 9809 and *L. hilgardii* IOEB 9649 were also found to produce phenylethylamine (Moreno-Arribas et al., 2000). Moreno-Arribas et al. (2002) reported the production of tyramine by three *Leuconostoc mesenteroides* strains. Two of them (BIFI-61 and BIFI-70) were isolated from wine and one (BIFI-60) from grape must. In addition, it has been demonstrated that *L. hilgardii* X_1B is able to produce putrescine and agmatine (Arena and Manca de Nadra, 2001). Two strains of *Lactobacillus buchneri* have been associated with putrescine production (Moreno-Arribas et al., 2002). Putrescine production has also been observed in *O. oeni* IOEB 8419 (Lonvaud-Funel, 2001).

It seems that *O. oeni* and *L. brevis* strains are mostly involved in the production of histamine and tyramine respectively in wine. As the presence of biogenic amines in food products is a public health concern, more research is required to correlate amine production in wine with certain LAB species involved in the winemaking process. A better understanding of the range of microorganisms capable of producing biogenic amines should help to overcome this problem.

The most studied amino acid decarboxylase is histidine decarboxylase. By studying the amino acid and nucleotide sequences of the histidine decarboxylase genes in the bacteria *Lactobacillus* 30a and *Clostridium perfringens*, Le Jeune et al. (1995) were able to develop a polymerase chain reaction (PCR) detection system for the identification of histamine-producing LAB. The use of molecular tools for the rapid detection of undesirable bacteria is one of the most important developments in microbiology today. Tyrosine decarboxylases (TDCs) have been well characterised in eukaryotes, for example in parsley (*Petroselinum crispum*) (Kawalleck et al., 1993), opium poppy (*Papaver somniferum*) (Facchini and De Luca, 1994), *Arabidopsis thaliana* (Nass et al., unpublished) and *Drosophila virilis* (Sukhanova et al., 1997). To date, only one tyrosine decarboxylase (*tdc*) gene sequence has been described for prokaryotes (*Enterococcus faecalis*) (Connil et al., 2002). A partial TDC gene sequence from *L. brevis* IOEB 9809 has also been described (Lucas and Lonvaud-Funel, 2002). These authors developed a PCR amplification method to identify this partial TDC gene sequence. The PCR-based assay was also capable of identifying the TDC gene in a few other *L. brevis* strains. When TDC genes from other LAB species are sequenced, it should be possible to design primers in conserved regions and to develop a rapid detection method for tyramine-producing LAB in food products. Consequently, the formation of tyramine in food may be prevented.

In this study, we have investigated the ability of LAB isolated from South African wine to produce histamine, tyramine, putrescine and cadaverine. We also describe

the use of the PCR-based assay developed by Lucas and Lonvaud-Funel (2002) for the identification of the TDC gene in some of the LAB investigated and the subsequent sequencing of the amplified partial gene sequences in order to enhance the development of a rapid detection method for tyramine-producing LAB.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIAL STRAINS, PLASMIDS AND CULTURE CONDITIONS

All lactic acid bacterial strains (*Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus pentoses*, *Lactobacillus brevis*, *Lactobacillus hilgardii*, *Lactobacillus vermiforme* and *Oenococcus oeni*) used in this study were isolated from South African brandy base wines (Du Plessis et al., 2002b), except for strains of the genus *Pediococcus*, which were isolated from Pinotage wine. Some of the isolates were identified by total soluble cell protein patterns, 16S rRNA sequence analysis and a PCR method using species-specific primers (Du Plessis et al., 2002a). The other isolates were identified preliminarily (Du Plessis, personal communication). All LAB strains were cultured in MRS medium (Biolab, Merck, South Africa) (De Man et al., 1960) at 30°C. *O. oeni* strains were also cultured at 30°C, but in MRS medium supplemented with 20% apple juice (Pure Joy), after which the pH was adjusted to 5.5. All broth cultures were incubated under static conditions.

Escherichia coli strain DH5α (GIBCO-BRL/Life), used for the cloning procedures, was grown in Luria-Bertani (LB) medium (Biolab, Merck, South Africa) at 37°C. Plasmid pGEM-T Easy (Plasmid pGEM-T Easy System I, Promega) was used as a general vector for cloning and sequencing. Clones were grown at 37°C in LB medium containing 100 μg ml^{-1} ampicillin. IPTG (isopropyl-β-D-thiogalactoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were included at 30 μg ml^{-1} in LB agar medium containing 100 μg ml^{-1} ampicillin to enable blue/white screening of the clones.

3.2.2 QUALITATIVE DETECTION OF AMINE FORMATION BY LAB STRAINS

The screening of the LAB strains for amino acid decarboxylase activity was done on agar plates as described by Bover-Cid and Holzapfel (1999). In order to induce the decarboxylase enzyme before the actual screening test, the strains were subcultured five to 10 times in MRS broth supplemented with 0.1% of each precursor amino acid and 0.005% of pyridoxal-5-phosphate (Sigma, Aldrich, Germany). The precursor amino acids included L-histidine, tyrosine disodium salt, L-ornithine hydrochloride, L-arginine (Sigma, Aldrich, Germany) and L-lysine monohydrochloride (Biolab, Merck, South Africa). Pyridoxal-5-phosphate was included as a decarboxylase cofactor for its enhancing effect on amino acid decarboxylase activity (Recsei et al., 1985). The concentration of each amino acid in the screening medium was 1%. Bromocresol

purple was used at 0.006% as a pH indicator. The pH of the medium was adjusted to 5.3 and autoclaved.

The strains spotted onto the decarboxylase medium plates with and without amino acids (as control) were incubated at 30°C for four to six days. Tests were repeated for all the positive strains.

3.2.3 PCR AMPLIFICATION OF THE TDC GENE FRAGMENTS

TDC gene fragments were obtained by PCR (PCR Express, Hybaid, USA) using genomic DNA as template, *Ex Taq* DNA polymerase (TaKaRa; Takara Bio Inc., Japan) and two primers (P2-for and P1-rev) (**Table 3.1**) that were chosen by studying the amino acid sequences of three peptides of the purified TDC enzyme from *L. brevis* IOEB 9809 (Lucas and Lonvaud-Funel, 2002). The reaction conditions were 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension at 72°C for 20 min. Amplification products were analysed on 1% agarose gels.

3.2.4 INVERSE PCR AMPLIFICATION OF THE TDC GENE FRAGMENT OF *L. BREVIS* M58

Restriction fragments (3.5 to 10.5 kb) resulting from a *Sau*3AI (Roche, Germany) digest of genomic DNA from *Lactobacillus brevis* M58 were fractionated and purified through a sucrose gradient (Ausubel et al., 1994). DNA fragments with a size of about 5 kb were ligated and used as a template for inverse PCR amplification of the TDC gene fragment of *L. brevis* M58 with TaKaRa. Oligonucleotides designed from the partial TDC gene sequence that had been amplified with the primer pair P2-for and P1-rev (Lucas and Lonvaud-Funel, 2002) were used as primers (M58 Inv 5' and Inv 3') (**Table 3.1**). The reaction conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 10 min and a final extension at 72°C for 20 min. Amplification products were analysed on 1% agarose gels.

Table 3.1
Primers used for PCR amplification

Primer	Sequence
P2-for	5'-GAYATIATIGGIATIGGIYTIGAYCARG-3'
P1-rev	5'-CCRTARTCIGGIATIGCRAARTCIGTRTG-3'
M58 Inv 5'	5'-CGGGCATTGTTCTTGGACGAGGACGATCAG-3'
M58 Inv 3'	5'-GCACCTTCTTCAGTTGATCCGGCCACACC-3'

3.2.5 ISOLATION OF GENOMIC DNA

Genomic DNA was extracted from the LAB according to the isolation procedure of Dellaglio et al. (1973). Some changes were made to the procedure. After the cells were harvested, washed and resuspended in solution A and lysozyme, they were

incubated for 30 min at 55°C, instead of for 3 hours at 37°C.

3.2.6 DNA SEQUENCE ANALYSIS

Amplified DNA was purified from agarose gels by means of a standard freeze-squeeze procedure, cloned into plasmid pGEM-T Easy (Plasmid pGEM-T Easy System I, Promega) and sequenced.

3.3 RESULTS AND DISCUSSION

3.3.1 BIOGENIC AMINE PRODUCTION BY LAB STRAINS

A total of 400 LAB strains, consisting of *L. paracasei* sp. *paracasei*, *L. casei*, *L. plantarum*, *L. pentoses*, *L. brevis*, *L. hilgardii*, *L. vermiforme*, *O. oeni* and *Pediococcus* spp., were investigated for their potential to produce histamine, tyramine, putrescine and cadaverine. The LAB strains that were shown to produce biogenic amines with the amino acid decarboxylase screening plate method are listed in **Table 3.2**. Biogenic amine production was observed in 14 (3.5%) of the tested LAB strains. Tyramine (10 strains) and putrescine (four strains) were the only amines produced. None of the strains tested had the ability to produce histamine or cadaverine. Positive reactions were recorded when a purple colouration occurred in the decarboxylase screening medium (**Fig. 3.1**). The change of the medium colour to purple is a response of the indicator, bromocresol purple, to a pH shift. The pH shift is dependent on the production of the more alkaline biogenic amines from the amino acids initially included in the medium. In the case of tyramine production, a clear halo was observed due to the disappearance of tyrosine precipitate (**Fig. 3.1**). The decarboxylase screening medium presents some limitations in terms of sensitivity in detecting amine-producing microorganisms. It has a detection limit estimated around 350 mg l^{-1} and allows a rapid preliminary selection of strains with decarboxylase activity (Bover-Cid and Holzapfel, 1999).

In bacteria, putrescine can be produced either by the decarboxylation of ornithine or by the decarboxylation of arginine into agmatine, which is then converted into putrescine. Arginine can also be metabolised to ornithine via the arginine deiminase pathway (Arena and Manca de Nadra, 2001) (**Fig. 3.2**). Knowing that arginine is one of the most important amino acids in grape musts and wines (Sponholz, 1991; Moreno-Arribas et al., 1998), we checked the ability of the four putrescine-producing strains to produce amines in the decarboxylase media supplemented with arginine. All four strains gave a positive result. However, we cannot yet be sure if putrescine, agmatine or both amines were produced. To verify which amine was produced, a quantitative detection method, such as high performance liquid chromatography, has to be used. It has been reported that, under specific culture conditions, some strains might possess more than one amino acid decarboxylase activity (Gale, 1946).

Table 3.2
Biogenic amine-producing lactic acid bacteria strains

Tyramine-producing strains	Putrescine-producing strains
Lactobacillus brevis M58	*Lactobacillus hilgardii* B74
Lactobacillus brevis W78	*Lactobacillus hilgardii* G79
Lactobacillus hilgardii G81	*Lactobacillus hilgardii* K24
Lactobacillus hilgardii I24	*Lactobacillus hilgardii* M59
Lactobacillus hilgardii K56	
Lactobacillus hilgardii M2	
Lactobacillus hilgardii M30	
Lactobacillus hilgardii M32	
Lactobacillus hilgardii W16	
Lactobacillus hilgardii W53	

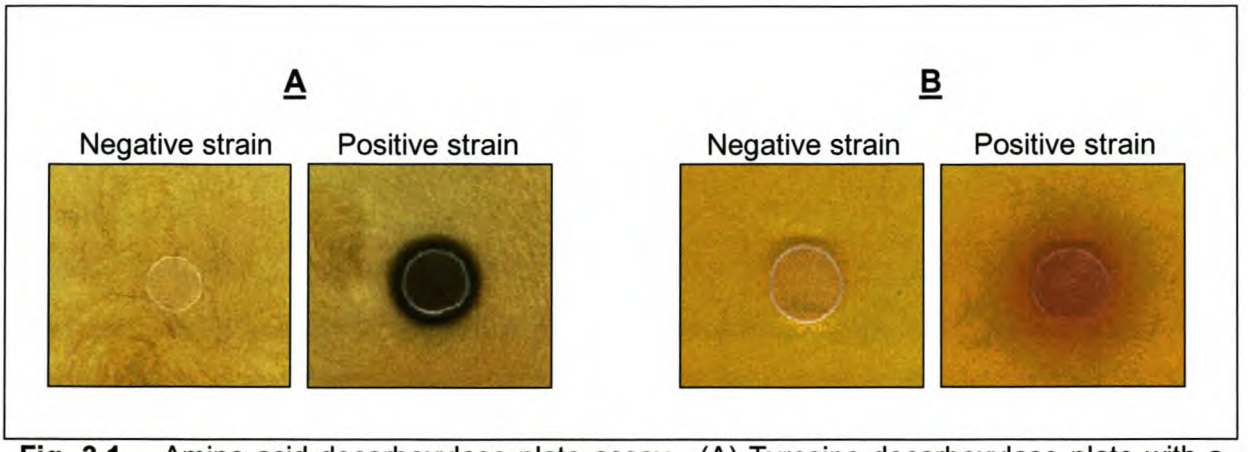

Fig. 3.1. Amino acid decarboxylase plate assay. (A) Tyrosine decarboxylase plate with a positive and negative strain. Colonies surrounded by a clear halo due to tyrosine precipitate disappearance were identified as tyramine producers. (B) Ornithine decarboxylase plate with a positive and negative strain. Putrescine production was recorded when a purple colouration of the medium occurred around the colony.

Strains of *L. brevis* and *L. hilgardii* have the ability to produce biogenic amines. Tyramine production was observed in two *L. brevis* strains and eight *L. hilgardii* strains and putrescine production was seen in four *L. hilgardii* strains. Previously, tyramine production in wine was associated mainly with *L. brevis* strains (Moreno-Arribas and Lonvaud-Funel, 1999; Moreno-Arribas et al., 2000). Only one *L. hilgardii* strain (IOEB 9649) isolated from wine has previously been associated with tyramine production (Moreno-Arribas et al., 2000) and one (X_1B) with putrescine production (Arena and Manca de Nadra, 2001).

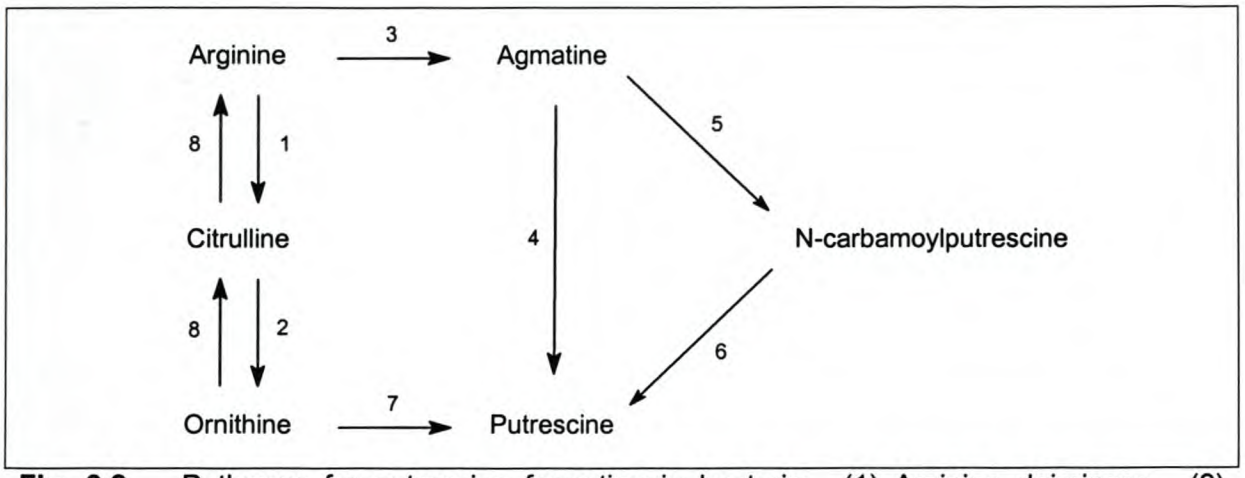

Fig. 3.2. Pathways for putrescine formation in bacteria. (1) Arginine deiminase. (2) Ornithine transcarbamylase. (3) Arginine decarboxylase. (4) Agmatine deiminase. (5) Agmatinase. (6) N-carbamoylputrescine hydrolase. (7) Ornithine decarboxylase. (8) Anabolic system (Arena and Manca de Nadra, 2001).

The ability of some *L. hilgardii* strains to produce putrescine is of particular interest because of the importance of its practical implications. Besides being a potentiator of histamine toxicity and its ability to form carcinogenic nitrosamines in the presence of nitrite, putrescine is also the amine generally present at the highest concentration in wines (Soufleros et al., 1998). Concentrations of 15 to 20 and 20 to 30 mg l^{-1} putrescine have been reported to cause a significant decrease in the sensorial quality of white and red wines respectively (Arena and Manca de Nadra, 2001).

3.3.2 IDENTIFICATION OF THE TDC GENE FRAGMENTS

To study fragments of the TDC genes of the tyramine-producing LAB in this study, PCR amplification with primer pair P2-for and P1-rev (Lucas and Lonvaud-Funel, 2002) was used. PCRs were performed on the genomic DNA of six of the 10 tyramine-producing strains (two *L. brevis* and four *L. hilgardii* strains) and on an *L. brevis* strain that is unable to produce tyramine as a control (**Fig. 3.3**). PCR products of ~890 bp were amplified for all six tyramine-producing strains. The size of these amplification products is approximately in agreement with the size (~800 bp) reported by Lucas and Lonvaud-Funel (2002). These authors only tested the primer pair P2-for and P1-rev in *L. brevis* strains. Here we show that the primer pair can also be used to detect TDC genes in *L. hilgardii* (M2, M30, G81 and K56) strains.

In order to examine the amplified gene fragments of some of the LAB strains, the PCR products of *L. brevis* M58, *L. brevis* W78 and *L. hilgardii* M2 were sequenced. A 926 bp fragment was sequenced for all three strains and all three encoded 307 amino acids of a single open reading frame.

In order to describe the complete genetic sequence of another TDC gene in bacteria, apart from *E. faecalis*, inverse PCR amplification was applied to ligated *Sau*3AI restriction fragments of genomic DNA from *L. brevis* M58. The inverse PCR procedure was not optimal and yielded unspecific products (results not shown).

However, we sequenced 1 kb of a 5 kb amplified product and were able to lengthen the single open reading frame of *L. brevis* M58 by 31 amino acids (**Fig. 3.4**).

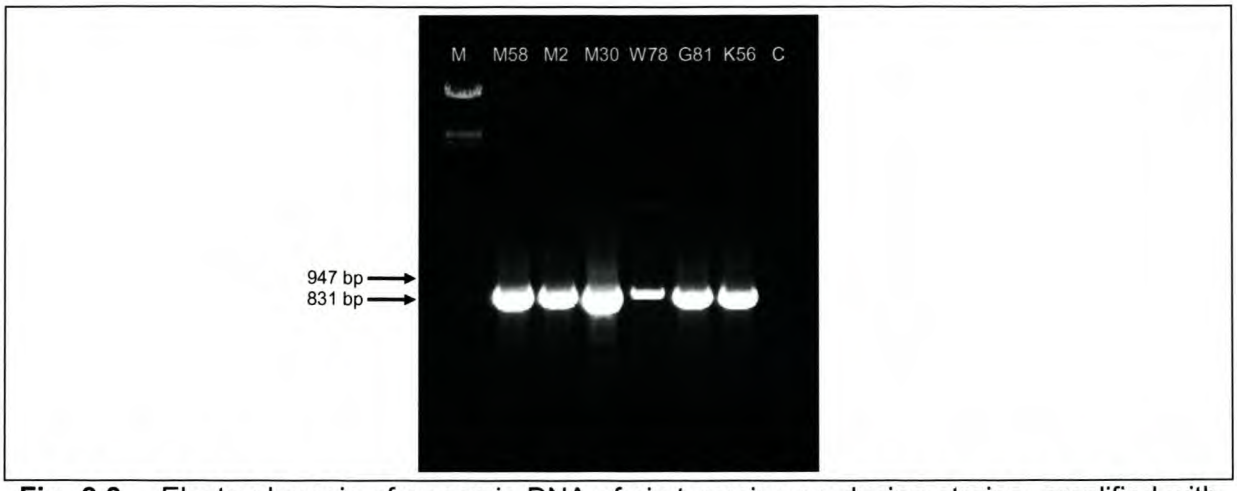

Fig. 3.3. Electrophoresis of genomic DNA of six tyramine-producing strains, amplified with primer pair P2-for and P1-rev on a 1% agarose gel. A negative tyramine-producing *L. brevis* strain was used as a control (C). The size marker contains λ DNA digested with *Eco*RI and *Hind*III (M). The bacterial strains are listed in **Table 3.2**.

3.3.3 COMPARISON OF THE AMINO ACID SEQUENCES OF THE TDC FRAGMENTS WITH KNOWN TDC SEQUENCES

An amino acid sequence alignment of the TDC fragments of *L. brevis* M58, *L. brevis* W78 and *L. hilgardii* M2 with the TDC fragment of *L. brevis* IOEB 9809 and *tdc* gene of *E. faecalis* is shown in **Fig. 3.4**. The results obtained show that a very high degree of homology exists among the *tdc* gene and the gene fragments of the LAB. All five sequences show an overall identity of 55.8% (170 amino acids) along the 307 amino acids of the gene fragments.

The TDC protein fragments contain two conserved regions that have been observed for pyridoxal-phosphate-dependent decarboxylases (Connil et al., 2002). The one region is the attachment site for the cofactor pyridoxal-phosphate (S-[LIVMFYW]-X(5)-K-[LIVMFYWGH]-[LIVMFYWG]-X(3)-[LIVMFYW]-X-[CA]-X(2)-[LIVMFYWQ]-X(2)-[RK]). Lysine (K) serves as the attachment site for pyridoxal-phosphate. The other region, the VHVDAAY motif, has been shown to be particularly conserved in pyridoxal-phosphate-dependent decarboxylases (**Fig. 3.4**).

Blast analysis (Altschul et al., 1997) showed that the protein fragments of the three LAB are closely related to bacterial pyridoxal-phosphate-dependent decarboxylases, mainly glutamate-, diaminobutyrate- and dopa decarboxylases. The best homology scores were observed with two hypothetical proteins from *Enterococcus faecium* and with the tyrosine decarboxylase from *Enterococcus faecalis*. Blast searches did not reveal any alignment with the TDCs in eukaryotes.

The Blast analysis and the presence of typical features (two conserved regions) of pyridoxal-phosphate-dependent decarboxylases show that the TDC protein fragments of the LAB strains in this study, like the two known TDC enzymes of

E. faecalis (Børresen et al., 1989) and *L. brevis* IOEB 9809 (Moreno-Arribas and Lonvaud-Funel, 2001), belong to the group of pyridoxal-phosphate-dependent decarboxylases.

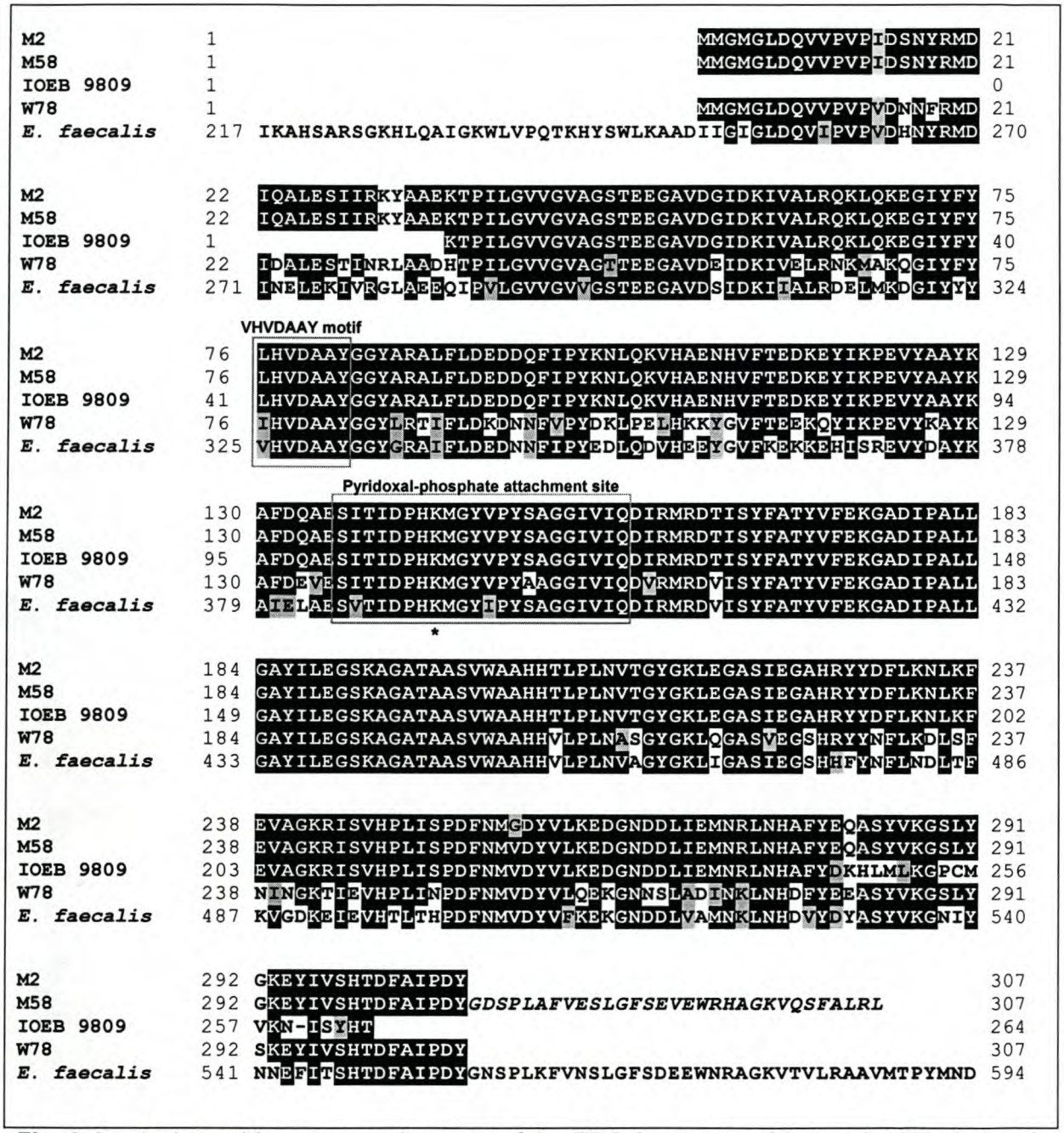

Fig. 3.4. Amino acid sequence alignment of the TDC fragments of *L. brevis* M58, *L. brevis* W78 and *L. hilgardii* M2 with the TDC fragment of *L. brevis* IOEB 9809 (AF446085) and the *tdc* gene of *E. faecalis* (AF354231). Identical amino acid residues are shown on a black background, while similar residues are shown on a gray background. The boxes represent the VHVDAAY motif and the pyridoxal-phosphate attachment site. The lysine (K) residue, which provides the attachment site for pyridoxal-phosphate, is indicated by an asterisk (*). The italic residues show the 31 amino acids added to the partial sequence of *L. brevis* M58 obtained with inverse PCR amplification.

3.4 CONCLUSIONS

On the basis of the present and previous results on biogenic amine formation by LAB in wine, it is clear that this metabolic feature is not rare. These results suggest that *Lactobacillus* may be responsible for tyramine and putrescine production and that it can contribute significantly to the overall biogenic amine content in wines. It also suggests that amine production is strain dependent and not species specific. It is important to remember that these results for biogenic amine production in synthetic media do not necessarily imply similar behaviour in wine. Wine represents a complex environment with a wide number of factors influencing microbial growth and decarboxylase activity and, thus, further investigation is necessary to determine if these amine-producing bacteria behave similarly in wine conditions.

In prokaryotes, the *tdc* gene sequence of *E. faecalis* (Connil et al., 2002) and the partial TDC gene sequence of *L. brevis* IOEB 9809 (Lucas and Lonvaud-Funel, 2002) have been described. Here we report the partial TDC gene sequences of *L. brevis* M58, *L. brevis* W78 and *L. hilgardii* M2. The PCR primers used to amplify the partial sequences in this study were developed by Lucas and Lonvaud-Funel (2002) after studying the sequences of three peptides of the purified TDC enzyme from *L. brevis* IOEB 9809. These authors used the primers to detect the TDC gene in tyramine-producing *L. brevis* strains. We used the primers to amplify a fragment of the TDC gene in tyrosine-decarboxylating *L. brevis* and *L. hilgardii* strains. Thus, our results show that the primers can also be used to detect tyramine-producing *L. hilgardii* strains.

An analysis of the sequences revealed that the three TDC protein fragments, like the two known TDC enzymes of *E. faecalis* and *L. brevis* IOEB 9809, belong to the group of pyridoxal-phosphate-dependent decarboxylases. Blast analysis indicated that bacterial pyridoxal-phosphate-dependent decarboxylases are closely related. In addition, we can conclude that the three partial prokaryotic TDCs are not closely related to the TDCs in eukaryotes.

Since the primer pair P2-for and P1-rev that were used to detect the TDC gene in *L. brevis* and *L. hilgardii* strains and the partial amino acid sequences of the TDC genes in the four LAB are highly conserved (**Fig. 3.4**), it seems that these primers could be used to detect tyramine-producing LAB. It is possible that the conservation only exists in pyridoxal-phosphate-dependent TDCs and that the primers would not be successful in detecting LAB with pyridoxal-phosphate-independent TDCs. As the *tdc* gene of *E. faecalis* also shows high homology with the TDC gene fragments in the LAB, which are all gram-positive bacteria, it may suggest the possibility that the primers could detect pyridoxal-phosphate-dependent TDCs in gram-positive bacteria. However, to report the suitability of these primers to rapidly detect tyramine-producing LAB, they should be tested in more bacteria.

The results of this study provide knowledge about which LAB in the winemaking process could contribute to the production of biogenic amines and the sequencing of

additional partial TDC genes could possibly assist in the development of a rapid detection method for tyramine-producing LAB in food products.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

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4.1 CONCLUDING REMARKS

In the past, the definition of a quality wine was controlled by the winemaker. Globalisation and worldwide access to information has resulted in a more knowledgeable and affluent consumer with a greater understanding of product value and a discriminating demand for quality. Thus, the control of the definition of quality is now in the hands of the consumer. To be successful in the modern marketplace, consumer preference will have to be used to guide production decisions as well as the marketing of wines (Bisson et al., 2002). One of these consumer demands that is of great importance is the healthful property of wine.

It is generally accepted that moderate wine consumption has health benefits. Wine has antimicrobial and anticancer activity, is effective in the management of stress and reduces cardiac and neurodegenerative diseases (Frankel et al., 1993; Weisse et al., 1995; Miloso et al., 1999; Soleas et al., 2002). Some of the protective compounds found in wine are the phenolic compounds resveratrol, quercetin, catechin and alcohol. However, some undesirable compounds, such as ethyl carbamate, a carcinogen, and biogenic amines, can also be found in wine.

The demand of consumers for better and healthier foods has led to renewed interest in studies on biogenic amines. The presence of these compounds in food and beverages is becoming an economic problem directly linked to their influence on human health. If large amounts of these amines are ingested, they can cause health problems, such as hypertension, respiratory distress, migraine and psychiatric disorders, such as schizophrenia (Ten Brink et al., 1990; Buckland et al., 1997). Histamine and tyramine are the most studied biogenic amines due to their toxicological effects. Secondary amines, such as putrescine and cadaverine, have the ability to form carcinogenic nitrosamines in the presence of nitrite and, like ethanol, potentiate the toxicity of other amines (Ten Brink et al., 1990).

Biogenic amines occur in a wide variety of food products, such as cheese, dried sausage, sauerkraut, fishery products, chocolates, wine and beer (Shalaby, 1996; Silla Santos, 1996). This thesis focussed mainly on the presence of biogenic amines in wine. Histamine, tyramine and putrescine are the amines found most frequently in wine and are believed to be produced mainly by lactic acid bacteria (LAB) during malolactic fermentation. The species *Oenococcus oeni* (Lonvaud-Funel and Joyeux, 1994; Coton et al., 1998; Guerrini et al., 2002), the main LAB responsible for malolactic fermentation, and *Lactobacillus brevis* (Moreno-Arribas and Lonvaud-Funel, 1999; Moreno-Arribas et al., 2000) have been associated primarily with the production of histamine and tyramine respectively in wine. To help with the prevention of amine formation, it is necessary to relate amine production with certain LAB species involved in the winemaking process and more research therefore is required.

Another possible way to prevent amine formation is to develop methods for the rapid detection of bacteria carrying amino acid decarboxylase genes. In this regard, a polymerase chain reaction (PCR) detection system already exists for the identification of histidine-decarboxylating LAB. This method was developed by Le Jeune et al. (1995) after studying the amino acid and nucleotide sequences of the histidine decarboxylating genes of *Lactobacillus* 30a and *Clostridium perfringens*. Histidine decarboxylating genes have been studied in a number of different bacteria. Only one tyrosine decarboxylase (*tdc*) gene sequence has been described for bacteria (*Enterococcus faecalis*) (Connil et al., 2002), in addition to the description of a partial tyrosine decarboxylase (TDC) gene sequence from *L. brevis* IOEB 9809 (Lucas and Lonvaud-Funel, 2002). To identify the partial TDC gene sequence from *L. brevis* IOEB 9809, Lucas and Lonvaud-Funel (2002) developed PCR primers (P2-for and P1-rev) from the sequences of three peptides of the purified TDC enzyme from this bacteria. The primers were then used to detect the TDC gene in a few other tyramine-producing *L. brevis* strains. When more lactic acid bacterial TDC genes are sequenced, it would most likely be possible to design primers in conserved regions and to develop a rapid detection method for tyramine-producing LAB in food and beverages.

In the first part of this study, we investigated the ability of LAB isolated from South African wine to produce biogenic amines. We found that *Lactobacillus* (*L. brevis* and *L. hilgardii*) may be the LAB that contributes to tyramine and putrescine production in wine. Future work would be necessary to determine if these amine-producing bacteria behave similarly in wine conditions. The ability of the LAB to produce biogenic amines has only been investigated in synthetic media and wine represents a complex environment with a wide number of factors, such as temperature, ethanol, pH and sulphur dioxide levels, that could influence microbial growth and decarboxylase activity.

The next part of this study focussed on the use of the PCR primers developed by Lucas and Lonvaud-Funel (2002) to identify tyramine-producing LAB, the sequencing of additional TDC gene fragments and a comparison of these sequences with the known *tdc* gene of *E. faecalis* and the TDC gene fragment of *L. brevis* IOEB 9809. Lucas and Lonvaud-Funel (2002) used the primer pair P2-for and P1-rev to detect the TDC gene in tyramine-producing *L. brevis* strains. We used the primers to amplify a fragment of the TDC gene in tyrosine-decarboxylating *L. brevis* and *L. hilgardii* strains. Thus, our results show that the designed primers can also be used to detect tyramine-producing *L. hilgardii* strains. Sequencing of the amplified products of three of the LAB in this study enabled us to determine the partial TDC gene sequences of two *L. brevis* strains (M58 and W78) and of a *L. hilgardii* strain (M2). An amino acid sequence alignment of these three TDC gene fragments with the known TDC gene fragment of *L. brevis* IOEB 9809 and the *tdc* gene of *E. faecalis* showed a high degree of relatedness and conserved regions. The results also revealed that the three TDC protein fragments, like the two known TDC enzymes of

E. faecalis (Børresen et al., 1989) and *L. brevis* IOEB 9809 (Moreno-Arribas and Lonvaud-Funel, 2001), belong to the group of pyridoxal-phosphate-dependent decarboxylases.

Since the primer pair P2-for and P1-rev used to detect the TDC gene in the *L. brevis* and *L. hilgardii* strains and the partial amino acid sequences of the TDC genes in the four LAB is highly conserved, it seems that these primers could be used to detect tyramine-producing LAB. It is possible that the conservation only exists in pyridoxal-phosphate-dependent TDCs and that the primers would not be successful in detecting LAB with pyridoxal-phosphate-independent TDCs. As the *tdc* gene of *E. faecalis* also shows high homology with the TDC gene fragments in the LAB, which are all gram-positive bacteria, it may suggest the possibility that the primers can detect pyridoxal-phosphate-dependent TDCs in gram-positive bacteria. However, to report the suitability of these primers to rapidly detect tyramine-producing LAB, they should be tested in more bacteria.

Consumers are demanding healthier foods and, as hazardous amine compounds occur in our everyday diet, procedures are necessary to prevent their formation. LAB, which are believed to be the main amine producers, are frequently used as starter cultures in food fermentations. In wine, the commercially available malolactic starters are used to carry out controlled and consistent malolactic fermentations. The ability of these starter strains to produce biogenic amines has to be taken into consideration. Moreover, maximum allowable levels of biogenic amines in food products can be established. Limits already exist for histamine levels in fish. At present there are no regulations regarding the level of biogenic amines in wines, but it may happen in the future and this will affect winemakers directly. Thus, research on biogenic amines in wine is necessary. The results of this study provide knowledge about which LAB in the winemaking process could contribute to the production of biogenic amines. In addition, the sequencing of additional partial TDC genes could possibly assist in the development of a rapid detection method for tyramine-producing LAB in food products.

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