Food Spoilage Characteristics of *Chryseobacterium* Species

by

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DECLARATION

I declare that the dissertation hereby submitted by me for the MSc.Agric. degree in the Faculty of Natural and Agricultural Science at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

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A. Mielmann

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection, Rockville, Maryland
C.	*Chryseobacterium*
E.	*Elizabethkingia*
e.g.	for example
Fig.	Figure
Figs.	Figures
GN	Gram negative
h	hour(s)
LMG	Laboratory of Microbiology, University of Ghent, Belgium
$mg.l^{-1}$	milligram per litre
min	minute(s)
ml	millilitre
NCTC	National Collection of Type Cultures, Central Public Health Laboratory, London, UK
nm	nanometre
ppm	parts per million
µl	microlitre
µm	micrometre
w/v	weight per volume
cfu	colony forming unit
g	gram
cm^2	square centimetre
cm^3	cubic centimetre

CHAPTER 1

INTRODUCTION

Food spoilage has been a continuing problem since humans first discovered they could produce more food than could be consumed in a single meal (Edward, 1990). Food spoilage is any organoleptic change – that is, any tactile, visual, olfactory, or flavour change – that the consumer considers to be an unacceptable departure from the normal state (Ayres *et al*., 1980). Micro-organisms are capable of producing a wide range of chemicals associated with their metabolic activities (metabolic by-products) giving off-odours and off-flavours that are unacceptable or highly objectionable to the consumer (Garbutt, 1997). Off-odours and off-flavours are a common cause of spoilage in all branches of the food industry and the economic consequences can be serious (Dainty, 1996). Consumption of microbially contaminated food can also cause serious infections or poisoning (Madigan *et al*., 2000).

Flavobacteria, together with *Pseudomonas*, have been shown to cause spoilage in food and food products (Forsythe, 2000). Metabolites produced by flavobacteria include alcohols, sulphur compounds, ketones, aldehydes, esters and amines and the resultant odours can be described as fishy, foul, sulphuric and ammonia-like (Nychas and Drosinos, 1999). Most of the food spoiling flavobacteria have, however, been grouped in the new *Chryseobacterium* genus (Bernardet *et al*., 1996). *Chryseobacterium* species are widely distributed in water, soil, the clinical environment and food commodities, such as milk, meat, poultry and fish (Jooste and Hugo, 1999). Two *Chryseobacterium* species, *Chryseobacterium meningosepticum* and *Chryseobacterium miricola*, have recently been transferred to the novel genus *Elizabethkingia*, as *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* (Kim *et al.,* 2005b). Organisms that have not been included in the present study are the newest validated species of the *Chryseobacterium* genus, namely *C. formosense* (Young *et al.,* 2005), *C. daecheongense* (Kim *et al.,* 2005a), *C. taichungense* (Shen *et al.,* 2005), *C. vrystaatense* (De Beer *et al*., 2005), *C. soldanellicola* (Park *et al.,* 2006) and *C. taeanense* (Park *et*

al., 2006) and also *C. proteolyticum*', a species that has not been validly published (Bernardet *et al.,* 2002).

The role and significance of flavobacteria in food and their proven and potential significance as food spoilage bacteria has been the main reason for further research. The measurement of microbial metabolites associated with microbial growth has not been studied in similar detail to the taxonomy and nomenclature of the *Chryseobacterium* genus. The main purpose of this study, therefore, was to determine the potential food spoilage characteristics of the genus *Chryseobacterium*. This was regarded as necessary to obtain a better understanding of their characteristics and food spoilage potential, so as to inform the food microbiologist and to broaden the knowledge on these aspects of members of the *Chryseobacterium* genus.

The first objective of this study was to investigate the potential food spoilage characteristics of *Chryseobacterium* strains by examining the utilisation of BIOLOG (Biolog, Inc., Hayward, California) carbon sources, performing additional metabolic activity tests and determining the ability to produce biogenic amines at different temperatures and sodium chloride concentrations, using a modified Niven medium (Niven *et al*., 1981). The results are presented in Chapter 3 of this thesis.

The second objective was to examine the growth and hydrolytic activities of *Chryseobacterium* strains by using different microbiological tests, to embark on a preliminary study of volatile compounds produced in milk using headspace gas chromatography (GC) and to determine the spoilage level in inoculated milk samples, using sensory analysis. The results are presented in Chapter 4 of the thesis.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Microbial growth destroys vast quantities of food, causing economic problems and loss of significant nutrient sources (Madigan *et al*., 2000). Growth of microorganisms in foods can cause spoilage by producing an unacceptable change in taste, odour, appearance, texture, and a combination of the above (Garbutt, 1997).

Spoilage is not only due to the visible growth of microorganisms, but also to the production of end metabolites which result in off-odours, gas and slime production (Forsythe, 2000). The spoilage potential of a microorganism is the ability of a pure culture to produce the metabolites that are associated with the spoilage of a particular product. In general, several of the organisms isolated from a food product will be able to produce spoilage metabolites when allowed unlimited growth. It is crucial that quantitative considerations are introduced, since the spoilage activity of an organism lies in its quantitative ability to produce spoilage metabolites. These considerations in general, are the implementation of a careful combination of microbiology, sensory analysis and chemistry (Gram *et al.*, 2002).

During the past decade, the *Flavobacteriaceae* family has emerged as a taxonomic grouping for a variety of Gram negative yellow-pigmented rods and *Flavobacterium* has become only one of several genera in this family (Bernardet *et al*., 1996). Many of the *Flavobacterium* species that were associated with food spoilage and pathogenicity in the past, have now been grouped into other genera, such as *Bergeyella, Chryseobacterium, Empedobacter, Myroides* and *Weeksella*, in the *Flavobacteriaceae* family (Holmes, 1992; Hugo and Jooste, 2003). Due to this fairly new reclassification, literature with regard to food spoilage still refers to

psychrotrophic bacteria of this group involved in spoilage as *Flavobacterium* or flavobacteria or CDC Group IIb organisms (De Beer, 2005).

2.2. Definition of food spoilage

Food spoilage is defined as any change in the visual appearance, smell, or taste of a food product that makes it unacceptable to the consumer (Madigan *et al*., 2000). The concept of a spoiled food is subjective and associated with individual taste. Personal preferences, ethnic origin and family background may play a role in an individual deciding whether a food is spoiled (Garbutt, 1997).

2.3. The nature of food spoilage

Spoilage can be microbial or mechanical in origin (Ayres *et al*., 1980). Microbial spoilage is by far the most common cause of spoilage of perishable foods and may manifest itself as visible growth (slime, colonies), as textural changes (degradation of polymers) or as off-odours and off-flavours. Despite chill chains, chemical preservatives and a much better understanding of microbial food spoilage, it has been estimated that 25% of all foods produced globally is lost *post harvest* or *post slaughter* due to microbial spoilage (Gram *et al*., 2002).

The physical and chemical characteristics of the food and how it is stored, determine its degree of susceptibility to microbial attack (Madigan *et al*., 2000). Although the total microbial flora may increase during storage, it is specific spoilage organisms which cause the chemical changes and the production of off-odours (Forsythe, 2000). This is because the chemical properties of foods vary widely, and different foods are colonized by the indigenous spoilage organisms that are best able to use the nutrients available. Microbial growth in foods follows the standard pattern for a bacterial growth curve. It is only when the microbial population density reaches a substantial level that harmful spoilage effects are usually observed (Madigan *et al*., 2000).

Off-odours or off-flavours can often be detected soon after 10^6 organisms/g or per ml or per cm^2 of food surface have been produced. This level can be considered as the cut-off point between spoiled and unspoiled (level of incipient spoilage) (Garbutt, 1997). Indeed, throughout much of the exponential phase of growth, population densities may be too low to observe any perceptible effect, but because of the nature of exponential growth, it is only the last doubling or two of the population that leads to observable spoilage (Madigan *et al*., 2000).

2.4. The most important factors affecting the growth of food spoilage bacteria

2.4.1. Temperature

One of the most crucial factors affecting microbial growth in food is temperature (Madigan *et al*., 2000). Growth is restricted to those temperatures at which an organism's cellular enzymes and membranes can function (Garbutt, 1997). As the temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates, and growth becomes faster. However, above a certain temperature, particular proteins may be irreversibly damaged. Thus, as the temperature is increased within a given range, growth and metabolic functions increase up to a point where inactivation reactions set in. Every food spoilage bacteria has cardinal temperatures namely, a minimum temperature below which growth no longer occurs, an optimum temperature at which growth is most rapid, and a maximum temperature above which growth is not possible (Madigan *et al*., 2000).

Since the first observation of bacterial growth at 0°C, many terms were used for these organisms. The term "psychrotroph" was introduced by Eddy (1960) to replace the misnomer "psychrophilic". The latter term indicates organisms that have a preference for growing at low temperatures, while psychrotrophs should rather be regarded as cold tolerant being able to grow at 7°C or less but having optimum temperatures of 25 to 35°C. In 1976 the International Dairy Federation (IDF) adopted the following definition: A psychrotroph is a

micro-organism which can grow at 7°C or less, irrespective of its optimum growth temperature (IDF, 1976).

Many psychrotrophic bacteria, when present in large numbers, can cause a variety of off-flavours as well as physical defects in foods. Raw foods held under refrigeration prior to processing, as well as non-sterile heat processed foods that rely on refrigeration for shelf life, are subject to quality loss and possible spoilage by psychrotrophic bacteria. Although psychrotrophic bacteria will not grow in frozen foods, they can grow and cause spoilage if the food is allowed to thaw partially, and is subsequently held at too high a temperature (i.e., unfrozen, but still refrigerated) (Gilliland *et al*., 1976). Studies have revealed that the most common bacteria isolated on dairy equipment surfaces are Gram negative psychrotrophs (e.g. flavobacteria), which are responsible for growth and spoilage in milk at refrigeration temperatures (Koutzayiotis *et al*., 1993). Jooste *et al*. (1986) investigated the role of flavobacteria as causative agents of the putrid butter defect and found that the optimum growth temperature for the six *Flavobacterium* strains from butter tested, was 25°C and that these strains were capable of multiplication in cream both at 6°C and 25°C.

2.4.2. pH

pH is one of the main factors affecting the growth and survival of micro-organisms in culture media and in foods. All micro-organisms have a pH range in which they can grow and an optimum pH at which they grow best. Bacteria generally have a minimum pH for growth of around 4.0 – 4.5 and an optimum pH between 6.8 and 7.2, (that is, more or less neutral), and pH maxima between 8.0 and 9.0 (Garbutt, 1997). Organisms that thrive at low pH values are called acidophiles. Organisms that have very high pH optima for growth, are known as alkaliphiles, which can produce hydrolytic enzymes, such as proteases and lipases (Madigan *et al*., 2000).

The pH minimum for an organism is determined by the temperature of the environment (e.g. the incubation temperature in the laboratory), the nutrients

that are available, the water activity and the presence or absence of inhibitors (Garbutt, 1997). Despite the pH requirements of a particular organism for growth, the optimal growth pH represents the pH of the extracellular environment only, the intracellular pH must remain near neutrality in order to prevent destruction of acid-or alkali-labile macromolecules in the cell. For the majority of microorganisms, whose pH optimum for growth is between 6 and 8 (referred to as neutrophiles), the cytoplasm remains neutral or very nearly so (Madigan *et al*., 2000). When the microbial cell is subjected to extreme pH values, cell membranes become damaged. The pH minimum for an organism depends on the type of acid present. Generally, the minimum is higher if any organic acid is responsible for the environmental pH rather than an inorganic acid. Foods are quite variable in terms of their pH values. Most are acidic, ranging from the very acidic to almost neutral in reaction. pH changes in foods due to the activity of micro-organisms are common. Meat becomes more alkaline when spoilage is caused by Gram negative rods such as *Pseudomonas* spp.. The organism uses amino acids as its carbon source which leads to the production of ammonia, making the cell environment more alkaline (Garbutt, 1997). Shimomura *et al.* (2005) found that the pH range of *Chryseobacterium shigense* for growth was 5-8. According to Park *et al.* (2006) the pH range for growth of *Chryseobacterium soldanellicola* is pH 5-7 and that for optimal growth is pH 5. The pH range for growth of *Chryseobacterium taeanense* is pH 5-9 and that for optimal growth is pH 5.

2.4.3. Water activity and Sodium chloride

Water availability not only depends on the water content of an environment, that is, how moist or dry a solid microbial habitat may be, but is also a function of the concentration of solutes such as salts, sugars, or other substances that are dissolved in water. This is because dissolved substances have an affinity for water, which makes the water associated with solutes unavailable to organisms. Water availability is generally expressed in physical terms such as water activity (a_w) (Madigan *et al*., 2000). The water content of a food may bear little relationship to its water activity. Foods may have a low salt content but a low water activity. Each specific organism has its own range of water

activity in which it will grow. Most organisms have an optimum approaching 1.0, where the water activity is high but, where there is also sufficient dissolved nutrients to support rapid growth (Garbutt, 1997).

An added complication is the reaction that some organisms show towards sodium chloride (NaCl). Halophiles are organisms that require sodium ions in order to grow. Moderate halophiles are organisms that require NaCl but will grow only at moderate concentrations, i.e. between 1 and 10%. Sodium ions are believed to be involved with the transport mechanisms associated with the cell membrane and the uptake of materials from the environment. Extreme halophiles are organisms that will only grow at high sodium chloride concentrations (Garbutt, 1997) and generally require 15-30% NaCl, depending on the species, for optimum growth. Halotolerant organisms can tolerate some reduction in the a_w of their environment, but generally grow best in the absence of the added solute (Madigan *et al*., 2000).

In a study by Jooste *et al*. (1986), the *Flavobacterium* strains tested in NaCl Broth were able to grow in 1% (w/v) NaCl, but not in 4% NaCl Broth. Mudarris *et al.* (1994) found that *Chryseobacterium scophthalmum* was able to grow in the presence of 0 to 4% NaCl, but not in the presence of 5% NaCl. According to Jooste and Hugo (1999) no growth of *Chryseobacterium indologenes* and *Chryseobacterium meningosepticum* occurred in Nutrient Broth with the addition of 6% NaCl. De Beer *et al*. (2005) found that *Chryseobacterium gleum* and *Chryseobacterium indologenes* exhibited growth in the presence of 3% NaCl. According to Park *et al.* (2006) *Chryseobacterium soldanellicola* exhibited growth in the presence of 0 to 4% (w/v) NaCl within 14 days and *C. taenense* exhibited growth in the presence of 0 to 6% (w/v) NaCl within 14 days.

2.5. Spoilage caused by flavobacteria

Flavobacteria have been associated with spoilage of food, but information about the incidence and role of flavobacteria in food deterioration is difficult to obtain, mainly due to the history of faulty classification or reclassification of

these organisms. They are, however, accepted as common contaminants of protein-rich foods and under refrigerated storage, they are in competition with the pseudomonads (García-López *et al.*, 1999; De Beer, 2005).

Undesirable flavours and odours, possible slime production and/or toxic metabolic end products are detrimental and apart from an economical loss to industry and consumers, may also have a health impact on consumers. Even if the spoilage bacteria are not pathogenic *per sé*, changes in the biochemical status of stored food due to deterioration by such bacteria, may make conditions favourable for other bacteria, or even pathogens, to grow in (De Beer, 2005).

Studies on the proteolytic activities of flavobacteria (Cousin, 1982; Jooste, 1985) have indicated that flavobacteria may possibly produce pasteurisation resistant extracellular enzymes and that they may in this way contribute to the psychrotrophic spoilage of milk and dairy products. Although psychrotrophs secrete other enzymes with spoilage potential, e.g., glycosidases, the most important enzymes from the viewpoint of food spoilage are extracellular proteinases, lipases, and phospholipases on which this review will concentrate (Fox *et al.,* 1989).

2.5.1. Proteolytic activity

All enzymes that catalyse hydrolysis of proteins to peptones, polypeptides, and amino acids, are called proteolytic enzymes. These enzymes hydrolytically cleave the peptide linkage with the formation of a free amino and carboxylic acid group. Animal proteinases include such enzymes as pepsin, rennet, trypsin, chromotrypsin, and cathepsin (Mountney and Gould, 1988).

Continued proteolysis results in putrid off-flavours associated with lower-molecular-weight degradation products such as ammonia, amines, and sulphides (Frank, 1997). Proteinase production by psychrotrophs is normally at a maximum in the late exponential or stationary phase of growth. Bitter peptides are normally characterised by large numbers of hydrophobic amino

acids (Chen *et al*., 2003). Proteases produced by psychrotrophs have been shown to hydrolyse casein, but whey proteins were more resistant against hydrolysis (Venter, 1997). The optimum pH and temperature for protease production depends on the species and strain. The most common proteolytic activity in milk was reported as clotting (Cousin, 1982).

Roussis *et al*. (1999) found that *Flavobacterium* MTR3 proteinases were active at 32-45°C, and exhibited considerable activity at 7°C. The enzyme was active at pH 6.0-8.0, and exhibited considerable activity at pH 6.0 in the presence of 4% NaCl.

2.5.2. Lipolytic activity

Lipolytic enzymes can be defined as carboxylesterases that hydrolyse acylglycerols (Chen *et al*., 2003). Most bacterial lipases are extracellular and are produced during the late log and early stationary phases of growth (Fox and Stepaniak, 1983). True lipases act on insoluble substrates such as micelles in emulsion or surface monolayers (Stepaniak and Sorhaug, 1989).

Lipolysis is known to contribute both desirable and undesirable flavours to dairy products, initially through hydrolysis of milk triacylglycerols. Short-chain fatty acids, such as butyric acid (C4:0), caproic acid (C6:0) and caprylic acid (C8:0), impart sharp and tangy flavours. Medium-chain fatty acids, such as capric (C10:0) and lauric acid (C12:0) tend to impart a soapy taste, while long-chain fatty acids, such as myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0), contribute little to flavour (Al-Shabibi *et al*., 1964). Unsaturated fatty acids released during lipolysis are susceptible to oxidation and the concomitant formation of aldehydes and ketones, which give rise to off-flavours described as “oxidised card-boardy” (tallowy), or metallic (Chen *et al*., 2003).

The lipases from many of the psychrotrophic bacteria are remarkably heat stable and may, therefore, contribute to lipolysis in dairy products, even when they are heat treated. The microbial lipases can attack intact fat globules and

may cause lipolysis without any prior activation (Mottar, 1989). Other unpleasant flavours, such as ‘’rancid, butyric, bitter, unclean, soapy and astringent’’ in milk and milk products, have also been attributed to lipolysis (Deeth and Fitz-Gerald, 1994). In general, flavobacteria are less well-known for lipase production. However, significant lipase production by some *Flavobacterium* strains have been reported by some researchers (Roussis *et al*., 1999).

Optim al temperature for extracellular Gram negative bacterial lipases is found in the temperature range of 30 to 40°C. Bacterial lipases appear to be very stable at temperatures below 8°C. The optimum pH of most extracellular Gram negative bacterial lipases appears to be at neutral or alkaline pH values between seven and nine. It has been suggested that the optimum pH depends upon the nature of the substrate, the buffer solution, and other external conditions (Mottar, 1989).

2.5.3. Phospholipase C production

The production of phospholipases, especially type C or lecithinase by some *Flavobacterium* strains have been reported by some researchers (Fox *et al.,* 1976; Cousin, 1989). Phospholipases are a complex group of enzymes which act on phospholipids. Most bacterial phospholipases are of the C-type, that is, they hydrolyse phospholipids to diglycerides and substituted phosphoric acid. There are at least two subclasses of phospholipase C (Fox *et al*., 1989):

(1) those that hydrolyse phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS) and
(2) those that hydrolyse phosphatidylinositol (PI).

Phospholipases are potentially important in milk and milk products because of their ability to degrade the phospholipids of the milk fat globule membrane, thereby increasing the susceptibility of the milk fat (triglycerides) to lipolytic attack. Extracellular phospholipases produced by psychrotrophs growing in stored raw milk have the potential to exaggerate the problem of rancidity (Cousin, 1989; Mottar, 1989).

2.6. Flavobacteria in the perishable food environment

The group of Gram negative bacteria collectively known as the flavobacteria have, over time, been assigned various roles. In the food environment they have increasingly become associated with the spoilage of food and food products (De Beer, 2005). According to Madigan *et al.* (2000), foods can be classified as highly perishable foods, semi-perishable foods and stable or non-perishable foods. This review will concentrate on highly perishable foods.

2.6.1. Milk and milk products

Flavobacteria are frequently found in the dairy processing environment and they are responsible for several defects in dairy products (Jooste and Hugo, 1999). In milk they produce heat resistant proteolytic (and possibly lipolytic) enzymes responsible for off-flavours in pasteurised milk and cream, surface taint in butter and thinning in creamed rice. They are also responsible for reduction in cheddar cheese yield and bitterness in milk due to the production of phospholipase C (Fox *et al.*, 1976; García-López *et al.*, 1999; Jooste and Hugo, 1999; Bernardet *et al.*, 2002). Common defects caused by protease activity in milk are the development of unclean and bitter flavours and gelation of the milk. The amino acids formed may produce browning upon heating and lower the nutritional value of the milk (Venter, 1997). Rancid and fruity flavours result from lipolysis. Milliere and Veillet-Pancet (1985), however, found *Flavobacterium* to be the most abundant caseolytic psychrotroph in raw milk (53.3%).

2.6.2. Red Meat

Spoilage of raw red meat will result in off-odours, possible slime production, discoloration of a specific area and undesirable flavours due to metabolic end-products formed (De Beer, 2005). Psychrotrophic organisms continue to dominate the spoilage flora up to temperatures of about 25ºC (Garbutt, 1997). Metabolites produced by flavobacteria include alcohols such as methanol and ethanol, sulphur compounds such as dimethylsulphide, methylmercaptan and

methanethiol, ketones, aldehydes, esters and amines from amino acid metabolism (Banwart, 1989). Off- or mal-odours can be described as fishy, foul, sulphuric and ammonia-like (Nychas and Drosinos, 1999). The presence of flavobacteria have also been demonstrated in processed meats (McMeekin *et al*., 1971; McMeekin *et al.,* 1972). Similarly, in chilled meats and poultry, flavobacteria are a constant part of the initial flora, but are unable to compete with pseudomonads during storage (McMeekin, 1982).

2.6.3. Poultry

Spoilage of poultry is generally restricted to the outer surfaces of the skin and cuts and has been characterized by off-odours, which appears at a bacterial load between 10^6 and 10^8/cm^2 (Banwart, 1989), sliminess generally occurs shortly after the appearance of off-odours, with log counts/cm^2 of about 8 (Jay, 1992), and various types of discolorations (Jackson *et al*., 1997). The inner portions of poultry tissue are generally sterile, or contain relatively few organisms, which generally do not grow at low temperatures. Poultry legs are more perishable. This is due to the slightly higher pH (6.2 to 6.4) of leg muscle compared to the 5.7 to 5.8 of breast meat (Mossel *et al*., 1995).

Hinton *et al*. (2004), found that there was a significant increase of psychrotrophic spoilage bacteria during processing due to cross-contamination and that these bacteria were responsible for spoilage of poultry during refrigerated storage. The source of origin of these organisms, may be from the poultry itself or from the abattoir environment (Hang'ombe *et al*., 1999). Mai and Conner (2001) found that the incidence of *Pseudomonas* and *Flavobacterium* on chicken carcasses were 17% and 16% respectively. According to Nychas and Drosinos (1999), the incidence of flavobacteria on poultry is much higher than on fresh red meat, while De Beer (2005) found that *Chryseobacterium* species were present throughout the processing unit of a poultry processing plant. Environmental sources such as dust, most likely contributed to contamination levels of psychrotrophic, yellow-pigmented colonies and especially *Chryseobacterium*, in raw chicken meat.

2.6.4. Fish

The normal spoilage flora of fish at chill temperatures consists largely of psychrotrophic Gram negative bacteria (Mossel *et al*., 1995). Bacteria can be detected in fresh fish in the slime coat on the skin (10^3 – 10^5 cfu/cm^2), gills (10^3 – 10^4 cfu/g) and the intestines (10^2 – 10^9 cfu/g). Fish spoilage bacteria apparently have little difficulty in growing in the slime and on the outer integument of fish. Slime is composed of mucopolysaccharide components, free amino acids, trimethylamine oxide (TMAO), which plays an important role in microbial degradation of freshly caught fish (De Beer, 2005), piperidine derivatives, and other related compounds (Jay, 1992).

Flavobacteria occur regularly on fish and shellfish, but their role in spoilage of chilled fish is minor compared to that of *Pseudomonas* (Engelbrecht, 1992). The catabolites produced by spoilage organisms in fish include ammonia, amines and sulphides (Gram and Dalgaard, 2002). Other typical fish spoilage odours are fruity, pungent and musty and are mainly produced by Gram negative bacteria such as the pseudomonads and flavobacteria (Engelbrecht *et al*., 1996).

2.7. Microbial deterioration of food components

The type and extent of microbial colonization of a food only partly affects its ultimate deterioration, because the biochemical activities of the microbial community structure at the time of the onset of spoilage are also decisive (Mossel *et al*., 1995). Organoleptic deterioration may, however, occur before any marked chemical changes take place in the food. This is because some odiferous metabolites can be detected organoleptically at very low levels. Less than 1 ppm dimethyl sulphide or methyl mercaptan is sufficient to cause off-odours (Fields *et al*., 1968). Even at the maximum cell concentration usually achieved (about 10^9 cfu g^{-1} or ml^{-1}), metabolising at the optimum rate would only produce about 2 ml g^{-1} h^{-1} of carbon dioxide. At lower temperatures this rate would be much less. Conversely, high levels of

microbes may be present in a food that shows no obvious organoleptic change (Mossel *et al*., 1995).

The growth of microbes in foods inevitably causes chemical changes. Bacteria, the predominating organisms in the microbial ecology of most foods, are extremely small: a rod of 2 x 0.8 µm has a volume of about 10^{-12} cm^3. Although they have a high metabolic potential per cell, large numbers of bacteria are required before they can cause measurable chemical changes (Mossel *et al*., 1995).

2.7.1. Microbial metabolites

Biological as well as fabricated food structures will possess receptors to which microorganisms can absorb. The resulting colonization of such structures may occur in a stratified way, leading to relatively high local concentrations of microbial metabolites (Marshall, 1985; Delaquis *et al*., 1988). The metabolites formed by a given spoilage association will once again depend on the prevailing intrinsic, extrinsic and implicit conditions. These include the limiting factors influencing: (1) the type of spoilage, determined by the relative amounts of metabolites formed; and (2) the rate at which these metabolites are produced during storage and distribution of the food. The latter is mostly expressed as the time to (onset of) spoilage, as detected by sensory evaluations – odour, colour, structure and taste (Mossel *et al*., 1995). The microbial metabolites depend not only on the storage conditions but also on other environmental factors such as aeration, glucose and lactate availability, and pH (Dainty *et al*., 1985).

2.7.1.1. Carbohydrates

Carbohydrates, if available, usually are preferred by microorganisms to other energy-yielding foods (Mountney and Gould, 1988). The carbohydrates are divided into monosaccharides, disaccharides, and polysaccharides. The monosaccharides are polyhydroxy aldehydes (aldoses), or polyhydroxy ketones (ketoses). For utilisation, bacteria first need to break down complex

carbohydrates such as starch into their constituent monosaccharides (Banwart, 1989). The random splitting of glycosidic bonds results in softening and liquefaction (Chesson, 1980). Several bacteria possess an extracellular enzyme, diastase or amylase, which hydrolyses the starch. The starch is then converted either directly to glucose or via intermediates such as maltose (Banwart, 1989).

Although flavobacteria do not degrade lignin and cellulose, it is possible that these organisms are involved in the breakdown of various proteins and carbohydrates (Shewan and McMeekin, 1983). Glucose is the main carbohydrate used as a carbon and energy source. The breakdown of this monosaccharide can proceed by several pathways. In aerobic respiration the glucose metabolite, pyruvate is converted into carbon dioxide (CO_2) and water (H_2O) by means of the tricarboxylic acid (TCA) cycle, Krebs cycle, or citric acid cycle. To enter the system, the pyruvate is converted to acetate activated by coenzyme A. Only the aerobic and some facultatively anaerobic microorganisms possess an intact TCA cycle. The pyruvic acid can be decarboxylated to form acetaldehyde and CO_2. The acetaldehyde can remain or be reduced to ethyl alcohol, oxidized to acetic acid, or condensed to form acetoin or acetylmethylcarbinol (AMC). The AMC can be oxidized to diacetyl, which has a butter flavour, or reduced to 2,3-butanediol. Pyruvate can be aminated to form alanine (Banwart, 1989). Boers *et al.* (1994) observed that the glucose concentration had decreased to a low level at the first signs of spoilage. It has been concluded also that glucose limitations caused a switch from a saccharolytic to an amino acid degrading metabolism in at least some bacterial species (Borch *et al*., 1991).

Foods with high levels of carbohydrates are preferentially colonized by glycolytic organisms and tend to ferment rather than putrefy. This leads to the production of acids (mainly lactic and acetic) and is accompanied by a reduction in pH. The lactate occurring in flesh foods due to post mortem glycolysis can often be differentiated by its optical rotation from lactic acid formed by microorganisms; this increases its reliability as an index of spoilage (Nychas *et al*., 1998). However, in some instances lactic acid may be

dissimilated and acetic acid may be a better indicator of microbial colonization and metabolism (Kakouri and Nychas, 1994).

2.7.1.2. Fats

The principle lipids in foods are fats. Fats are esters of glycerol and fatty acids and are called glycerides (Banwart, 1989), in the ratio of one molecule of glycerol to three molecules of fatty acids (Mountney and Gould, 1988). A pure fat is not attacked by microorganisms, since there must be a nutrient-containing aqueous phase in which the organism can grow. Lipase, an enzyme that hydrolyses fats to free fatty acids and glycerol, is present in many kinds of foods. Because milk contains an appreciable amount of this enzyme, milk fat often undergoes lipase-catalysed hydrolysis with the production of free fatty acids, diglycerides, monoglycerides, and in extreme cases, free glycerol (Mountney and Gould, 1988). Short-chain water-soluble fatty acids (butyric, caproic, and caprylic) cause obnoxious rancid flavours in milk (Banwart, 1989). Lipolysis in foods followed by ß-oxidation produce ketones, which always result in off-flavours (Mossel *et al*., 1995).

The oxidative deterioration of fats involves the reaction of unsaturated fatty acids with oxygen to yield hydroperoxides. The hydroperoxides are not flavour compounds, but readily decompose to carbonyl compounds resulting in off-flavours or -odours. The carbonyl compounds are mixtures of saturated and unsaturated aldehydes and produces ketones (Banwart, 1989).

2.7.1.3. Proteins

Microorganisms, through their proteolytic enzymes, break down protein into simpler substances. The breakdown usually follows the following pattern (Mountney and Gould, 1988): protein → peptones → polypeptides → peptides → amino acids → ammonia (NH_3) → elemental nitrogen (N).

Proteinases catalyse the hydrolysis of proteins to peptides, which may impart a bitter taste to foods. Peptidases catalyse the hydrolysis of

polypeptides to simpler peptides and finally to amino acids. The latter impart flavours, desirable or undesirable, to some foods; e.g., amino acids contribute to the flavour of ripened cheeses (Frazier, 1988). The products that are formed depend upon (1) the type of microorganism; (2) the types of amino acids; (3) temperature; (4) the amount of available oxygen; and (5) the types of inhibitors that might be present (Banwart, 1989).

Decomposition of protein by aerobic organisms is called decay. Proteins containing amino acids with sulphur, such as cystine and methionine, can be broken down with no unpleasant odour because the end products are completely oxidized and stabilized (Mountney and Gould, 1988). Sulphur compounds, however, are often associated with 'putrid' odours (Dainty *et al.*, 1985).

The metabolites produced by microorganisms in proteinaceous foods such as meat include ammonia, ethanol, lactate, acetate, indole and acetoin, with smaller quantities of higher fatty acids, amines and ethyl esters of the lower fatty acids, sulphides, hydrogen sulphide and mercaptans (Edwards and Dainty, 1987). Most of the esters, amines, ammonia and sulphur compounds are produced from amino acids. There is no significant degradation of protein proper until spoilage has progressed to obvious deterioration. Owing to production of amines and ammonia, the pH of proteinaceous foods tends to rise as spoilage progresses (Mossel *et al*., 1995). An increase in the pH of a protein food indicates protein degradation, just as a decrease in pH results from the fermentation of carbohydrates (Banwart, 1989).

2.7.2. Volatile compounds

In fresh products, such as fruit, vegetables and milk, flavour components are very abundant (Fedele *et al.,* 2005). Chang (1973) stated that while the odour of some foods may be accounted for by single key compounds, most food odours are the result of complex mixtures. Dainty *et al.* (1989) stated that the variability of individual chemicals found in the aroma of spoiled samples was not significant. A better understanding of the complexity can be gained if the

volatile compounds are grouped into classes: sulphur compounds, ketones, esters, aromatic hydrocarbons, aliphatic hydrocarbons, aldehydes and alcohols, but according to Fedele *et al.* (2005), not all of these compounds have significant effects on the overall odours. Examination of volatile compound profiles indicated there were at least 3 requirements for development of putrid odours. These requirements are that: (1) the total volatile compound peak area must be appreciably high, (2) with exception of aliphatic hydrocarbons, the sulphur compounds must be the major constituents of the profile and (3) large quantities of other classes, if present, may modify the effects of the sulphur compounds (Dainty *et al.*, 1989).

The determination of total 'volatile bases', which include ammonia, trimethylamine and other compounds, correlates well with organoleptic judgment in a number of species of fish (Mossel *et al*., 1995). Stutz *et al.* (1991) found that the concentration of four of the volatile compounds, acetone, methyl ethyl ketone, dimethyl sulphide and dimethyl disulphide increased continuously during storage of minced meat stored aerobically at 5, 10, or 20°C. Hydrogen sulphide and ammonia are formed as a result of the conversion of cysteine to pyruvate by the enzyme cysteine desulphydrase (Nychas *et al*., 1998). Acetoin is the major volatile compound produced on raw and cooked meats in O_2-containing atmospheres (Jay, 1992). According to Overton and Manura (1999) milk samples were found to contain numerous straight and branched chain hydrocarbons, aldehydes, alcohols, ketones, fatty acids, esters, phenolic compounds and lactones.

2.7.3. Biogenic amines

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. Biogenic amines in food and beverages are formed by the enzymes of raw material or are generated by microbial decarboxylation of amino acids, but it has been found that some of the aliphatic amines can be formed "in vivo" by amination from corresponding aldehydes (Santos, 1996). Koessler *et al*. (1928) proposed that biogenic amine formation is a protective

mechanism for bacteria against acidic environments. The production of amines requires the availability of free amino acids and appropriate status of environmental factors such as pH and temperature (Maijala *et al*., 1993). The precursors of the main biogenic amines involved in food poisoning are (Santos, 1996): histidine → histamine, tyrosine → tyramine, hydroxytryptophane → serotonin, tryptophane → tryptamine, lysine → cadaverine, ornithine → putrescine, arginine → spermine, and arginine → spermidine.

The prerequisites for biogenic amine formation by microorganisms are (Santos, 1996): (1) availability of free amino acids, but not always leading to amine production; (2) presence of decarboxylase-positive microorganisms; and (3) conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity.

Biogenic amines are present in a wide range of food products including fish products, meat products, dairy products, wine, beer, vegetables, fruits, nuts and chocolate (Santos, 1996). Virtually all foods that contain proteins or free amino acids and are subject to conditions enabling microbial or biochemical activity, are conducive to the production of biogenic amines. The total amount of the different amines formed strongly depends on the nature of the food and the microorganisms present (Brink *et al*., 1990).

Different biogenic amines (histamine, putrescine, cadaverine, tyramine, spermine, spermidine) have been detected in fish such as mackerel, herring, tuna, and sardines. Other amines, such as trimethylamine and dimethylamine are present in fish and fish products at levels depending on the fish freshness (Santos, 1996). Bacterial-produced histamine has also been found in dairy products and vegetables (Actis *et al*., 1999). Amines (e.g. histamine, tryptamine, tyramine) are also important because of their role in causing spoilage of dairy products by producing typical off-flavours and putrid odours (Chander *et al*., 1989). Putrescine, cadaverine, histamine, tyramine, spermine and spermidine were found to be present in minced pork, beef and poultry stored at chill temperatures (Nychas *et al*., 1998).

Histamine has been recognised as the causative agent of scombroid poisoning (histamine intoxication), as well as nausea, vomiting, gastrointestinal distress and headache (lenistea, 1973; Niven *et al.*, 1981), whereas tyramine has been related to food-induced migraines and hypertensive crisis in patients under antidepressive treatment with monoamine oxidase inhibitor (MAOI) drugs. Secondary amines such as putrescine and cadaverine can react with nitrite to form heterocyclic carcinogenic nitrosamines, nitrosopyrolidine and nitrosopiperidine (Santos, 1996).

The levels reported for histamine and its potentiators in food would not be expected to pose any problem if normal amounts were consumed. Sandler *et al*. (1974) reported that 3 mg of phenylethylamine causes migraine headaches in susceptible individuals, while 6 mg total tyramine intake was reported to be a dangerous dose for patients receiving monoamine oxidase inhibitors (Shalaby, 1993). The level of 1000 mg kg^{-1} (amine/food) is considered dangerous for health. This level is calculated on the basis of food borne histamine intoxications related to amine concentration in food (Taylor, 1985). The European Community has recently proposed that the average content of histamine should not exceed 10-20 mg/100 g of fish (Santos, 1996).

2.8. The genus *Chryseobacterium*

2.8.1. Ecology

Flavobacteria (including *Chryseobacterium, Cytophaga, Flavobacterium* and *Flexibacter*) are widely distributed in soil and aquatic environments, raw meat, and milk, and have been found in human clinical material (Dugas *et al.,* 2001). CDC Group IIb strains (also now known to contain most *Chryseobacterium* species), have been found to be the most common of the *Flavobacterium* isolates from clinical specimens and the hospital environment in the UK (Owen and Holmes, 1981). The clinical role of *Flavobacterium,* including Group IIb, has been reviewed by Von Graevenitz (1981), and several reports have drawn attention specifically to the role of Group IIb in a case of meningitis and in various cases of bacteraemia (Holmes *et al*., 1984b).

In a study of yellow pigmented Gram negative bacteria from environmental sources by Hayes (1977), phenon 1 was found to be the largest single cluster. Representative strains of this phenon which were subsequently examined by Owen and Holmes (1981) were found to resemble Group IIb strains. In a study by Jooste *et al*. (1985), it was found that their cluster 1A comprised 43 % of the total isolates. This cluster was regarded as Group IIb-like organisms, since it contained the reference strains NCTC 10795 and strain M15/1 of Hayes phenon 1. It would appear, therefore, that this group of bacteria is generally the most prevalent flavobacterial taxon in both clinical and non-clinical environments (Hugo, 1997).

2.8.2. Taxonomy of Chryseobacterium

The genus *Chryseobacterium* was proposed in the mid nineties by Vandamme *et al.* (1994) for the species in Group A of Holmes (1992), which included *Flavobacterium* CDC Group IIb strains. *Flavobacterium* species that have since been renamed include: *Chryseobacterium* [*Flav*.] *indologenes, Chryseobacterium* [*Flav*.] *gleum, Chryseobacterium* [*Flav*.] *indoltheticum*, *Chryseobacterium* [*Flav*.] *balustinum*, *Chryseobacterium* [*Flav*.] *meningosepticum* with *C. gleum* as type species (Bernardet *et al*., 2002), as both its genotypic and phenotypic characteristics have been studied in detail (Holmes *et al*., 1984b). *Chryseobacterium* [*Flav*.] *scophthalmum* was also included in this genus in 1994 (Mudarris *et al*., 1994).

Since the publication of Bernardet *et al*. (2002), new species have been validated. *Chryseobacterium defluvii*, isolated from sewage water (Kämpfer *et al*., 2003) and *C. miricola*, isolated from condensation water in a Russian space station (Li *et al*., 2003) were also introduced to the study. Studies by Hugo (1997) and Hugo *et al*., (2003) have shown that yellow pigmented flavobacterial strains from raw milk were actually members of the genus *Chryseobacterium*. These studies also led to the description of a new species, *Chryseobacterium joostei* , isolated from raw milk.

The latest validated species of this genus are *C. formosense* (Young *et al*., 2005), *C. daecheongense* (Kim *et al*., 2005a), *C. taichungense* (Shen *et al*., 2005), *C. shigense* (Shimomura *et al*., 2005), *C. vrystaatense* (De Beer *et al*., 2005), *C. soldanellicola* (Park *et al.,* 2006) and *C. taeanense* (Park *et al.,* 2006). The species validated in 2005 and 2006 were not included in this study. One strain, *'C. proteolyticum'* has been described by Yamaguchi and Yokoe (2000) but has not been validly published (Bernardet *et al*., 2002). Studies by Kim *et al*. (2005b) proposed that *C. meningosepticum* and *C. miricola* should be transferred to a new genus, *Elizabethkingia*. Of the currently validated species of *Chryseobacterium*, only *C. balustinum*, *C. gleum*, *C. indologenes* and *C. joostei* are often associated with food (Hugo and Jooste, 2003). Table 2.1 contains a summary of the species of the genus and their sources of isolation.

2.8.3. Description of Chryseobacterium

Chry.se.o.bac.te'ri.um. Gr. adj. *chryseos*, golden; Gr. neut. n. *bakterion*, a small rod; N.L. neut. n. *Chryseobacterium*, a yellow rod.

Cells are Gram negative, nonmotile, non-spore-forming rods with parallel sides and rounded ends; typically the cells are 0.5 µm wide and 1 to 3 µm long. Intracellular granules of poly-ß-hydroxybutyrate are absent (Vandamme *et al*., 1994). All *Chryseobacterium* species are aerobic chemoorganotrophs; their metabolism is strictly respiratory, not fermentative, except for *C. scophthalmum*, which also exhibits a fermentative metabolism (Mudarris *et al*., 1994). However, some such as *C. gleum*, *C. indologenes* and other CDC Group IIb strains can grow anaerobically in the presence of nitrate by using nitrate as a terminal electron acceptor and reducing it to nitrogen (N_2) (Holmes *et al*., 1984b). *Chryseobacterium indologenes* strains can also grow under anaerobic conditions in the presence of fumarate (Yabuuchi *et al*., 1983). All strains grow at 30°C; most strains grow at 37°C. Growth on solid media is typically pigmented (yellow to orange), but nonpigmented strains occur. Colonies are translucent (occasionally opaque), circular, convex or low

Table 2.1 Currently known *Chryseobacterium* species, their sources of isolation and reference(s).

Species (former name)	Isolation	Reference(s)
Chryseobacterium balustinum	Blood of fresh water fish, France	Holmes et al., 1984a
C. defluvii	Activated sewage sludge, Germany	Kämpfer et al., 2003
C. gleum	High vaginal swab, London, UK	Holmes et al., 1984b
C. indologenes	Trachea at autopsy, USA	Yabuuchi et al., 1983
C. indoltheticum	Marine mud	Campbell and Williams, 1951
C. joostei	Raw tanker milk, RSA	Hugo et al., 2003
Elizabethkingia meningoseptica (*C. meningosepticum*)	Spinal fluid, USA	King, 1959; Holmes et al., 1984a
Elizabethkingia miricola (*C. miricola*)	Space station, Russia	Li et al., 2003
C. scophthalmum	Gills of diseased turbot, Scotland, UK	Mudarris et al., 1994
C. daecheongense	Sediment, freshwater lake	Kim et al., 2005a
C. formosense	Rhizosphere of garden lettuce, Taiwan	Young et al., 2005
C. shigense	Lactic acid beverage, Japan	Shimomura et al., 2005
C. vrystaatense	Raw chicken, RSA	De Beer et al., 2005
C. soldanellicola	Roots of sand-dune plants, Korea	Park et al., 2006
C. taeanense	Roots of sand-dune plants, Korea	Park et al., 2006

convex, smooth, and shiny, with entire edges. The strains are positive for catalase, oxidase and phosphatase activities. Several carbohydrates, including glycerol and trehalose, are oxidized. Strong proteolytic activity occurs. Esculin is hydrolysed, but agar is not digested. These organisms are resistant to a wide range of antimicrobial agents (Vandamme *et al*., 1994). Branched-chain fatty acids (i.e., 15:0 iso, iso 17:1?9c, 17:0 iso 3OH, and summed feature 4 [15:0 iso 2OH or 16:1?7t or both]) are predominant. Sphingophospholipids are absent. Menaquinone 6 is the only respiratory quinone. Homospermidine and 2-hydroxyputrescine are the major polyamines in *C. indologenes*, whereas putrescine and agmatine are minor components (Vandamme *et al*., 994).

Most *Chryseobacterium* species exhibit a rather high tolerance to sodium chloride (NaCl), except *C. balustinum*. All *Chryseobacterium* species are able to grow on marine agar (e.g., Difco Marine Agar 2216 [1.95% NaCl in addition to several other salts]), although only members of two species (*C. balustinum* and *C. indoltheticum*) were actually isolated from marine environments (Bernardet *et al*., 2002). The DNA base composition ranges from 33 to 38 mol% guanine plus cytosine. *Chryseobacterium* are widely distributed in soil, water, and clinical sources (Vandamme *et al*., 1994).

2.8.4 Contamination of foods with Chryseobacterium

Jooste *et al*. (1985) were the first to isolate *Flavobacterium* CDC Group IIb strains from milk and butter. In a subsequent study (Jooste *et al*., 1986), it was suggested that these *Flavobacterium* species caused putrefaction in salted butter by growing in cream prior to churning. In another study in which *Flavobacterium* CDC Group IIb and *C. balustinum* strains were isolated, Jooste and Britz (1986) found that the practical importance of dairy flavobacteria lies as much in their psychrotrophic growth and consequent proteinase production in refrigerated milk as in their contamination of milk via poorly sanitized pipelines and equipment. A study by Welthagen and Jooste (1992) indicated that CDC Group IIb isolates comprised the largest part of pigmented bacteria from raw milk. In subsequent investigations (Hugo and

Jooste, 1997; Hugo *et al*., 1999), a large group of the CDC Group IIb milk isolates evaluated in the above mentioned studies were identified as *C. indologenes* and one isolate as *C. gleum*. Among the remaining milk isolates, two new genomic groups [including the new species *C. joostei*; (Hugo *et al*., 2003)] were identified. In a study by Venter *et al*. (1999), a metalloprotease from a strain of *C. indologenes* was purified and characterized. This protease was very heat-stable and its affinity for casein may play a role in the spoilage of milk and milk products. Several *Chryseobacterium* species are associated with spoilage of dairy products during cold storage and include *C. balustinum, C. gleum* and *C. joostei* (Bernardet *et al*., 2002).

Chryseobacterium balustinum had initially been isolated from the scales of freshly caught halibut (*Hippoglossus hippoglossus*) in the Pacific Ocean (Harrison, 1929; Bernardet *et al*., 2006). Since this organism produced a yellowish slime on the skin, it was considered a fish spoilage agent rather than a pathogen (Austin and Austin, 1999). Although, recently isolated again from the skin and muscle of wild and farmed freshwater fish, *C. balustinum* was not regarded as an important contributor of the spoilage of the fish because of its low incidence (<1% of all isolates; González *et al*., 2000). The five *C. balustinum* strains, isolated in the latter study and identified following a rather extensive phenotypic characterization, were not found in freshly caught fish, but in fish stored more than three days in melting ice.

Gennari and Cozzolino (1989) isolated 39 strains of flavobacteria from the skin and gills of fresh and ice-stored Mediterranean sardines (*Sardina pilchardus*). Analysis of their phenotypic traits, however, could not place the isolates in any known species of the flavobacteria, but the authors found that four strains had characteristics resembling those of Holmes' group A. Most of the species in this group are now known as *Chryseobacterium.*

Flavobacteria have been frequently isolated from meat and poultry products (García-López *et al*., 1998), although they have seldom been accurately identified. When Hayes (1977) divided a large collection of flavobacteria and related Gram negative yellow pigmented rods into nine phena, the first five

phena were found to belong to the genus *Flavobacterium*. In a study by Owen and Holmes (1981), the conclusion was drawn that Hayes' phenon 1 corresponded closely to CDC Group IIb; and some members of this taxon were later attributed to *C. indologenes* and *C. gleum*. The 53 strains in this phenon were isolated from the following sources: raw beef carcasses (6 isolates), raw lamb carcasses (7 isolates), raw pig carcasses (8 isolates), raw eviscerated chicken carcasses (13 isolates), raw milk (13 isolates), water from rivers or streams (2 isolates), and soil (3 isolates). Consequently *Chryseobacterium* strains clearly are found in a variety of meat products, but no mention was made about their spoilage role in these products. *Chryseobacterium gleum* and *C. indologenes* are often present on raw meat (Bernardet *et al.*, 2006). *Chryseobacterium vrystaatense* was present on raw chicken, obtained from a chicken-processing plant (De Beer, 2005).

2.9. Changes in foods caused by *Chryseobacterium*

The non-diffusible, non-fluorescent bright yellow to orange pigments produced by most *Chryseobacterium* strains belong to the flexirubin type (Yabuuchi *et al.*, 1983; Holmes *et al.*, 1984b; Mudarris *et al.*, 1994; Yamaguchi and Yokoe, 2000; Hugo *et al.*, 2003; Bernardet *et al.,* 2006). The production of pigment may depend on the culture medium (Hugo and Jooste, 2003); it may also be more pronounced at low temperatures, in the presence of daylight, and in the presence of compounds such as casein, milk and starch (Holmes *et al.*, 1984a).

The production of extracellular slimy substances after prolonged incubation has been reported in *C. defluvii* (Kämpfer *et al.*, 2003) and contributes to the increased hydration capacity that accompanies low-temperature meat spoilage (Jay, 1992). A strong odour, reminiscent of that emitted by cultures of some *Empedobacter*, *Myroides* and *Sphingobacterium* strains, is produced by most *Chryseobacterium* strains in liquid and solid culture; it has been described as "fruity" (Yabuuchi *et al.*, 1983; Yabuuchi *et al.*, 1990). Fruity odours arise from the degradation of the amino acids glycine, leucine and serine to form lower fatty acid esters (Engelbrecht *et al.,* 1996). In a study by

Engelbrecht *et al*. (1996) to determine spoilage characteristics of bacteria isolated from Cape hake, the odours produced were arranged into four off-odour categories: stale, pungent, fruity and sulphidy. Thirty percent of the *Flavobacterium* isolates produced no odours. Twenty six percent of the *Flavobacterium* isolates were able to produce H_2S. Of the *Flavobacterium balustinum* (now *C. balustinum*) strains tested for odour production, 75% produced odours. Of the unidentified *Flavobacterium*-like isolates tested, 58% produced odours. The genus *Flavobacterium* (29%) had spoilage potential on the basis of off-odour production (fruity, pungent, sulphidy) under the specified test conditions.

Various proteolytic activities (i.e., degradation of skim milk, casein, and gelatin in agar media) and H_2S production were also found in *Chryseobacterium balustinum*, *C. gleum* and *C. indologenes* strains isolated from Cape marine fish in South Africa (Engelbrecht *et al.*, 1996). When off-odour production of the *Chryseobacterium* strains was evaluated in fish muscle extract, four of the eight *C. balustinum* strains were found to produce a pungent odour, two a stale odour, and two no odour. Four of the five *C. gleum* strains produced a stale odour, and one *C. indologenes* strain produced a fruity odour (Engelbrecht *et al*., 1996).

Flavobacteria are generally considered to be of low significance in fish and other food spoilage (Gennari and Cozzolino, 1989). Off-odours such as sweet and fruity, putrid, sulphur and cheesy, characterize aerobically stored meat (Dainty and Mackey, 1992). Off-odours of poultry have been listed as sulphide-like, fruity, fishy, and like evaporated milk. Milk spoilage is associated with a bitter flavour and rancidity as well as a fruity odour. The fruity odour is due to a mixture of ethyl butyrate and ethyl hexanoate (Banwart, 1989).

Homospermidine is the major polyamine in *C. balustinum*, *C. gleum*, *C. meningosepticum* and *C. indologenes*, trace amounts of putrescine and agmatine are also detected in the three former species (Hamana and Mastuzaki, 1990), whereas diaminopropane and 2-hydroxyputrescine are

minor components in *C. indologenes* (Hamana and Matsuzaki, 1991). Recently described *Chryseobacterium* species have not been investigated for their polyamine composition, except *C. defluvii*, which contains sym-homospermidine as the major component and spermine and spermidine as minor components (Kämpfer *et al*., 2003). Contrary to the statement by Kämpfer *et al*. (2003), previous publications have shown that no polyamine pattern can be considered characteristic of members of the genus *Chryseobacterium*.

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CHAPTER 3

POTENTIAL FOOD SPOILAGE CHAR ACTERISTICS OF *Chryseobacterium* SPECIES

Abstract

The utilisation of BIOLOG carbon sources by 9 *Chryseobacterium* spp. was investigated for their potential to produce food spoilage defects. Forty three carbon sources gave differentiation between the species of which the miscellaneous carbon substrates (49.1%) were the most utilised. The metabolic activity of *Chryseobacterium* spp. was investigated, using phenotypic tests. All the species hydrolysed aesculin and 89% of the species produced indole from tryptophan. A modified Niven medium was used to investigate the ability of *Chryseobacterium* spp. to produce biogenic amines at different temperatures and sodium chloride concentrations. Temperatures at and below 15°C seemed to have a definite inhibitory effect on the production of biogenic amines in some strains. The ability of other strains to produce biogenic amines at low temperatures (e.g. 7°C) is however of great significance to the food industries. An increase in salt concentration resulted in fewer strains being able to produce biogenic amines.

3.1. Introduction

Spoilage is characterised by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. Knowledge of the micro-organisms involved in spoilage and the metabolites associated with spoilage is needed to develop microbiological and chemical methods for evaluation of quality and shelf life. Such knowledge is important, e.g., if a chemical spoilage index is to be developed or may be used to eliminate or prevent a particular spoilage promoting compound in foodstuffs (Gram *et al.,* 2002).

Specific micro-organisms are associated with food spoilage and resultant metabolic activities resulting in slime production or the production of ammonia

and sulphur compounds, are responsible for undesirable flavours and odours (Gram *et al.*, 2002). As part of the Gram negative rod, psychrotrophic, proteolytic group, the genus *Chryseobacterium* is considered to be an active food spoilage organism (Hugo and Jooste, 2003; Bernardet *et al.,* 2006). Hydrolysis of proteins, fats and polysaccharides can cause changes in the texture of foods. Incomplete metabolism of the amino acids and fatty acids and fermentations of simple sugars can contribute to changes in flavour. Foods of mixed composition frequently undergo several simultaneous changes in odour, flavour and texture in the process of spoilage (Ayres *et al*., 1980).

It is known that biogenic amines can cause migraines and headaches and that these amines may sometimes be present in dairy products, especially products like cheese and yoghurt (Edwards and Sandine, 1981). The determination of biogenic amines in foods is consequently important not only from the point of view of their toxicity, but also because they can be used as food spoilage indicators (Santos, 1996).

The aim of this study was to use BIOLOG MicroPlates to identify the specific carbon sources utilised by the *Chryseobacterium* species, since the microbial degradation of similar carbon sources in foods can lead to the production of microbial metabolites, which could produce potential spoilage defects. An array of phenotypic tests was performed to investigate the metabolic activity of the *Chryseobacterium* species, as well as metabolites not included in the BIOLOG system. The ability to produce biogenic amines at different temperatures and sodium chloride concentrations by the *Chryseobacterium* species, was investigated by using a modified Niven medium (Niven *et al*., 1981).

3.2. Materials and methods

3.2.1. Strains and growth conditions used

The reference strains used in this study are listed in Table 3.1. The type strains of seven *Chryseobacterium* spp. and two *Elizabethkingia* spp. were used for purposes of comparison. All the freeze-dried reference strains were reactivated in Nutrient Broth (Oxoid CM67) and checked for purity on Nutrient Agar (Oxoid CM3) at 25ºC for 24-48 h. The strains were maintained on Nutrient Agar slants and stored at 4ºC.

3.2.2. Utilisation of carbon sources

The reference strains were streaked on to Nutrient Agar (Oxoid CM3) plates and incubated at 25ºC for 24 h. Gram staining, oxidase and catalase tests were performed on the reference strains as described by Hugo *et al*. (2003), to verify that the strains are Gram negative, oxidase positive and catalase negative. The strains were then inoculated on to Triple Sugar Iron Agar (TSI) slants as described by Fankhauser (2001). The strains were then subjected to testing on BIOLOG GN2 MicroPlates (Biolog, Inc., Hayward, California) according to the manufacturer's protocol to differentiate between the different species and to identify the carbon sources (Table 3.3) utilised by the different species. The strains were classified in a Gram negative non-enteric (GN-NENT) group, inoculated on BIOLOG Universal Growth Agar (BUG) and incubated at 25ºC for 24 h. For identification purposes, the GN2 MicroPlates were visually read (well by well) after 16 h and 24 h of incubation at 25ºC.

3.2.3. Phenotypic characterisation of the isolates

In order to supplement metabolites in the BIOLOG system and due to a lack of relevant phenotypical test results in literature, an extra battery of metabolic activity tests was performed to give further differentiation of the Gram negative, yellow-pigmented species at 25°C using the methods described by MacFaddin (1980), Gerhardt *et al*. (1981), Barrow and Feltham (1993) and Hugo *et al.* (2003).

Table 3.1 Reference strains used in this study.

Strain	Code
Chryseobacterium gleum	NCTC 11432^T
Chryseobacterium balustinum	LMG 8329^T
Chryseobacterium defluvii	LMG 22469^T
Chryseobacterium indologenes	LMG 8337^T
Chryseobacterium indoltheticum	ATCC 27950^T
Chryseobacterium joostei	LMG 18212^T
Chryseobacterium scophthalmum	LMG 13028^T
Elizabethkingia meningoseptica	NCTC 10016^T
Elizabethkingia miricola	LMG 22470^T

Abbreviations: LMG, Laboratory of Microbiology, University of Ghent, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; ATCC, American Type Culture Collection, Rockville, Maryland.

3.2.4. Production of biogenic amines

The ability of the bacteria to produce amines from amino acids was determined by using the method described by Bester *et al*. (1993). The cultures were streaked out on a modified Niven medium (Niven *et al*., 1981) to which either histidine (2.7% (m/v)), tyrosine (0.1%), tryptophan (1.1%), arginine (1.0%) or lysine (1.0%) was added as substrate for decarboxylation. Unless otherwise indicated, incubation was at 25°C for 48 h.

The effect of incubation temperature on amine production was examined by incubating the inoculated plates at 7, 15, 20, 25 or 30°C. The effect of sodium chloride (NaCl) on amine production was examined by using the modified Niven medium (Niven *et al*., 1981) containing each of the five amino acids separately in combination with each of 0% (control), 1%, 2%, 3%, 4% or 5% (m/v) NaCl used to evaluate the effect of salt concentration on amine production. The plates were incubated for 48 h at 25°C.

3.3. Results and discussion

3.3.1. Carbon source utilisation

All the reference strains were Gram negative rods, oxidase positive, catalase negative and produced an alkaline/acid reaction on the TSI slants, which indicated that only glucose was fermented and peptone utilised (Fankhauser, 2001). Although the BIOLOG supplier recommended an incubation temperature of 30ºC for the GN-NENT group, optimum growth for the range of reference strains tested was obtained at 25ºC as the optimum growth temperature for *Chryseobacterium* species is 25ºC.

After 16 h of incubation, *C. gleum, C. indoltheticum* and *E. meningoseptica* produced a 100% probability according to BIOLOG identification (Table 3.2a). After 24 h of incubation, *C. indologenes, C. scophthalmum* and *C. balustinum* produced a 100% probability according to BIOLOG identification (Table 3.2b). At 16 h to 24 h of incubation, the similarity index (SIM) must be at least 0.50

Table 3.2a Species identification on the BIOLOG system after 16 h.

Strain	PROB	SIM	BIOLOG ID	Correct ID
NCTC 11432^T	100	0.78	*C. gleum*	*C. gleum*
LMG 8329^T	64	0.51	*C. balustinum*	*C. balustinum*
LMG 22469^T	-	0.46	*Chryseobacterium*	*C defluvii*
LMG 8337^T	88	0.75	*Chryseobacterium*	*C. indologenes*
ATCC 27950^T	100	0.86	*C. indoltheticum*	*C. indoltheticum*
LMG 18212^T	-	0.46	*Chryseobacterium*	*C. joostei*
LMG 13028^T	99	0.89	*C. scophthalmum*	*C. scophthalmum*
NCTC 10016^T	100	0.74	*C. meningosepticum*	*E. meningoseptica*
LMG 22470^T	-	0.43	*Chryseobacterium*	*E. miricola*

Abbreviations: PROB, Probability; SIM, similarity; ID, identification.

Table 3.2b Species identification on the BIOLOG system after 24 h.

Strain	PROB	SIM	BIOLOG ID	Correct ID
NCTC 11432^T	100	0.83	*C. gleum*	*C. gleum*
LMG 8329^T	100	1.00	*C. balustinum*	*C. balustinum*
LMG 22469^T	-	0.43	*Chryseobacterium*	*C defluvii*
LMG 8337^T	100	1.00	*C. indologenes*	*C. indologenes*
ATCC 27950^T	100	0.92	*C. indoltheticum*	*C. indoltheticum*
LMG 18212^T	-	0.46	*Chryseobacterium*	*C. joostei*
LMG 13028^T	100	0.92	*C. scophthalmum*	*C. scophthalmum*
NCTC 10016^T	100	0.80	*C. meningosepticum*	*E. meningoseptica*
LMG 22470^T	-	0.43	*Chryseobacterium*	*E. miricola*

Abbreviations: PROB, Probability; SIM, similarity; ID, identification.

to be considered acceptable (Biolog Inc.). The similarity index was determined by Biolog's MicroLog computer software. Only 33% of the reference strains produced a similarity index of less than 0.50 and produced no probability according to BIOLOG identification after 16 h to 24 h of incubation (Table 3.2a, 3.2b). This is because *C. defluvii, C. joostei* and *E. miricola* are not present in the BIOLOG system's current database and therefore only the genus name was identified.

Adequate and stable patterns on the MicroPlates were formed after 24 h of incubation. Some of the reactions on the MicroPlates were intermediate and classified as "borderline". Although it is normal for certain genera to produce a light or faint purple colour reaction in some of the microplate wells, most reference strains gave clear "positive" reactions (Biolog, Inc.), which the researcher focused on after 24 h of incubation to identify the different carbon sources utilised by the reference strains.

The percentage of species that utilised the carbon sources are shown in Table 3.3. All the *Chryseobacterium* species and the two *Elizabethkingia* species utilised gentiobiose, a-D-glucose, D-mannose and succinic acid mono-methyl ester. Seventy-eight percent of the species utilised D-trehalose and L-asparagine. Sixty-seven percent of the species utilised L-serine, L-threonine, uridine and glycerol. Fifty-six percent of the species utilised dextrin, maltose, acetic acid, L-aspartic acid and glycyl-L-glutamic acid. Forty-four percent of the species utilised a-cyclodextrin, L-alanyl glycine, inosine and thymidine. Thirty-three percent of the species utilised Tween 40, Tween 80, D-fructose, a-ketobutyric acid, a-ketovaleric acid, L-glutamic acid, L-leucine and L-ornithine. Twenty-two percent of the species utilised glycogen, D-celliobiose, D-psicose, formic acid, propionic acid, glycyl-L-aspartic acid, L-alanine, L-phenylalanine and L-proline. Only 11% of the species were able to utilise N-acetyl-D-glucosamine, D-arabinose, D-mannitol, ß-methyl-D-glucoside, citric acid, 2,3-butanediol and D,L-a-glycerol phosphate.

Instead of discussing the utilisation of each carbon source individually, the carbon sources that produced clear positive reactions after 24 h of incubation,

Table 3.3 The carbon substrates, utilised by the reference strains, divided into chemical guilds.

Chemical guild	Substrate utilised	Species									% of + reactions
		1	2	3	4	5	6	7	8	9	
Amino acids	Glycyl-L-aspartic acid	+	-	-	+	-	-	-	-	-	22
	Glycyl-L-glutamic acid	+	+	-	+	-	-	-	+	+	56
	L-Alanine	+	-	-	+	-	-	-	-	-	22
	L-Alanyl-glycine	+	-	-	+	-	-	+	+	-	44
	L-Asparagine	+	+	-	+	-	+	-	+	+	78
	L-Aspartic acid	+	+	-	-	-	+	-	+	+	56
	L-Glutamic acid	+	-	-	-	+	-	-	+	-	33
	L-Leucine	+	-	-	+	-	-	+	-	-	33
	L-Ornithine	+	+	-	+	-	-	-	-	-	33
	L-Phenylalanine	+	-	-	+	-	-	-	-	-	22
	L-Proline	+	-	-	+	-	-	-	-	-	22
	L-Serine	+	+	-	+	-	-	+	+	+	67
	L-Threonine	+	+	-	+	+	-	+	+	-	67

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative; %, percentage.

Table 3.3 continued

Chemical guild	Substrate utilised	Species									% of + reactions
		1	2	3	4	5	6	7	8	9	
Carbohydrates	a-D-Glucose	+	+	+	+	+	+	+	+	+	100
	ß-Methyl-D-glucoside	+	-	-	-	-	-	-	-	-	11
	D-Arabinose	-	-	-	+	-	-	-	-	-	11
	D-Cellobiose	-	-	-	-	+	-	+	-	-	22
	D-Fructose	+	-	-	+	-	-	-	+	-	33
	D-Mannitol	-	-	-	-	-	-	-	+	-	11
	D-Mannose	+	+	+	+	+	+	+	+	+	100
	D-Psicose	+	-	-	+	-	-	-	-	-	22
	D-Trehalose	+	+	+	+	-	-	+	+	+	78
	Gentiobiose	+	+	+	+	+	+	+	+	+	100
	Maltose	+	-	+	+	+	-	-	+	-	56
	N-Acetyl-D-glucosamine	-	-	-	-	-	-	-	+	-	11

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative; %, percentage.

Table 3.3 continued

Chemical guild	Substrate utilised	Species									% of + reactions
		1	2	3	4	5	6	7	8	9	
Carboxylic acids	a-Keto butyric acid	-	-	-	+	+	-	+	-	-	33
	a-Keto valeric acid	+	-	-	+	+	-	-	-	-	33
	Acetic acid	+	+	-	+	-	-	+	+	-	56
	Citric acid	+	-	-	-	-	-	-	-	-	11
	Formic acid	+	-	-	+	-	-	-	-	-	22
	Propionic acid	+	-	-	+	-	-	-	-	-	22
Miscellaneous	2,3-Butanediol	+	-	-	-	-	-	-	-	-	11
	Inosine	+	-	-	+	-	+	-	+	-	44
	Succinic acid mono-methyl	+	+	+	+	+	+	+	+	+	100
	Thymidine	+	-	-	+	+	-	-	+	-	44
	Uridine	+	-	-	+	+	+	-	+	+	67
	Glycerol	+	+	-	+	-	+	-	+	+	67
	D,L-a-Glycerol phosphate	-	-	-	+	-	-	-	-	-	11

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative; %, percentage.

Table 3.3 continued

Chemical guild	Substrate utilised	Species									% of + reactions
		1	2	3	4	5	6	7	8	9	
Polymers	a-Cyclodextrin	+	-	+	+	-	-	-	+	-	44
	Dextrin	+	+	+	+	-	-	-	+	-	56
	Glycogen	+	-	-	+	-	-	-	-	-	22
	Tween 40	+	+	-	-	-	-	-	+	-	33
	Tween 80	+	+	-	-	-	-	-	+	-	33

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative; %, percentage.

a could be assigned to chemical guilds (Table 3.3) of carbohydrates, carboxylic acids, polymers, amino acids and miscellaneous carbon sources (Preston-Mafham *et al*., 2002).

Forty-three of the carbon sources could differentiate between the species and these carbon sources included 13 amino acids, 12 carbohydrates, six carboxylic acids, seven miscellaneous carbon substrates and five polymers (Table 3.3). *Chryseobacterium gleum* utilised 37 carbon sources, followed by *C. indologenes* with 33 and *E. meningoseptica* with 25. *Chryseobacterium balustinum* utilised 16 carbons sources, followed by *C. indoltheticum* and *C. scophthalmum* with 12 and *E. miricola* with 11. *Chryseobacterium joostei* and *C. defluvii* utilised only nine and eight carbon sources, respectively.

When comparing the chemical guilds, all the species utilised amino acids, except *C. defluvii*. *Chryseobacterium defluvii, C. joostei* and *E. miricola* were not able to utilise carboxylic acids, and *C. joostei, C. indoltheticum, C. scophthalmum,* and *E. miricola* could not utilise the polymers either. The average of each chemical guild, utilised by the species was calculated by taking the sum of the % positive reactions (Table 3.3), divided by the number of carbon sources, present in the chemical guild. The *Chryseobacterium* and *Elizabethkingia* species utilised the miscellaneous carbon substrates (49.1%) the most, followed by the carbohydrates (46.3%), amino acids (42.7%), the polymers (37.6%) and the carboxylic acids (29.5%).

Anaerobic decomposition of amino acids may result in the production of obnoxious odours and is then called putrefaction. It results in foul-smelling, sulphur-containing products, such as hydrogen, methyl, and ethyl sulphides and mercaptans, plus ammonia, biogenic amines (e.g. putrescine), indole, skatole, and fatty acids (Frazier, 1988). The oxidation/reduction of amino acids results in two organic acids, ammonia, and carbon dioxide, e.g. glutamic acid, yields acetic acid, butyric acid, carbon dioxide (CO_2), ammonia and hydrogen (Banwart, 1989). According to Ayres *et al.* (1980), the incomplete metabolism of amino acids can result in putrescence. The formation of ammonia from amino acids in meats and milk can result in alkalinisation while

the liberation of hydrogen sulphide (H_2S) from amino acids can result in sulphide spoilage. Examination of the tastes of the amino acids that occur in protein hydrolysates showed that bitterness is exclusively a characteristic of the hydrophobic L-amino acids (e.g. leucine) (Coultate, 1984).

The selection of carbon sources in GN plates is biased towards simple carbohydrates (Preston-Mafham *et al*., 2002). Complex di-, tri-, or polysaccharides usually are hydrolysed to simple sugars before utilisation. Monosaccharides (e.g. a-D-glucose, D-mannose) would aerobically be oxidised to CO_2 and water (H_2O) (Frazier, 1988). Some of the metabolic products resulting from carbohydrates (e.g. D-trehalose, gentiobiose) include organic acids, alcohols, CO_2, hydrogen (H_2) and H_2O (Banwart, 1989). The microbial production of polysaccharides (e.g. dextrin) from various disaccharides, present in food can form unpleasant slime in and on food, causing the food to be both unpalatable and unacceptable to the consumer. Ayres *et al.* (1980) stated that the microbial fermentation of sugars can lead to souring and butyric spoilage defects.

The carboxylic acids (e.g. acetic acid) are organic compounds containing oxygen and are weak acids (Ebbing and Gammon, 1999). Many of these organic acids are oxidised by micro-organisms to carbohydrates, causing the medium to become more alkaline. Aerobically the organic acids may be oxidised completely to CO_2 and H_2O. Acids may also be oxidised to other, simpler acids or to other products similar to those from sugars (Frazier, 1988). The production of acids leads to sourness, but according to Coultate (1984), a-acids can be responsible for bitter tastes in foods.

The polymers include polysaccharides and fatty acid esters of a polyoxyalkylene derivative of sorbitan (Holding and Collee, 1971). The polysaccharides can cause changes in food texture (Ayres *et al*., 1980). Tween mixtures provide suitable conditions for the activation of both lipase and other esterases (Harrigan and McCance, 1976), which can cause spoilage of the final products during storage (Chen *et al.,* 2003). The

hydrolysis of esters (e.g. succinic acid mono-methyl ester) leads to the production of a carboxylic acid and alcohol (Ebbing and Gammon, 1999).

3.3.2. Phenotypic characteristics

As shown in Table 3.4, all the *Chryseobacterium* species and the two *Elizabethkingia* species tested negative for the production of ammonia from peptone and arginine. *Chryseobacterium gleum, C. indologenes, C. joostei, C. scophthalmum* and *C. miricola* produced ammonia from urea. Ammonia is usually formed in appreciable amounts in the advanced stages of protein decomposition (Nychas *et al.,* 1998). The production of ammonia tends to cause an increase in pH (Banwart, 1989). In addition to the formation of malodorous compounds, the release of large amounts of ammonia also contributes to the development of spoilage odours (Nychas *et al.,* 1998).

As shown in Table 3.5, all the *Chryseobacterium* species and the two *Elizabethkingia* species hydrolysed aesculin. This glycoside is hydrolysed to aesculetin and glucose (Holding and Collee, 1971). *Chryseobacterium indologenes* and *C. joostei* hydrolysed starch, while *C. balustinum*, *C. defluvii* and *Elizabethkingia miricola* only hydrolysed starch weakly. Starch is attacked by the hydrolytic action of extracellular amylases. a-Amylase rapidly liquefies starch, simultaneously attacking many 1,4-glycosidic bonds, including those in the centre of the chain. This results in the production of maltose, glucose and oligomers with three to seven glucose residues. Because of its rapid breakdown of the macromolecular structure, the viscosity of the solution and its ability to react with iodine also declines rapidly, whilst fermentable sugars (glucose, maltose, maltobiose) appear gradually (Schlegel, 1993). *Chryseobacterium indologenes, C. indoltheticum* and *E. miricola* produced hydrogen sulphide (H_2S). This may result from the decomposition of organic sulphur compounds, e.g. cysteine and cystine, or from the reduction of inorganic sulphur compounds, e.g. sulphite (Harrigan and McCance, 1976). Hydrogen sulphide combines with muscle pigment in meat, to give a green discoloration (Nychas *et al.,* 1998). According to Ayres

Table 3.4 The production of ammonia by species of *Chryseobacterium* and *Elizabethkingia*.

Characteristic	Species 1	2	3	4	5	6	7	8	9
Production of									
Ammonia from peptone	-	-	-	-	-	-	-	-	-
Ammonia from arginine	-	-	-	-	-	-	-	-	-
Ammonia from urea	+	-	-	+	-	+	+	-	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative.

Table 3.5 Phenotypic characteristics of reference strains of *Chryseobacterium* and *Elizabethkingia*.

	Species								
Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Hydrolysis of									
Aesculin	+	+	+	+	+	+	+	+	+
Starch	-	w	w	+	-	+	-	-	w
Production of									
Hydrogen Sulphide	-	-	-	+	+	-	-	-	+
Phenylpuryvic acid	-	-	-	-	-	-	-	w	-
Indole from tryptophan	+	+	+	+	+	+	-	+	+
Reduction of nitrate	+	+	-	-	-	-	-	-	-
Reduction of nitrite	+	-	-	-	-	-	-	-	-
Acid production from ethanol	-	+	-	-	-	-	w	-	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive -, negative

et al. (1980), the liberation of H_2S from amino acids results in sulphide spoilage. The production of H_2S is recognised by its foul odour and is very toxic (Ebbing and Gammon, 1999).

Elizabethkingia meningoseptica was the only species to produce phenylpyruvic acid weakly (Table 3.5). The deamination of phenylalanine leads to the production of phenylpyruvic acid and ammonia (Frazier, 1988), which can contribute to putrescence and alkalinisation (Ayres *et al*., 1980). Only *C. scophthalmum* was not able to produce indole from tryptophan (Table 3.5). Organisms containing the enzyme, tryptophanase, break down tryptophan into indole, pyruvic acid, and ammonia. Indole imparts disagreeable odours associated with putrefaction (Mountney and Gould, 1988). According to Ayres *et al*. (1980), the formation of indole from tryptophan can lead to "unclean" flavours.

Chryseobacterium gleum and *C. balustinum* reduced nitrate and only *C. gleum* reduced nitrite (Table 3.5). According to Schlegel (1993), the reduction of nitrate and nitrite can lead to the production of gaseous nitrogen dioxide (NO_2) and molecular nitrogen (N_2). NO_2 can cause irritating odours and is very toxic, while N_2 is odourless and non-toxic (Ebbing and Gammon, 1999).

Chryseobacterium balustinum and *E. miricola* produced acid from ethanol while *C. scophthalmum* produced acid weakly from ethanol (Table 3.5). According to Ayres *et al*. (1980), the oxidation of ethanol to acetic acid can produce a souring acetic type of spoilage in the presence of low pH foods.

3.3.3. Biogenic amines

The effect of incubation temperature on the ability of *Chryseobacterium* species to produce spermine, cadaverine, histamine, tyramine and tryptamine on modified Niven medium, is shown in Tables 3.6a – 3.6d.

Spermine, cadaverine and tyramine were produced by all the strains at 25 °C and 30°C. Only *Elizabethkingia meningoseptica* produced histamine well

Table 3.6a The effect of incubation temperature on spermine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	Temperature				
	7°C	15°C	20°C	25°C	30°C
1	-	w	+	+	+
2	w	+	+	+	+
3	-	w	+	+	+
4	-	w	+	+	+
5	w	w	+	+	+
6	w	+	+	+	+
7	w	+	+	+	+
8	-	w	+	+	+
9	-	w	+	+	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.6b The effect of incubation temperature on cadaverine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	Temperature				
	7°C	15°C	20°C	25°C	30°C
1	-	w	+	+	+
2	w	+	+	+	+
3	-	-	w	+	+
4	-	w	+	+	+
5	w	+	+	+	+
6	w	+	+	+	+
7	-	w	+	+	+
8	-	w	+	+	+
9	-	w	+	+	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.6c The effect of incubation temperature on histamine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	Temperature				
	7°C	15°C	20°C	25°C	30°C
1	-	-	-	w	w
2	-	-	-	w	-
3	-	-	-	w	-
4	-	-	-	w	w
5	-	-	w	w	w
6	-	-	w	w	w
7	w	-	w	w	-
8	-	-	w	+	w
9	-	-	w	w	w

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.6d The effect of incubation temperature on tyramine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	Temperature				
	7°C	15°C	20°C	25°C	30°C
1	w	+	+	+	+
2	+	+	+	+	+
3	-	w	+	+	+
4	w	w	+	+	+
5	w	+	+	+	+
6	+	+	+	+	+
7	w	+	+	+	+
8	-	-	w	+	+
9	-	w	+	+	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.6e The effect of incubation temperature on tryptamine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	Temperature				
	7°C	15°C	20°C	25°C	30°C
1	-	w	+	+	+
2	-	-	w	-	-
3	-	-	w	w	+
4	-	-	w	+	+
5	-	w	w	+	-
6	-	w	+	w	+
7	-	w	w	w	-
8	-	w	+	+	+
9	-	w	+	+	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

at 25°C. *Chryseobacterium gleum*, *C. indologenes, C. indoltheticum, E. meningoseptica* and *E. miricola* produced tryptamine at 25°C and *C. gleum, C. defluvii, C. indologenes, C. joostei, E. meningoseptica* and *E. miricola* produced histamine at 30°C. Temperatures at and above 25°C seemed to have a definite inhibitory effect on the production of some biogenic amines such as histamine and tryptamine.

Spermine, cadaverine and tyramine were produced by all the strains at 20 °C, except for *C. defluvii* which produced cadaverine weakly at 20°C and *E. meningoseptica* produced tyramine weakly at 20°C. Spermine was produced by *C. balustinum*, *C. joostei* and *C. scophthalmum* at 15°C. Cadaverine was produced by *C. balustinum, C. indoltheticum* and *C. joostei* at 15°C. None of the strains were able to produce histamine at 15°C. Tyramine was produced by *C. gleum, C. balustinum, C. indoltheticum, C. scophthalmum* and *C. joostei* at 15°C, while 67% of the species produced tryptamine weakly.

Only tyramine was produced at 7°C by *C. balustinum* and *C. joostei.* In a study by Bester *et al.* (1993), flavobacterial strains of cluster 1A (CDC Group IIb), were able to produce tyramine at 7°C. No species were however able to produce spermine, cadaverine, histamine and tryptamine at 7°C. Temperatures at and below 15°C seemed to have a definite inhibitory effect on the production of spermine, cadaverine, histamine, tyramine and tryptamine. However, the ability of some *Chryseobacterium* spp. to produce biogenic amines (e.g. tyramine), at low temperatures, i.e. 15°C and 7°C, is of great significance to the dairy and other food industries. Refrigerated products could cause amine poisoning if they were contaminated with amine-producing bacteria, e.g. *Chryseobacterium* spp. (Bester *et al*., 1993).

It must be taken note of that most amines, including histamine, are heat-stable and only partially destroyed in 3 h at 102°C or 90 min at 116°C (Ienistea, 1973). These findings stress the importance of the hygienic production and handling of food products and maintenance of the cold chain throughout production and distribution (Bester *et al*., 1993).

The production of spermine, cadaverine, histamine, tyramine and tryptamine on modified Niven medium to which various amounts of sodium chloride were added, is shown in Tables 3.7a – 3.7b.

Spermine, cadaverine, histamine and tryptamine were not produced by the strains tested in the presence of 4% and 5% NaCl. It would seem that an increase in the salt concentration resulted in fewer strains being able to produce the biogenic amines. Exceptions were *C. gleum, C. indoltheticum* and *E. miricola* that produced tyramine weakly in the presence of 4% NaCl. Only *C. gleum, C. indologenes, C. joostei* and *E. meningoseptica* produced tyramine in the presence of 3% NaCl.

Spermine was produced by *C. gleum, C. indoltheticum, C. joostei, E. meningoseptica* and *E. miricola* in the presence of 2% NaCl. *Elizabethkingia meningoseptica* produced cadaverine in the presence of 2% NaCl, while only *C. joostei* was able to produce histamine weakly in the presence of 2% NaCl. Tyramine was produced by *C. joostei* and *E. meningoseptica* in the presence of 2% NaCl. Spermine, cadaverine and tyramine were produced by all the strains in the presence of 1% NaCl, with the exception of *C. defluvii* that produced cadaverine and tyramine weakly. It would seem that the decarboxylation of histidine and tryptophan were very sensitive to NaCl concentrations, because only *C. joostei* was able to produce histamine in the presence of a 1% NaCl, while *C. gleum, C. defluvii* and *C. indologenes* were able to produce tryptamine at this concentration.

These results show that salt concentrations in excess of 4% would be needed to prevent amine production of *Chryseobacterium* species in food products. This is in contrast to a study by Bester *et al*. (1993), who showed that 7% of the flavobacterial strains of cluster 1A (CDC Group IIb) tested by them were able to produce tyramine even in the presence of 5% NaCl.

Table 3.7a The effect of sodium chloride concentration on spermine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	% Sodium chloride (NaCl)				
	1%	2%	3%	4%	5%
1	+	+	-	-	-
2	+	w	-	-	-
3	+	w	-	-	-
4	+	w	w	-	-
5	+	+	w	-	-
6	+	+	w	-	-
7	+	w	-	-	-
8	+	+	-	-	-
9	+	+	w	-	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.7b The effect of sodium chloride concentration on cadaverine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	% Sodium chloride (NaCl)				
	1%	2%	3%	4%	5%
1	+	w	-	-	-
2	+	-	-	-	-
3	w	-	-	-	-
4	+	w	-	-	-
5	+	w	-	-	-
6	+	w	w	-	-
7	+	w	-	-	-
8	+	+	-	-	-
9	+	w	-	-	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.7c The effect of sodium chloride concentration on histamine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	% Sodium chloride (NaCl)				
	1%	2%	3%	4%	5%
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	+	w	-	-	-
7	w	-	-	-	-
8	w	-	-	-	-
9	w	-	-	-	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.7d The effect of sodium chloride concentration on tyramine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	% Sodium chloride (NaCl)				
	1%	2%	3%	4%	5%
1	+	w	+	w	-
2	+	w	-	-	-
3	w	-	-	-	-
4	+	w	+	-	-
5	+	w	w	w	-
6	+	+	+	-	-
7	+	w	-	-	-
8	+	+	+	-	-
9	+	w	w	w	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Table 3.7e The effect of sodium chloride concentration on tryptamine production by *Chryseobacterium* and *Elizabethkingia* species.

Species	% Sodium chloride (NaCl)				
	1%	2%	3%	4%	5%
1	w	w	-	-	-
2	w	-	-	-	-
3	w	-	-	-	-
4	w	w	-	-	-
5	w	-	-	-	-
6	+	w	w	-	-
7	w	-	-	-	-
8	+	w	-	-	-
9	+	w	w	-	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

3.4. Conclusions

The ability to utilise carbon sources by *Chryseobacterium* spp. tested in this study does not directly reflect the probability of food spoilage defects, but the BIOLOG system can be used as an effective screening method for identifying the carbon sources that could be investigated further for their potential to produce food spoilage defects. Phenotypic tests on *Chryseobacterium* spp. can be used as an alternative method to investigate the hydrolysis of food components and the production of metabolites, which could result in potential food spoilage defects such as putrefaction and alkalinisation, which are usually associated with disagreeable odours. Some *Chryseobacterium* spp. have the ability to decarboxylate some of the precursors of biogenic amines at different temperatures and in the presence of different sodium chloride concentrations. This results in the formation of biogenic amines which could cause amine poisoning and these organisms should consequently be regarded as significant spoilage organisms in food products.

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CHAPTER 4

GROWTH AND HYDROLYTIC ACTIVITIES OF *Chryseobacterium* SPECIES IN MILK

Abstract

Growth capacity was tested at 4°C, 25 °C, 32°C and 37°C. *Chryseobacterium* spp. were able to grow at a pH of 1-4 and a sodium chloride (NaCl) concentration of 1-3%. None of the species showed growth on ß-polyhydroxybutyrate, while growth was observed on Cetrimide Agar, MacConkey Agar and Simmons Citrate Agar. The optimum growth temperature for the four *Chryseobacterium* spp. tested was 25°C. Casein, gelatin, lecithin, olive oil and tributyrin were hydrolysed by some of the species, while 56% of the species produced phospholipase C. A preliminary study was made to determine volatile compounds in milk samples inoculated with *Chryseobacterium* spp. using headspace gas chromatography (GC). A total of seven volatile compounds were tentatively identified in the fat free milk and five compounds in the full cream milk. Sensory evaluation was done on milk samples inoculated with three cultures, resulting in the identification of odour descriptors, such as “fruity” and “smelly feet”.

4.1. Introduction

Several *Chryseobacterium* species which include *C. balustinum, C. gleum* and *C. joostei* are associated with the spoilage of dairy products during cold storage (Bernardet *et al.,* 2002). In milk they produce heat resistant proteolytic and lipolytic enzymes responsible for off-flavours (Bernardet *et al*., 2002). They also produce off-odours (García-López *et al.,* 1999) in pasteurised milk and cream, surface taint in butter, thinning in creamed rice and bitterness due to the production of phospholipase C (Jooste and Hugo, 1999; Bernardet *et al*., 2002). The production of these spoilage enzymes are dependent *inter alia* on temperature and growth phase (Jooste and Fischer, 1993).

Consumers depend upon their senses of sight, smell, taste and touch to evaluate food quality (Banwart, 1989). A careful combination of microbiological-, sensory- and chemical analysis are required to determine the spoilage potential of a microorganism. Despite the importance of microorganisms in food spoilage, the definition and assessment of spoilage relies on sensory evaluation since neither the "total count" of e.g. 10^7 cfu/ cm^2 nor the level of a specific spoilage organism *per se* can directly predict the sensory quality of a product (Gram *et al.,* 2002).

The growth and activity of spoilage microorganisms is mostly described and studied as a function of substrate base and of chemical and physical parameters such as temperature, pH, water activity and atmosphere (Gram *et al.,* 2002). The production of enzymes will always give valuable information on potential spoilage abilities (*e.g.* proteolysis and lipolysis) while production of metabolites containing alcoholic compounds contribute to certain flavour compounds (Banwart, 1989).

Volatile and semi-volatile organic compounds present both in the matrix and the headspace aroma are largely responsible for the flavour qualities of the foods we eat. Flavour is considered to be the most important factor of any of the quality categories when comparing various dairy products. Dairy products provide a great sense of eating pleasure as a result of their characteristic flavour and smooth texture and they play an important role in a well-balanced diet. Because milk possesses a bland and soft flavour, the appearance of an objectionable off-flavour or off-odour is readily noticeable. There is a delicate balance between many flavour compounds that contribute to a desirable milk flavour and if this balance is disturbed, off-flavours may occur (Overton and Manura, 1999). Flavobacteria can produce a putrid butter effect and a "sweaty feet" odour in skim milk (Jooste *et al.,* 1986).

The aim of this study was to investigate the different growth and hydrolytic characteristics of the type species of the *Chryseobacterium* genus. These characteristics included the optimum growth temperature and growth capacity at different temperatures, pH values and NaCl concentrations. The

proteolytic, lipolytic and phospholipase C production of the species was examined by using different microbiological culture media. The volatile compounds produced by the reference strains in milk, were examined by headspace gas chromatography (GC). An odour description of milk samples, inoculated with the bacterial cultures were given by a semi-trained sensory panel of ten persons.

4.2 Materials and methods

4.2.1. Strains investigated

The reference strains used in this study are listed in Table 3.1 in Chapter 3 of this thesis. Freeze-dried or agar slant cultures were reactivated in 10 ml Nutrient Broth (Oxoid CM67) and purity was checked by streaking on Nutrient Agar (Oxoid CM3). Incubation was at 25ºC for 24-48 h. The strains were maintained on Nutrient Agar slants and stored at 4ºC.

4.2.2. Growth characteristics

A battery of tests was performed to determine the different growth characteristics and to differentiate between Gram negative, yellow pigmented species, according to the methods described by MacFaddin (1980), Gerhardt *et al.* (1981), Barrow and Feltham (1983) and Hugo *et al.* (2003), unless indicated otherwise. All the tests were performed at 25°C, except where the growth of the reference strains at different temperatures, was examined on Nutrient Agar at 4°C, 25°C, 32°C, 37°C, 42°C and 55°C respectively.

4.2.2.1. Optimum growth temperature

The optimum growth temperature for four *Chryseobacterium* species was determined according to the method described by Jooste *et al.* (1986). Four consecutive overnight cultures (25°C) were prepared in tubes containing 10 ml each of Nutrient Broth. The *Chryseobacterium* species were incubated in a water-bath at each of the following temperatures: 20, 25, 30, 35, 40 and 45°C. Turbidity in the tubes, after 48 h, was read in a Milton Roy Spectronic 20D

spectrophotometer at 660 nm using uninoculated Nutrient Broth as control. Optical density values were converted to Klett units (1 Klett unit = Optical density / 0.002).

4.2.3. Hydrolytic activities

The proteolytic activity of the reference strains was determined by casein and gelatin hydrolysis, described by Harrigan and McCance (1976). The lipolytic activity was determined using lecithinase production (MacFaddin, 1980; Barrow and Feltham, 1983) and the hydrolysis of olive oil and tributyrin (Harrigan and McCance, 1976). Phospholipase C production was determined using the method of Chrisope *et al.* (1976).

4.2.4. Preliminary determination of volatile compounds in milk

Growth conditions were as follows: 100 ml samples of Clover, UHT process, fat free milk and full cream milk were measured aseptically into sterile Erlenmeyer flasks. Each milk sample was then inoculated with 1 ml of a 24 h old Nutrient Broth culture of *C. joostei*, *C. gleum* and *C. indologenes* respectively and incubated at 25°C for 72 h. Samples were prepared in duplicate.

Analytical procedures were as follows: The volatile compounds were determined according to Human (1998). Each of the inoculated milk samples (5 ml) and control samples (5 ml) were measured into a 10 ml headspace clear glass-vial and closed with crimpable headspace caps with silicone-PTFE seals. After incubation for 30 minutes at 70°C, a 1 ml headspace sample was withdrawn using a gas-tight syringe and injected into a Varian Chrompack CP-3800 gas chromatograph (GC) equipped with a flame ionisation detector (FID). A 30 m Zebron ZB-FFAP column (0.32 mm internal diameter), with a 25 µm film thickness and containing a nitroterephthalic acid modified polyethylene glycol liquid phase, with 40 to 250/260°C (isothermal) temperature limits (Phenomenex, USA) was used. The temperature profile used was: 80°C for 2 min, increased by 5 °C/ min to 250°C and held for 10 minutes. The total run time was 27 minutes. The carrier gas was hydrogen

UHP. The column flow was 2.0 ml/min, the linear velocity 50.9 cm/ sec and the total flow 27.8 ml/min. Identification of the unknown compounds was achieved by comparing their retention times to those of analytical grade standard compounds (Human, 1998). An ethylacetate stock solution was prepared as standard and contained 200 $mg.l^{-1}$ of each of propionic acid, valeric acid, 1-Heptanol, 1-Octanol, 1-Nonanol, 1-Decanol, 1-Pentanol, 1-Hexanol, 2-Octanone, 2-Butanone, 2-Heptanone, acetone, 4-Methyl-2-pentanol, 2-Methyl-1-butanol, ethyl acetate, methyl acetate, propyl acetate and *n*-buthanol (internal standard).

4.2.5. Sensory analysis of milk

Ten candidates for the semi-trained sensory odour descriptive panel were screened on the basis of their ability to differentiate among odours and are all scientists employed by, or post graduate students in the Food Science Division (Department of Microbial, Biochemical and Food Biotechnology, Free State University, Bloemfontein, South Africa). The panel consisted of seven female and three male persons, ranging from 23 to 60 years of age. As part of the training process, the panellists performed a paired comparison test and were asked to identify the spoiled sample by doing a sniffing test. The spoiled sample consisted of 10 ml fat free milk which was inoculated with *Chryseobacterium joostei* and incubated at 25°C for 72 h. The control sample was uninoculated fat free milk.

For the sniffing test, 10 ml samples of Clover, UHT Process, Fat Free and Full Cream milk in McCartney bottles were each inoculated with 0.1 ml of a 24 h old Nutrient Broth culture of *C. joostei*, *C. gleum* and *C. indologenes* respectively and incubated at 25°C for 72 h. An uninoculated milk sample of each type of milk was included to serve as controls. Thirty minutes before sensory evaluation, the milk samples were incubated at 50°C to allow better assessment of the volatile fraction. The milk samples were presented in the same McCartney bottles, in which the inoculations were done. These bottles had tight fitting caps to prevent any unnecessary odour release, which could influence results. Samples were coded with three digit numbers and served

randomly on white trays. The testing was done under red lights to mask any colour differences which occurred due to the characteristic yellow to orange pigmentation produced by *Chryseobacterium* species (Bernardet *et al.,* 2006). The inoculated samples, along with two control samples, one each of the fat-free and full cream milk, were presented to the panel in individual booths. Samples were uncapped, sniffed and removed immediately so, as not to cause exhaustion. When exhaustion did occur, panellists were instructed to smell their own skin or clothing. As part of the training, panellists were not allowed to wear any perfumed personal care products or smoke for 30 minutes prior to sniffing, as this could influence the results.

Three spoilage levels were chosen to define the intensity of the spoilage: level 1, no spoilage noted; level 2, weak spoilage noted; level 3, strong spoilage noted (Joffraud *et al.,* 2001). Panellists also described the type of odour, if any, produced by each of the three cultures. The samples were regarded as weakly spoiled and strongly spoiled when at least 50% of the panellists rated it at level 2 and level 3, respectively.

The sensory testing was done in individual booths at the sensory facility of the Food Science Division, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State.

4.3. Results and discussion

4.3.1. Growth activities

Growth at different temperatures are shown in Table 4.1. *Chryseobacterium balustinum, C. indologenes, C. indoltheticum* and *C. joostei* showed weak growth at 4°C. According to Bernardet *et al.* (2006), very poor growth or no growth at all occurs at 5°C. All the species were able to grow at 25°C, 32°C and 37°C, except *C. balustinum* which showed weak growth at 32°C and *C. indoltheticum* and *C. joostei* which showed weak growth at 37ºC. *Chryseobacterium gleum, C. indologenes, C. indoltheticum* and *Elizabethkingia meningoseptica,* showed growth at 42°C and none of the

Table 4.1 Growth of *Chryseobacterium* and *Elizabethkingia* species at different temperatures in Nutrient Broth.

Temperature	Species								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
4 ℃	-	w	-	w	w	w	-	-	-
25 °C	+	+	+	+	+	+	+	+	+
32 °C	+	w	+	+	+	+	+	+	+
37 °C	+	+	+	+	w	w	+	+	+
42 °C	+	-	+	+	-	-	-	+	-
55 °C	-	-	-	-	-	-	-	-	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

reference strains were able to grow at 55°C. Growth consequently seems to be inhibited at temperatures lower than 4°C and at 55°C or higher. Growth and metabolic activity are not pronounced as at optimum temperature but are not totally inhibited at 5, 10 and 15°C.

According to Hugo and Jooste (2003), the most used temperature range for *Chryseobacterium* species incubation is 20 – 30°C. At 42°C, most *Chryseobacterium* species will not grow, except some *C. gleum* and some *E. meningoseptica* strains (Bernardet *et al.,* 2006). *Chryseobacterium gleum* strains were reported to grow at 41°C in contrast with *C. indologenes* strains (Yabuuchi *et al.,* 1983; Bernardet *et al*., 2006). The critical growth temperature proposed for differentiating between *Chryseobacterium* species was 40°C (Ursing and Bruun, 1991), 41°C (Yabuuchi *et al.,* 1990), or 42°C (Holmes *et al.,* 1984a, 1984b; Vandamme *et al*., 1994; Bernardet *et al*., 2006).

Growth at different pH values is shown in Table 4.2. None of the reference strains could grow at a pH range of 1-4, but were able to grow at a pH range of 5-10. This is also the pH range of most foods which, makes food an ideal growth medium in which members of the genus *Chryseobacterium* can cause spoilage. According to Garbutt (1997), milk has a pH of 6.3-6.6 and meat can have a pH of 5.4 – 6.9. Madigan *et al*. (2000) stated that some alkaliphiles produce hydrolytic enzymes such as proteases and lipases. The activity of these enzymes can result in the production of off-odours and off-flavours in food products. pH values under five clearly had an inhibitory effect on the growth of the reference strains.

Growth at different NaCl concentrations are shown in Table 4.3. All the species, except *C. balustinum*, were able to grow at 1% (w/v) NaCl. *Chryseobacterium gleum, C. indologenes, C. scophthalmum, Elizabethkingia meningoseptica* and *E. miricola* were capable of growth at 2% NaCl, while *C. joostei, E. meningoseptica* and *E. miricola* were able to grow in the presence of 3% NaCl. No growth occurred at 4% and 5% NaCl, which indicated that NaCl concentrations above 3% had an inhibitory effect on the growth of the

Table 4.2 Growth of *Chryseobacterium* and *Elizabethkingia* species at different pH values.

pH	Species								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
pH 1	-	-	-	-	-	-	-	-	-
pH 2	-	-	-	-	-	-	-	-	-
pH 3	-	-	-	-	-	-	-	-	-
pH 4	-	-	-	-	-	-	-	-	-
pH 5	+	+	+	+	+	+	+	+	+
pH 6	+	+	+	+	+	+	+	+	+
pH 7	+	+	+	+	+	+	+	+	+
pH 8	+	+	+	+	+	+	+	+	+
pH 9	+	+	+	+	+	+	+	+	+
pH 10	+	+	+	+	+	+	+	+	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative.

Table 4.3 Growth of *Chryseobacterium* and *Elizabethkingia* species at different NaCl (w/v) concentrations.

NaCl Concentration	Species								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1%	+	-	+	+	+	+	+	+	+
2%	+	-	-	+	-	-	+	+	+
3%	-	-	-	-	-	+	-	+	+
4%	-	-	-	-	-	-	-	-	-
5%	-	-	-	-	-	-	-	-	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; -, negative.

species. In a study by Jooste *et al*. (1986), flavobacterial strains tested in NaCl Broth were able to grow at concentrations of 1% (w/v) NaCl but not at 4 % NaCl. Members of most *Chryseobacterium* species exhibit varying degrees of tolerance to NaCl, as shown by their ability to grow on Difco Marine Agar (1.95% NaCl, Bernardet *et al.,* 2002). Environmental strains may occur both in freshwater and seawater, as well as in freshwater and marine fish (Bernardet *et al*., 2006).

Growth on different culture media are shown in Table 4.4. *Chryseobacterium indologenes* and *C. joostei* were able to grow on Cetrimide Agar, while *Chryseobacterium joostei* and *Elizabethkingia miricola* exhibited growth on MacConkey Agar. According to Bernardet *et al.* (2006), most *Chryseobacterium* species do not grow or grow poorly on Cetrimide Agar while the ability to grow on MacConkey Agar varies among species. Hugo and Jooste (2003) reported that *C. balustinum* and *C. gleum* were unable to grow on Cetrimide Agar, while Yabuuchi *et al.* (1983) found that almost 50% of the *Chryseobacterium* strains were inhibited on MacConkey Agar. None of the species tested in this study were able to grow on ß-polyhydroxybutyrate. In previous studies growth on this medium by *C. balustinum* and *C. gleum* (Holmes *et al.,* 1984b) and *C. scophthalmum* (Bernardet *et al*., 2006) has been reported. The growth did not lead to the production of beta-hydroxybutyrate inclusion granules. It would seem that the above discrepancies between workers, in terms of the growth of *Chryseobacterium* strains on this medium, may be ascribed to the use of different techniques or conditions.

Elizabethkingia meningoseptica was positive for growth on Simmons Citrate Agar, which indicated that citrate was utilised as the sole carbon source. Utilisation of citrate and growth on citrate agar results in an alkaline reaction. According to Hugo and Jooste (2003), *C. balustinum* and *C. gleum* will not grow on Simmon's Citrate Agar. Members of the genus *Chryseobacterium* are generally not difficult to isolate and cultivate, since they are classic aerobic chemoorganotrophs. Hence, most of them grow readily on commercially

Table 4.4 Growth of *Chryseobacterium* and *Elizabethkingia* species on different culture media.

Growth medium	Species								
	1	2	3	4	5	6	7	8	9
Cetrimide Agar	-	-	-	+	-	+	-	w	w
ß-polyhydroxybutyrate	-	-	-	-	-	-	-	-	-
MacConkey Agar	w	-	w	-	w	+	w	w	+
Marine Agar	+	+	+	+	+	+	+	+	+
Simmons Citrate Agar	-	w	w	-	w	w	w	+	-

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

available organic media, usually making it unnecessary to use selective media and to add growth factors (Bernardet *et al*., 2006).

All the species were able to grow well on Marine Agar. Interestingly, enough although only two *Chryseobacterium* species originated from marine environments. Most species exhibit a rather high degree of halotolerance and are consequently also able to grow on Difco Marine 2216 Agar (1.95% NaCl among several other salts; Bernardet *et al*., 2002); *Chryseobacterium balustinum* is the only known exception. Conversely, the *Chryseobacterium* strains from marine origin grow as well on the above-mentioned non-marine media as on Marine 2216 Agar (Bernardet *et al*., 2006).

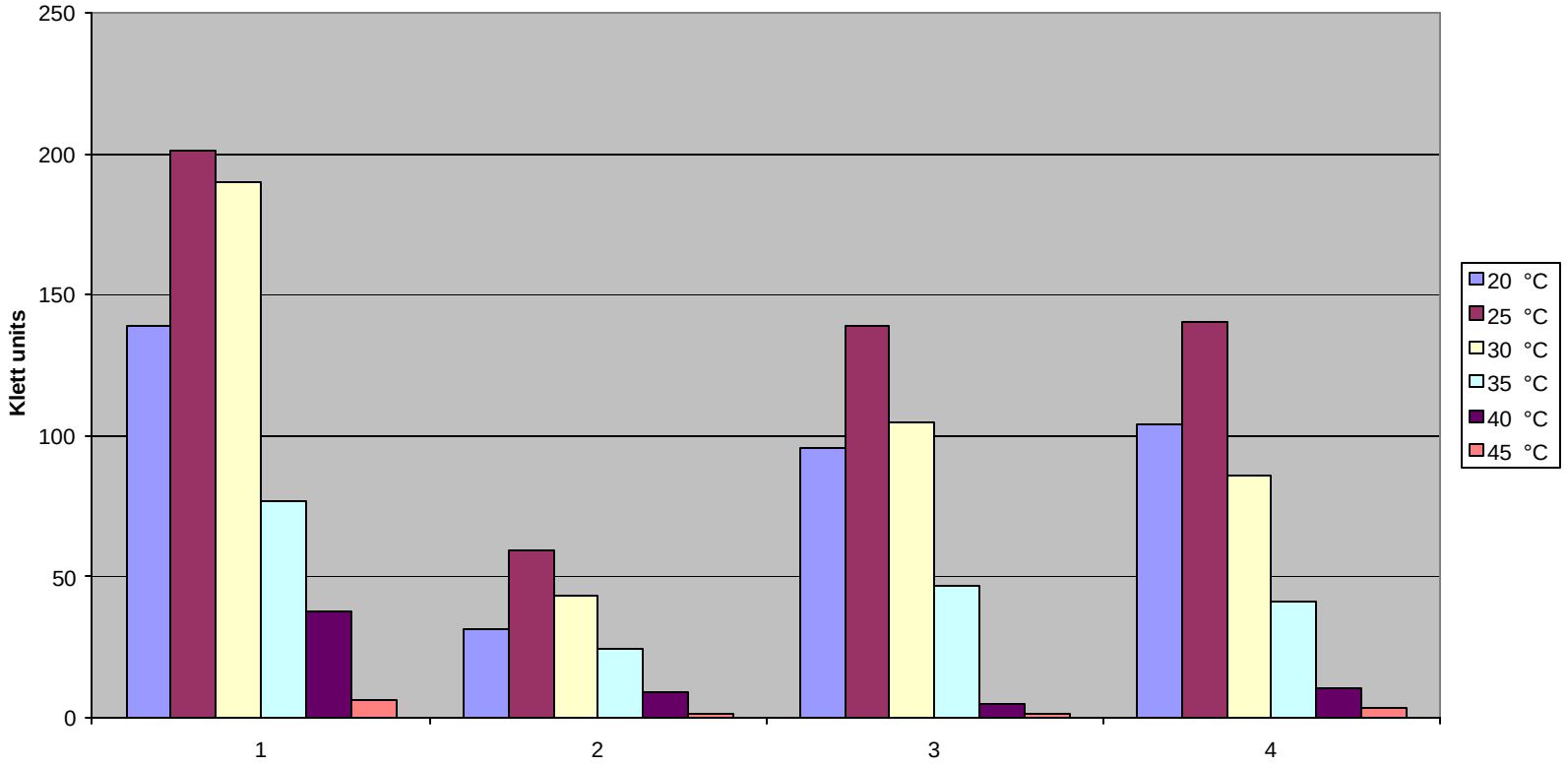
4.3.1.1. Optimum growth temperature

The optimum growth temperature for the four *Chryseobacterium* strains are shown in Fig. 4.1. All four strains gave the highest Klett values at 25°C. The lowest Klett values were given at 45°C. These results showed that the optimum temperature for the four strains was 25°C. It would seem that temperatures less than or more than 25°C are less suitable for growth.

4.3.2. Hydrolytic activities

According to the description of the genus *Chryseobacterium* by Vandamme *et al.* (1994), these organisms are capable of strong proteolytic activity. Table 4.5 shows that all the species hydrolysed casein. *Chryseobacterium defluvii, C. joostei*, *Elizabethkingia meningoseptica* and *E. miricola* hydrolysed gelatin (Table 4.5). In a study by Jooste and Britz (1986), it was found that a *Flavobacterium* strain, Ix 9a (which was shown by Hugo *et al.* (2003) to be a *C. indologenes* strain) was the most prolific producer of extracellular proteinase on Caseinate Agar at 22°C. Jooste *et al.* (1986), stated that six representative *Flavobacterium* isolates were all strongly proteolytic on Caseinate Agar. Various proteolytic activities (i.e., degradation of skim milk, casein, and gelatin on agar media) were also found in *C. balustinum, C. gleum* and *C. indologenes* strains isolated from Cape marine fish in South

Fig. 4.1 Optimum growth temperatures of *Chryseobacterium* strains after a 48 h incubation period in Nutrient Broth.



Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum* ; 3, *C. indologenes*; 4, *C. joostei*.

Table 4.5 Proteolytic and lipolytic activity and production of Phospholipase C by *Chryseobacterium* and *Elizabethkingia* species.

Characteristic	Species								
	1	2	3	4	5	6	7	8	9
Proteolytic activity									
Hydrolysis of casein	+	+	+	+	+	+	+	+	+
Hydrolysis of gelatin	-	-	+	-	-	+	-	+	+
Lipolytic activity									
Production of lecithinase	+	-	w	w	w	-	w	-	-
Hydrolysis of olive oil	+	+	+	+	+	+	+	+	+
Hydrolysis of tributyrin	+	w	w	+	+	+	+	+	+
Production of Phospholipase C	+	-	-	+	+	+	-	-	+

Species are listed as: 1, *Chryseobacterium gleum*; 2, *C. balustinum*; 3, *C. defluvii;* 4, *C. indologenes*; 5, *C. indoltheticum;* 6, *C. joostei;* 7, *C. scophthalmum*; 8, *Elizabethkingia meningoseptica*; 9, *E. miricola*. +, Positive; w, weakly positive; -, negative.

Africa (Engelbrecht *et al.,* 1996). In a study by Venter *et al*. (1999), a metalloprotease from a strain of *C. indologenes* was purified and characterised; this protease was very heat stable and its affinity for casein shows that it could play a role in the spoilage of milk and milk products.

Chryseobacterium defluvii, C. indologenes, C. indoltheticum and *C. scophthalmum* hydrolysed lecithin in Egg-Yolk Medium weakly. *Chryseobacterium gleum* in contrast was able to hydrolyse lecithin strongly. All the species hydrolysed olive oil and tributyrin, while *C. balustinum* and *C. defluvii* hydrolysed tributyrin weakly (Table 4.5). In a study by Jooste *et al.* (1986), not one of the six *Flavobacterium* isolates were found to be lipolytic. According to Bernardet *et al*. (2006), *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. joostei*, *C. scophthalmum* and *C. vrystaatense* were able to hydrolyse lecithin in a 10% Egg-Yolk Medium. *Chryseobacterium gleum, C. indologenes, C. joostei, Elizabethkingia meningoseptica* and *E. miricola* produced phospholipase C on Lecithin Agar. Chrisope and Marshall (1976) showed that the presence of phospholipase C enhanced lipolysis. According to Stead (1989), the psychrotrophic lipases generally act only on exposed lipids. Hence, the phospholipases play a role in degrading the phospholipids on the surface of the milk fat globule membrane exposing the milk lipids. This can enhance the lipolysis of the fat and cause defects such as rancidity (Mottar, 1989).

4.3.3. Preliminary determination of volatile compounds caused by Chryseobacterium species in milk

The headspace GC-FID chromatograms of the fat free milk samples, inoculated with three *Chryseobacterium* spp. are shown in Figs. 4.2a – 4.2c. All the species (*C. gleum*, *C. indologenes*, *C. joostei*) produced 1-Nonanol, 1-Decanol and 1-Dodecanol in the milk. 1-Heptanol, 1-Octanol, Propionic acid and Valeric acid was also present in low concentrations in the milk samples, inoculated with *C. gleum* and *C. indologenes*. Fat free milk, inoculated with *C. gleum,* yielded a higher concentration of 1-Nonanol than *C. indologenes* and

Fig. 4.2a GC-FID chromatogram of fat free milk, inoculated with *C. gleum*, after 72 h of incubation at 25°C.

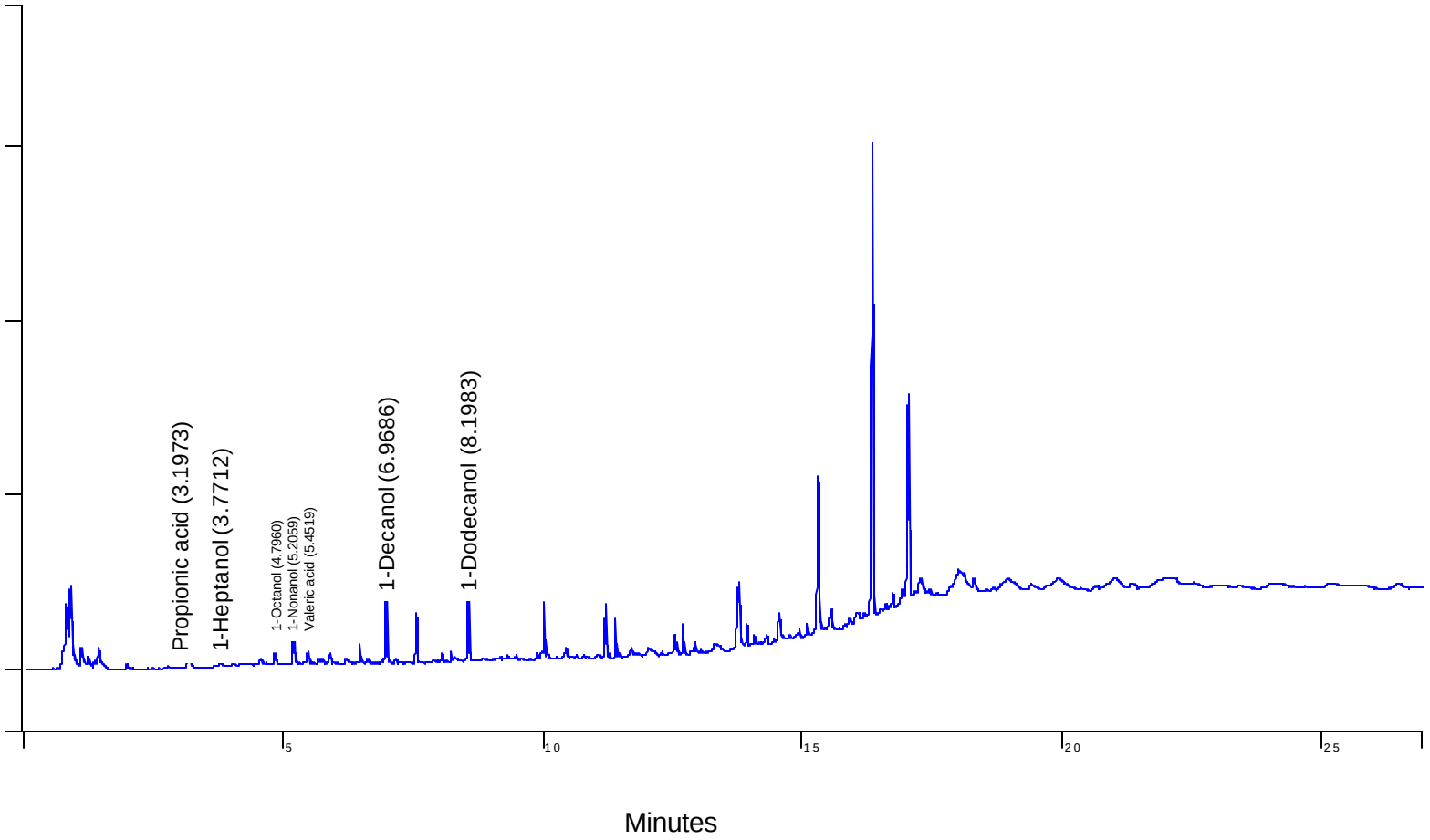


Fig. 4.2b GC-FID chromatogram of fat free milk, inoculated with *C. indologenes*, after 72 h of incubation at 25°C.

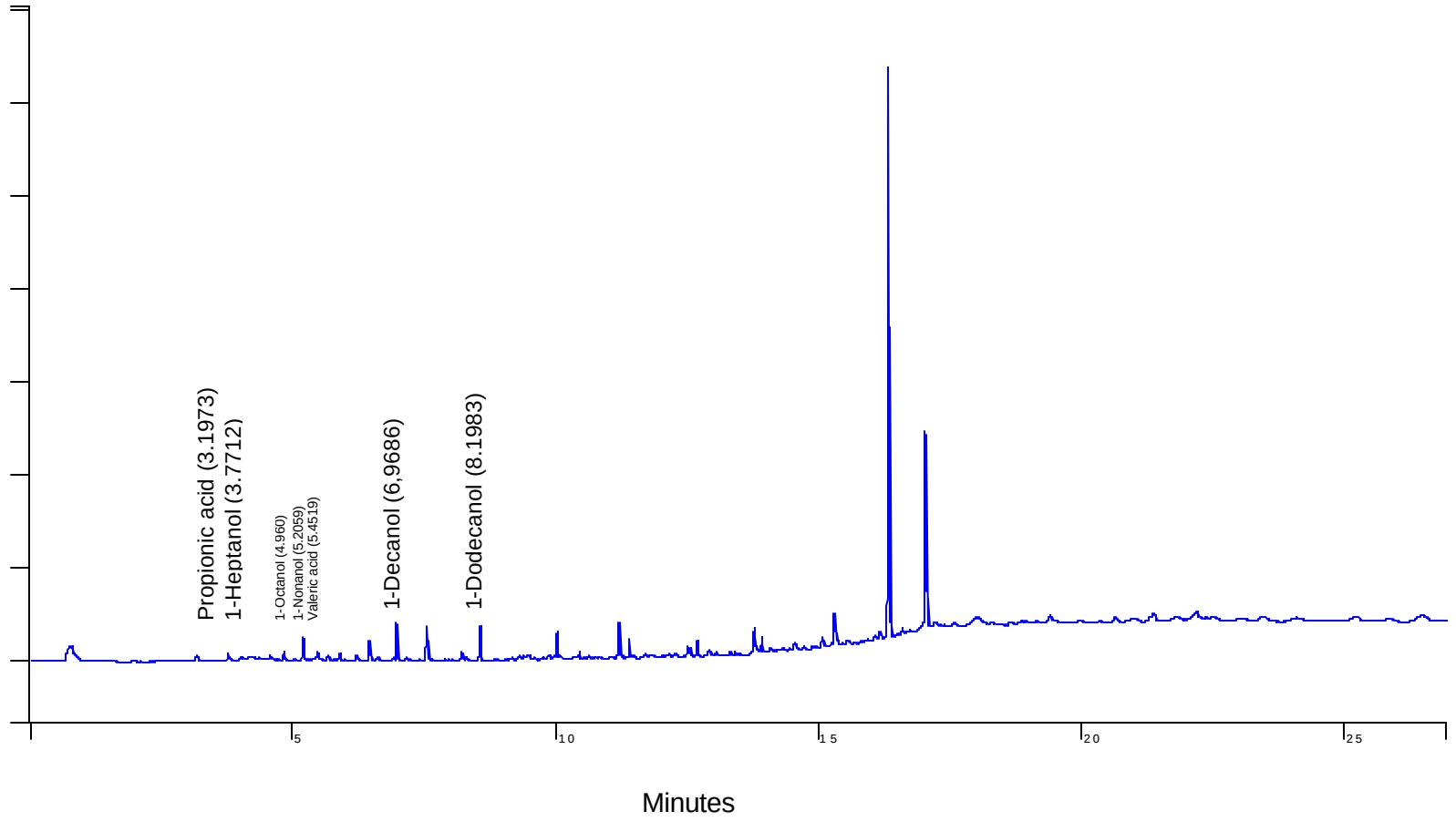
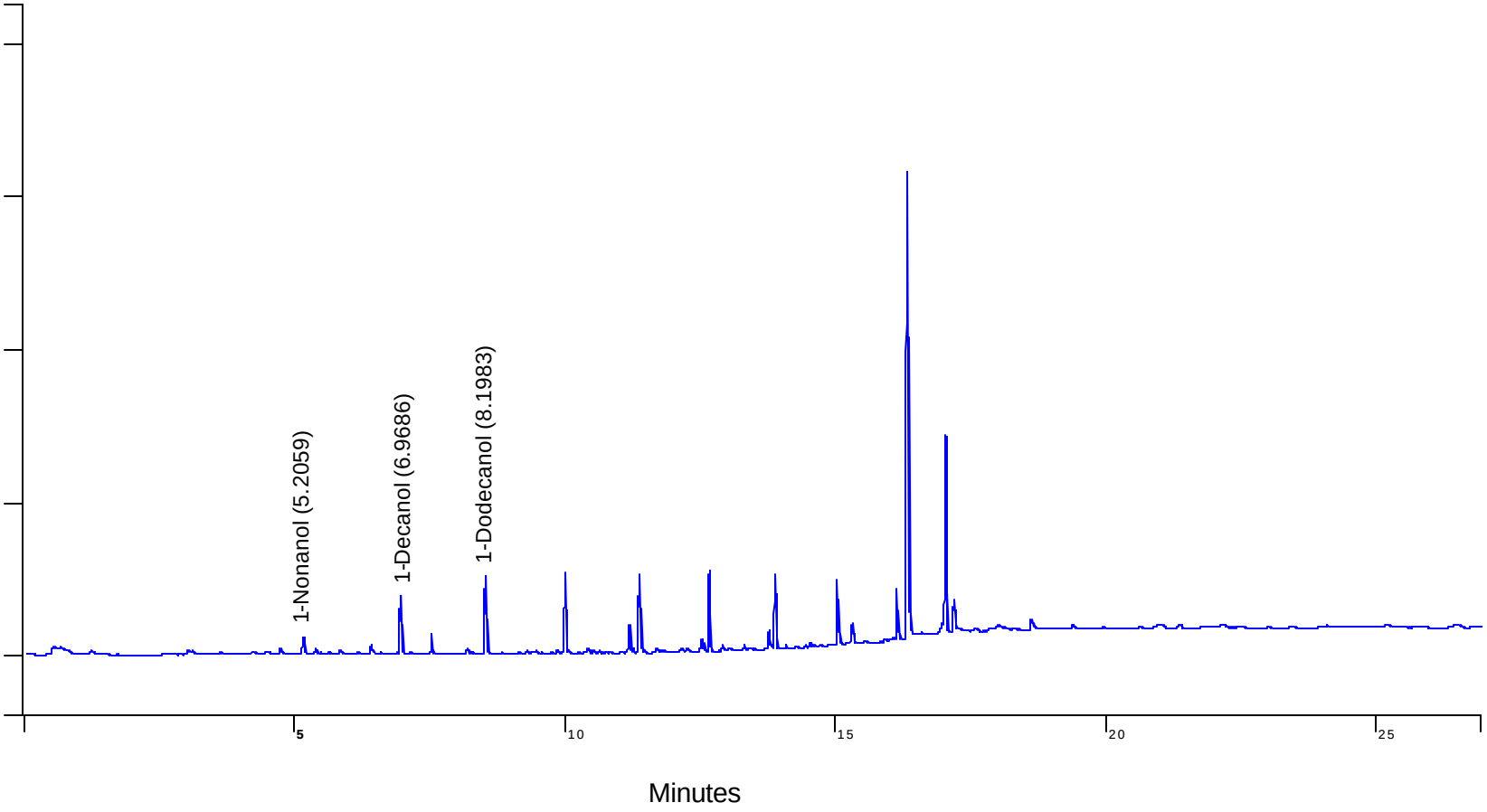


Fig. 4.2c GC-FID chromatogram of fat free milk, inoculated with *C. joostei*, after 72 h of incubation at 25°C.



C. joostei. Seven volatile compounds were presumptively identified in the fat free milk of which five were primary alcohols and two were carboxylic acids.

The headspace GC-FID chromatograms of the full cream milk, inoculated with three *Chryseobacterium* spp. are shown in Figs. 4.3a – 4.3c. *Chryseobacterium gleum, C. indologenes* and *C. joostei* produced 1-Nonanol, 1-Decanol, 1-Dodecanol, Propionic acid and Valeric acid in the milk. Valeric acid was present in very low concentrations in all the milk samples.

Full cream milk inoculated with *C. gleum* yielded a lower concentration of Propionic acid than *C. indologenes*, and *C. joostei* while milk inoculated with *C. indologenes* yielded higher concentrations of 1-Nonanol, 1-Decanol and 1-Dodecanol than *C. gleum* and *C joostei*. Five volatile compounds were presumptively identified in the full cream milk of which three were primary alcohols and two were carboxylic acids.

The primary alcohols (e.g. 1-Decanol) were probably produced by reduction of the corresponding aldehydes (Shiratsuchi *et al.,* 1994). In a study by Shiratsuchi *et al.* (1994), it was found that it is unlikely that primary alcohols contributed to the flavour of skim milk, because of their high flavour thresholds in milk (0.4-2.0 ppm). Jooste *et al.* (1986) found that flavobacteria gave rise to volatile fatty acids, identified as acetic, propionic, iso-butyric and iso-valeric acid in skim milk cultures.

Valeric acid (also called pentanoic acid) is found naturally in the perennial flowering plant valerian (*Valeriana officinalis*) from which it gets its name. It has a very unpleasant (Anonymous, 2006a), penetrating (Anonymous, 2006b) or pungent (Anonymous, 2006c) odour. Volatile esters of valeric acid are used as food additives because of their fruity flavours (Anonymous, 2006a).

Propionic acid (also called propanoic) has a pungent odour (Anonymous, 2006d) and slightly sweetish taste. It is found naturally in milk products, and as a product of bacterial fermentation (Anonymous, 2006e).

Fig. 4.3a GC-FID chromatogram of full cream milk, inoculated with *C. gleum*, after 72 h of incubation at 25°C.

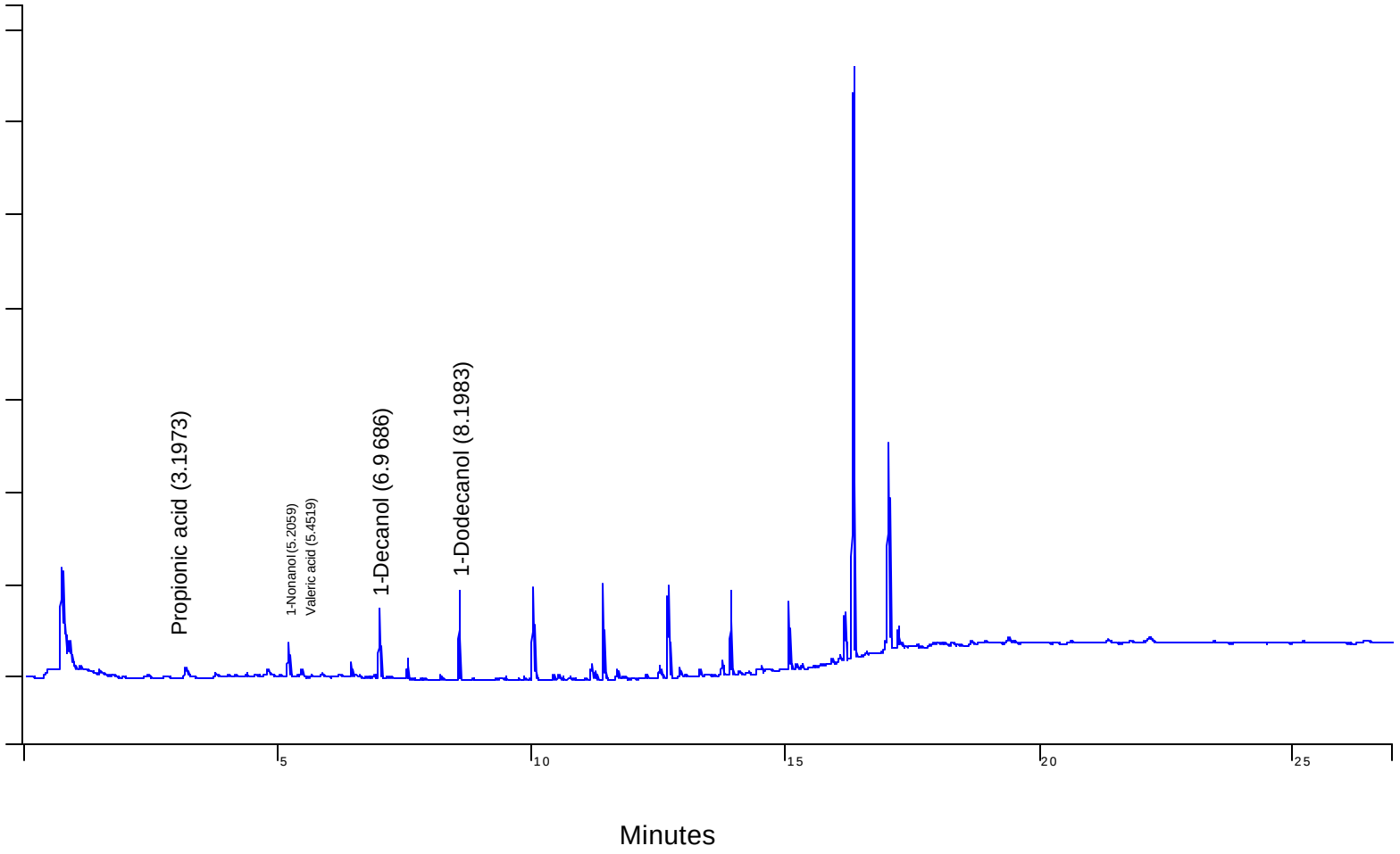


Fig. 4.3b GC-FID chromatogram of full cream milk, inoculated with *C. indologenes*, after 72 h of incubation at 25°C.

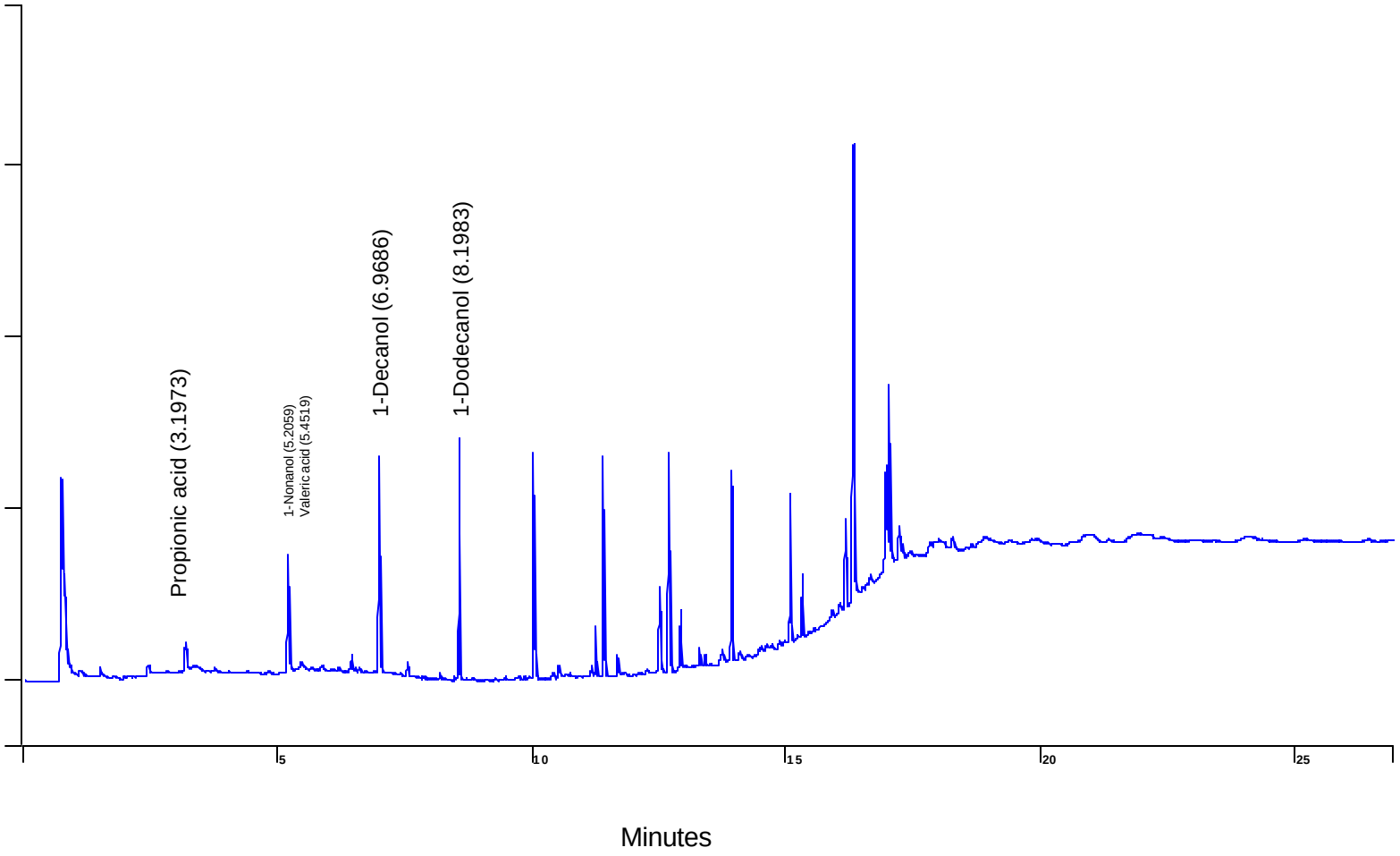
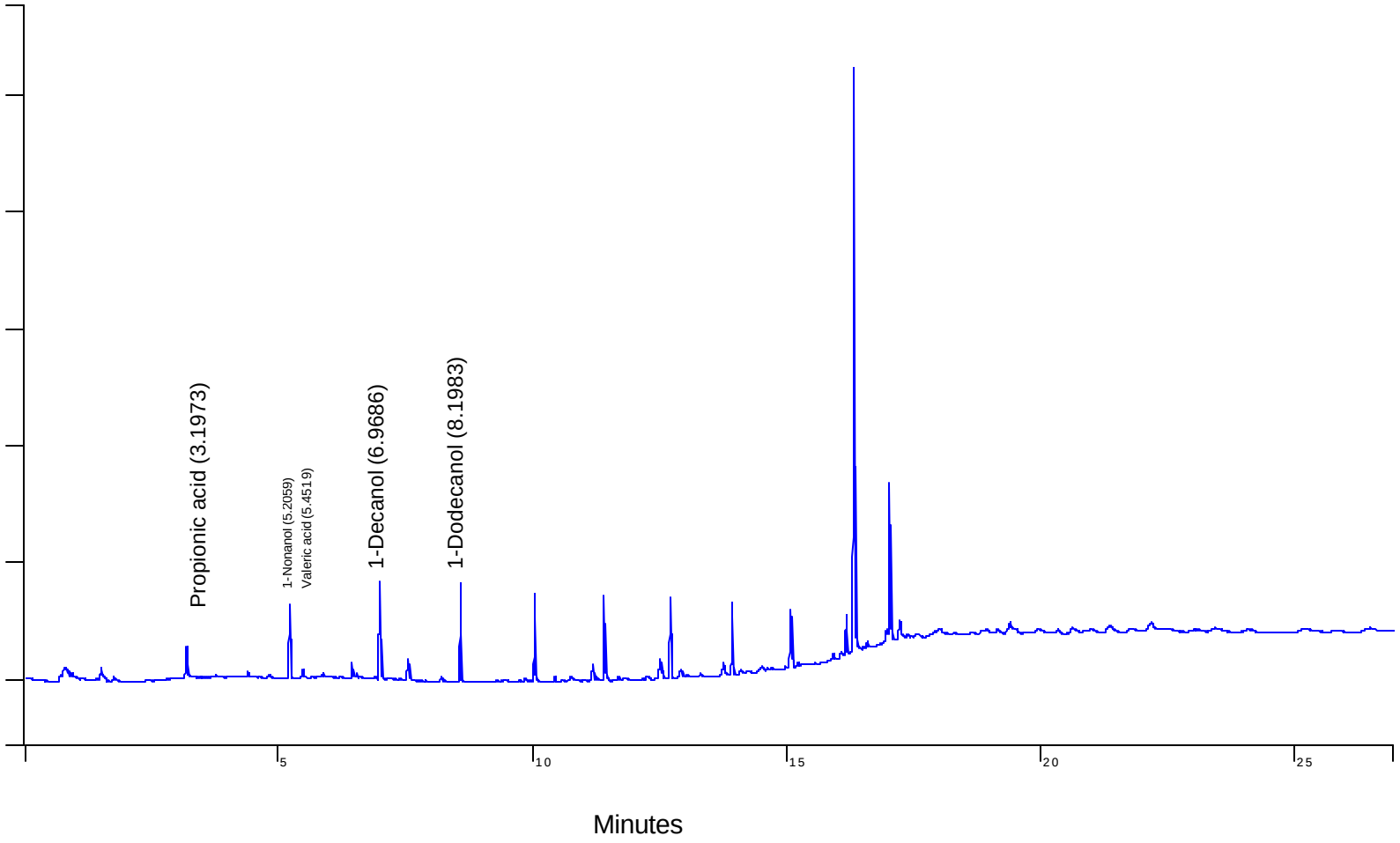


Fig. 4.3c GC-FID chromatogram of full cream milk, inoculated with *C. joostei*, after 72 h of incubation at 25°C.



The most notable difference when comparing the chromatograms of the two types of milk, was that the chromatogram peaks of the full cream milk were higher than the fat free milk and that more volatile compounds could be identified. Although many of the same volatiles were present in all the inoculated milk samples, there was a distinct variation in the concentrations as well as the variety of volatile compounds present. This variation could be due to the cow's diet, environment, heat treatment, bacterial spoilage, storage and the oxidation of lipids (Overton and Manura, 1999).

Although some ketones and esters were part of the analytical standard compounds used to compare retention times, only the primary alcohols and carboxylic acids were tentatively identified. The reason for this could be that the GC column was perhaps too polar and may not have picked up a possible homologue array of non-polar compounds. Other unidentified compounds could be the aliphatic compounds (e.g. octanal) which are typical products of lipid oxidation (Overton and Manura, 1999).

Fedele *et al.* (2005) observed that the volatile compound composition in milk from grazing goats in Italy, changed over the seasons. Alcohols increased significantly from winter to spring. This could explain the presence of mainly alcohols in the two milk types, since the milk, used in this study, was produced in the summer.

4.3.4. Sensory analysis

All the panellists were able to identify the spoiled sample in the paired comparison test. These results were analysed by consulting the Roessler table for Paired and Duo-trio tests (one-tailed) (Anonymous, 1995). It showed that, not only did the odour associated with spoiled milk differ significantly from that of the fresh milk sample ($P<0.001$), but that the panellists were acquainted with the specific odour. As indicated previously, the spoiled milk sample was inoculated with *C. joostei*, which revealed later on in the second part of the evaluation to have a strong odour, smelling ''sour'', ''flowery'', ''putrid'' and ''smelly feet'' like.

Further results of the sensory evaluations are shown in Tables 4.6a and 4.6b. Since milk possesses a bland and soft flavour, an occurrence of off-flavour is readily noticeable and objectionable (Shiratsuchi *et al.*, 1994). The fat free milk samples, inoculated with *C. gleum* and *C. joostei* were considered to be strongly spoiled, as they gave odours described as “fruity”, “flowery”, “smelly feet”, “blue cheese”, “sour” and “putrid”. Samples inoculated with *C. indologenes* were considered to be weakly spoiled, as it gave odours described as “sour”, “agar” and “putrid”.

Full cream milk samples inoculated with *C. gleum* were also considered to be strongly spoiled, as it yielded not only odours described as “fruity”, “flowery” and “blue cheese”, when compared to the fat free milk, but also “sour”,“rancid” and “putrid”. Samples inoculated with *C. joostei* and *C. indologenes* were both weakly spoiled and yielded odours described as “sour” and “putrid”, when compared to the fat free milk, as well as “agar” and “fruity” odours.

Fats are more difficult for microorganisms to attack than carbohydrates and proteins, although a few organisms do produce lipases (Mountney and Gould, 1988). This could explain why the fat free milk samples were more spoiled than the full cream milk samples. Lipolysis by flavobacteria is known to break down fatty acids, giving disagreeable and rancid off-odours and off-flavours in dairy products. Short-chain fatty acids, such as butyric acid (C4:0), caproic acid (C6:0) and caprylic acid (C8:0), give sharp and tangy flavours (Al-Shabibi *et al*., 1964). According to Cousin (1982) these short-chain fatty acids may be responsible for development of fruity flavours in milk, while Banwart (1989) described them as obnoxious rancid flavours. The oxidized flavour in milk is caused largely by the formation of aldehydes and ketones as the result of the oxidation of the unsaturated fatty acids such as oleic (Mountney and Gould, 1988). Therefore it could be possible that the formation of fatty acids were responsible for the “sour” and ‘‘fruity’’ odours that were observed. The aliphatic compounds octanal, nonanal and decanal are typical products of lipid oxidation. When these compounds pass a certain threshold, they are considered significant contributors to the development of rancid flavour

Table 4.6a Conditions and odours assigned to samples of fat free milk, inoculated with *Chryseobacterium* species and incubated at 25°C for 72 h.

Inoculum	Condition	Sensory descriptors
C. gleum	Strongly spoiled	Fruity, flowery, smelly feet, blue cheese
C. indologenes	Weakly spoiled	Sour, agar, putrid
C. joostei	Strongly spoiled	Sour, flowery, putrid, smelly feet

Table 4.6b Conditions and odours assigned to samples of full cream milk, inoculated with *Chryseobacterium* species and incubated at 25°C for 72 h.

Inoculum	Condition	Sensory descriptors
C. gleum	Strongly spoiled	Sour, fruity, rancid, flowery, putrid, butter, blue cheese
C. indologenes	Weakly spoiled	Sour, fruity, putrid
C. joostei	Weakly spoiled	Sour, agar, putrid

(Overton and Manura, 1999). Fruity odours arise from the degradation of the amino acids glycine, leucine and serine to form lower fatty acid esters (Engelbrecht *et al.,* 1996). According to Wilkes *et al.* (2000) fruity off-flavours may be primarily ascribed to ethyl butyrate and ethyl hexanoate. It has been reported that flavobacteria can cause an apple odour in butter (Wolochow *et al*., 1942). Bernardet *et al.* (2006) reported that a strong odour is produced by most *Chryseobacterium* strains in liquid and solid cultures; this has been described as ''fruity'' or ''cheesy''. Valeric acid could be responsible for the "fruity" odours that were observed by some of the panellists (Anonymous, 2006a).

The peptides produced as a result of proteolysis usually give rise to unclean and bitter flavours. Bitter peptides are normally characterised by large numbers of hydrophobic amino acids (Venter, 1997). Off-odours and off-flavours described as metallic, unclean, bitter and putrid are produced by the breakdown of amino acids used as energy sources by the organism (Garbutt, 1997). Continued proteolysis results in putrid off-flavours associated with lower-molecular-weight degradation products such as ammonia, amines, and sulphides (Frank, 1997). Therefore it could be possible that the breakdown of amino acids were responsible for the "putrid" odours that were observed.

Studies by Jooste *et al*. (1986) showed that flavobacteria were able to produce a "sweaty feet" odour in skim milk and concluded that iso-valeric acid was responsible for this off-odour. Therefore it could be possible that iso-valeric acid was responsible for the "smelly feet" odour that was observed.

4.4. Conclusions

In this study it was observed that *Chryseobacterium* species were able to grow at 4°C, at a pH of 5 to 10 and at sodium chloride concentrations of 1 to 3 %. It is known that *Chryseobacterium* species have the potential to spoil milk. For these reasons a decrease in temperature (below 4°C), in pH (below 5) and an increase in sodium chloride concentration (above 4%) will inhibit the growth of this genus and have a preservative effect in products with these

characteristics. Optimum growth was observed at 25°C, and it could be expected that spoilage defects in food products, kept at this temperature would develop most rapidly. The genus *Chryseobacterium* has the potential to produce spoilage defects due to proteolytic and lipolytic activity. Such activity could result in off-flavours and off-odours. Similarly the production of phospholipase C could enhance lipolysis and rancidity defects.

Chryseobacterium species were able to produce volatile compounds in milk. The primary alcohols produced were not likely to contribute to odour, while the carboxylic acids can be responsible for the production of a variety of odours (e.g. fruity). Sensory evaluation on inoculated milk samples resulted in the identification of odour descriptors, such as “putrid” and “smelly feet”. These descriptors can be used later as a starting point by a trained panel to profile the various odours produced by means of Quantitative Descriptive Analysis® (QDA). It is therefore advantageous to more completely identify the volatile compounds (using gas chromatography-mass spectrometry), and recognize their associated odours (using sensory analysis), as produced by the genus *Chryseobacterium* to more accurately evaluate the spoilage potential of this genus in a product such as milk.

4.5. Acknowledgments

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1. Food spoilage

Spoilt food is food that tastes and smells off. This is due to the undesirable growth of microorganisms that produce volatile compounds during their metabolism, which the human nose and mouth detect (Forsythe, 2000). Food spoilage is also a complex process and excessive amounts of food are lost due to microbial spoilage even with modern day preservation techniques. The spoilage potential of a microorganism is the ability of a pure culture of that organism to produce the metabolites that are associated with the spoilage of a particular product. A careful combination of microbial, sensory and chemical analyses are required to determine the spoilage potential of a microorganism. Knowledge of the microorganism involved in spoilage and the metabolites associated with spoilage is needed to develop microbiological and chemical methods for evaluation of quality and shelf life and to prevent particular spoilage promoting compounds (Gram *et al.,* 2002).

Flavobacteria which can cause spoilage in foods, have been grouped in the new *Chryseobacterium* genus (Bernardet *et al.,* 1996). Due to this fairly new reclassification, literature with regard to food spoilage still refers to flavobacteria or CDC Group IIb organisms. *Chryseobacterium* species are commonly encountered in food commodities, such as milk, meat, poultry and fish (Jooste and Hugo, 1999). With regard to practical significance and spoilage potential, it has been found that these organisms can produce heat resistant proteolytic and lipolytic enzymes responsible for off-flavours and off-odours in pasteurised milk and cream, surface taint and apple odour in butter, “thinning” in creamed rice, bitterness due to the production of phospholipase C and reduction in cheddar cheese yield (Fox *et al*., 1976; García-López *et al*., 1999; Jooste and Hugo, 1999; Bernardet *et al*., 2002). In eggs they are responsible for the coagulation of the liquid egg and the production of faecal, fishy, sulphuric and ammonia odours (García-López *et al.,* 1999). In fish they

can be associated with the production of yellow slime (Bernardet *et al.,* 2006), fruity, pungent and musty spoilage odours (Engelbrecht *et al.,* 1996). In a processing unit of a poultry processing plant, they contributed to contamination levels on raw chicken meat (De Beer, 2005). *Chryseobacterium joostei* was the first member of the *Chryseobacterium* genus to be isolated specifically from a food commodity. Other current *Chryseobacterium* species that are often associated with food are *C. balustinum, C. gleum* , *C. indologenes* and *C. joostei* (Hugo and Jooste, 2003). The most recently published new species; *C. formosense* (Young *et al.,* 2005), *C. daecheongense* (Kim *et al.,* 2005), *C. taichungense* (Shen *et al.,* 2005), *C. soldanellicola* (Park *et al.,* 2006) and *C. taeanense* (Park *et al.,* 2006) were not isolated from food. *Chryseobacterium shigense* however was isolated from a lactic acid beverage (Shimomura *et al.,* 2005) and *C. vrystaatense* was isolated from raw chicken (De Beer *et al.,* 2005).

The role and significance of the genus *Chryseobacterium* in food and their proven and potential significance as food spoilage bacteria have not been studied in equal detail as the taxonomy and nomenclature of this genus and this has been the main reason for the present research project. It was regarded as necessary to obtain a better understanding of the characteristics of these organisms pertaining to their food spoilage potential. The purpose of this work was to add to the knowledge on this genus and in the process inform the food microbiologist and technologist of the practical implications of food contamination by this group of microorganisms.

5.2. Utilisation of BIOLOG carbon sources

BIOLOG GN2 MicroPlates were used to identify the carbon sources, utilised by seven *Chryseobacterium* species and two *Elizabethkingia* species. Carbon sources that produced clear positive reactions were assigned to chemical guilds of carbohydrates, carboxylic acids, polymers, amino acids and miscellaneous (Preston-Mafham *et al*., 2002). Forty three carbon sources made it possible to differentiate between the species of *Chryseobacterium* studied. Of these species, *C. indologenes* utilised the

most carbon sources (37) and *E. miricola* the least (11). The carbon sources utilised most often were the miscellaneous carbon substrates (49.1%).

The incomplete metabolism of amino acids can result in putrescence (Ayres *et al.* ,1980). It results in foul-smelling, sulphur-containing products, such as hydrogen-, methyl-, and ethyl sulphides and mercaptans, plus ammonia, biogenic amines (e.g. putrescine), indole, skatole, and fatty acids (Frazier, 1988). The microbial production of polysaccharides (e.g. dextrin) from various disaccharides, present in food, can form unpleasant slime in and on food, causing the food to be both unpalatable and unacceptable to the consumer. The microbial fermentation of sugars can lead to souring and butyric spoilage defects (Ayres *et al.,* 1980). The production of acids leads to sourness, while a-acids can also result in bitter defects in foods (Coultate, 1984).

The BIOLOG system can be used as an effective screening method for identifying the carbon sources, utilised by the *Chryseobacterium* species which could then be investigated further for their potential to produce food spoilage defects.

5.3. Metabolic activity tests

A battery of additional metabolic activity tests was included to supplement possible metabolites that were not included in the BIOLOG system and to add to the list of detailed phenotypical test results in literature that may be relevant to food spoilage and deterioration. Some of the *Chryseobacterium* and *Elizabethkingia* species were able to produce ammonia from urea, hydrolyse aesculin and starch, produce hydrogen sulphide and indole from tryptophan, reduce nitrate and nitrite and produce acid from ethanol. The release of large amounts of ammonia can increase the pH (Banwart, 1989) and contributes to the development of spoilage odours (Nychas *et al.,* 1998). The liberation of hydrogen sulphide from amino acids results in sulphide spoilage (Ayres *et al*., 1980) and is very toxic (Ebbing and Gammon, 1999). The production of indole from tryptophan results in disagreeable odours associated with putrefaction (Mountney and Gould, 1988). The reduction of nitrate and nitrite

can lead to the production of gaseous nitrogen dioxide (NO_2) (Schlegel, 1993), which can cause irritating odours and is very toxic (Ebbing and Gammon, 1999). The oxidation of ethanol to acetic acid can produce a souring acetic type of spoilage (Ayres *et al*., 1980).

The results of the phenotypic tests proved that the production of food spoilage defects such as putrescence and alkalinisation, which are usually associated with undesirable odours, can be the result of the hydrolysis and production of metabolites by *Chryseobacterium* species.

5.4. Ability to produce biogenic amines

The ability of *Chryseobacterium* and *Elizabethkingia* species to produce biogenic amines at different temperatures and sodium chloride concentrations with a modified Niven medium (Niven *et al.,* 1981) was investigated. The results reported in Chapter 3 indicated that temperatures at and above 25°C had an inhibitory effect on the production of biogenic amines such as histamine and tryptamine. Temperatures at and below 15°C had an inhibitory effect on the production of biogenic amines, such as spermine, cadaverine, tyramine and tryptamine. Tyramine however could be produced at 7°C by *C. balustinum* and *C. joostei* which is of great significance to the dairy and other food industries. This could result in refrigerated products being the cause of amine poisoning if they were contaminated with these amine-producing *Chryseobacterium* species. The hygienic production and handling of food products and maintenance of the cold chain throughout production and distribution are therefore important (Bester *et al*., 1993).

An increase in the salt concentration of the growth medium resulted in fewer strains being able to produce biogenic amines. Exceptions were *C. gleum, C. indoltheticum* and *E. miricola* which produced tyramine weakly in the presence of 4% NaCl. The production of histamine and tryptamine by the species were very sensitive to NaCl concentrations, because only *C. joostei* was able to produce histamine in the presence of a 1% NaCl, while *C. gleum, C. defluvii* and *C. indologenes* produced tryptamine at this salt concentration.

These results show that salt concentrations in excess of 4% would be needed to prevent amine production of *Chryseobacterium* species in food products.

In conclusion it can be stated that the formation of biogenic amines at different temperatures and sodium chloride concentrations by some of the *Chryseobacterium* species could result in amine poisoning in foods. This can be ascribed to the ability of these organisms to decarboxylate some of the precursors of biogenic amines, making them important spoilage bacteria in food products.

5.5. Growth and hydrolytic activities

Growth activities of *Chryseobacterium* and *Elizabethkingia* species determined in this study included growth at different temperatures, pH values, sodium chloride concentrations and culture media. The optimum growth temperatures of four *Chryseobacterium* species were investigated. Temperatures under 4°C and at 55°C, pH values under five and sodium chloride concentrations above 3% inhibited the growth of the species tested. Growth was observed on Cetrimide Agar, MacConkey Agar, Simmons Citrate Agar and Marine Agar. It was observed again that most *Chryseobacterium* species grow readily on commercially available organic media, usually making it unnecessary to use selective media and to add growth factors (Bernardet *et al*., 2006). Temperatures under and above 25°C were less suitable for growth.

It was shown that *Chryseobacterium* and *Elizabethkingia* species were capable of hydrolysing casein, gelatin, olive oil and tributyrin and of producing lecithinase and phospholipase C. The genus *Chryseobacterium* was found to be strongly proteolytic (Vandamme *et al.*, 1994). All the species hydrolysed casein, while 44% of the species hydrolysed gelatin. Studies by Bernardet *et al.* (2006), showed that *Chryseobacterium* species were able to produce a precipitate on 10% Egg-Yolk Agar, which indicates lecithinase activity. In this study it was found that only *C. gleum* showed good hydrolysis of lecithin. All

the species hydrolysed olive oil, while 78% hydrolysed tributyrin. 55% of the species produced phospholipase C on the Lecithin Agar.

In conclusion it may be stated that it is known that *Chryseobacterium* species have the potential to spoil milk. Such potential spoilage can be prevented by a decrease in temperature (below 4°C) and pH (below 5) and an increase in sodium chloride concentration (above 4%) which inhibits the growth of this genus. Optimum growth was found to occur at 25°C, and allowing growth at this temperature in foods could enhance spoilage of food products. The genus *Chryseobacterium* had the ability to produce spoilage defects such as proteolysis and lipolysis, which result in off-flavours and off-odours. The production of phospholipase C by these organisms can enhance lipolysis and rancidity (Mottar, 1989).

5.6. Preliminary determination of volatile compounds caused by *Chryseobacterium* species in milk

The preliminary determination of volatile compounds in milk samples inoculated with three *Chryseobacterium* spp. (*C. gleum*, *C. indologenes*, *C, joostei*) was done using headspace gas chromatography (GC). Primary alcohols and carboxylic acids were identified both in the fat-free and full cream milk samples. The primary alcohols were probably produced by reduction of the corresponding aldehydes and it is unlikely that primary alcohols contributed to the flavour of milk, because of their high flavour thresholds in milk (Shiratsuchi *et al.,* 1994). Carboxylic acids identified can be responsible for the production of unpleasant, penetrating and pungent odours.

It is therefore advantageous to use a technique such as gas chromatography to identify volatile compounds produced by the genus *Chryseobacterium*. This will help in evaluating the quality of the milk and the spoilage potential of this genus.

5.7. Sensory analysis of milk

Sensory evaluation was done on fat-free and full cream milk samples, each inoculated with *C. gleum, C. indologenes* and *C. joostei* respectively. This resulted in the identification of odour descriptors, such as “fruity” and “smelly feet”. These descriptors can be used later as a starting point by a trained panel to profile the various odours produced by means of Quantitative Descriptive Analysis® (QDA).

The fat free milk samples, inoculated with *C. gleum* and *C. joostei* were considered to be strongly spoiled, while samples containing *C. indologenes* were weakly spoiled. Full cream milk samples inoculated with *C. gleum* were considered to be strongly spoiled, while *C. joostei* and *C. indologenes* were both weakly spoiled. Fats are more difficult for microorganisms to attack than carbohydrates and proteins (Mountney and Gould, 1988). This could explain why the inoculated fat free milk samples were more extensively spoiled than the inoculated full cream milk samples. Lipolysis by flavobacteria is known to break down fatty acids, giving off-odours in milk products. Short-chain fatty acids may be responsible for the production of sharp and tangy flavours (Al-Shabibi *et al*., 1964) or fruity flavours (Cousin, 1982) or obnoxious rancid flavours (Banwart, 1989). It is possible that the formation of fatty acids were responsible for the “sour” and ‘’fruity’’ odours that were described. Fruity odours may arise from the degradation of amino acids (Engelbrecht *et al.,* 1996) or derived primarily from ethyl butyrate and ethyl hexanoate (Wilkes *et al.,* 2000). Valeric acid could be responsible for the “fruity” and “flowery” odours that were described by some of the panellists (Anonymous, 2006a). Metallic, unclean, bitter and putrid odours are produced by the breakdown of amino acids (Garbutt, 1997) and therefore it is possible that the breakdown of amino acids were responsible for the “putrid” odour that was described. It is possible that iso-valeric acid was responsible for the “smelly feet” odour that was identified since Jooste *et al*. (1986) reported in their study that iso-valeric was responsible for a “sweaty feet” odour in skim milk cultures of flavobacteria.

It is clear that the definition and assessment of spoilage by the genus *Chryseobacterium* relies on sensory evaluation to predict the sensory quality of milk.

5.8. Recommendations for future research

Further research needs to investigate:

a. the potential food spoilage defects produced by the BIOLOG carbon sources utilised by the genus *Chryseobacterium.*
b. the amount of biogenic amines produced by *Chryseobacterium* species in food products.
c. the identification of the other unknown volatile compounds produced in milk and milk products by *Chryseobacterium* species.

The identified odour descriptors of inoculated milk samples may be used as a starting point by a trained panel to profile the various odours produced by means of Quantitative Descriptive Analysis® (QDA).

The role and significance as well as the potential food spoilage defects of *Chryseobacterium* species should be investigated in more detail in more practical scenarios.

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SUMMARY

The food spoilage potential of the genus *Chryseobacterium* is the ability of a pure culture of this genus to produce the metabolites that are associated with the spoilage of a particular food product. A careful combination of microbial, sensory and chemical analyses are required to determine the food spoilage potential of the genus *Chryseobacterium.* The role and significance of the genus *Chryseobacterium* in food and their proven and potential significance as food spoilage bacteria have not been studied in equal detail as the taxonomy and nomenclature of this genus and this has been the main reason for the present research project. It was regarded as necessary to obtain a better understanding of the characteristics of these organisms pertaining to their food spoilage potential. The purpose of this work would be to add to the knowledge on this genus and in the process inform the food scientist of the practical implications of food contamination by this group of microorganisms.

The ability to utilise carbon sources by *Chryseobacterium* species tested in this study does not directly reflect the probability of food spoilage defects, but the BIOLOG system can be used as an effective screening method for identifying the carbon sources that could be investigated further for their potential to produce food spoilage defects. Phenotypic tests on *Chryseobacterium* species can be used as an alternative method to investigate the hydrolysis of food components and the production of metabolites, which could result in potential food spoilage defects such as putrefaction and alkalinisation, which are usually associated with disagreeable odours. Some *Chryseobacterium* species had the ability to decarboxylate some of the precursors of biogenic amines at different temperatures and in the presence of different sodium chloride concentrations. This results in the formation of biogenic amines which could cause amine poisoning and these organisms should consequently be regarded as significant spoilage organisms in food products.

In this study it was observed that *Chryseobacterium* species were able to grow at 4°C, at a pH of 5 to 10 and at sodium chloride concentrations of 1 to 3%. It is known that *Chryseobacterium* species have the potential to spoil milk. For these reasons a decrease in temperature (below 4°C), in pH (below 5) and an increase in sodium chloride concentration (above 4%) will inhibit the growth of this genus and have a preservative effect in products with these characteristics. Optimum growth was observed at 25°C, and it could be expected that spoilage defects in food products, kept at this temperature would develop most rapidly. The genus *Chryseobacterium* has the potential ability to produce spoilage defects due to proteolytic and lipolytic activity. Such activity could result in off-flavours and off-odours. Similarly the production of phospholipase C could enhance lipolysis and rancidity defects.

Chryseobacterium species were able to produce volatile compounds in milk. The primary alcohols produced were not likely to contribute to flavour, while the carboxylic acids can be responsible for the production of a variety of flavours (e.g. fruity). Sensory evaluation on inoculated milk samples resulted in the identification of odour descriptors, such as “putrid” and “smelly feet”. It is therefore advantageous to use a technique such as gas chromatography to identify volatile compounds produced by the genus *Chryseobacterium*. This will help in evaluating the spoilage potential of this genus in a product such as milk more accurately. The role and significance as well as the potential food spoilage defects of *Chryseobacterium* species should be investigated in more detail in more practical scenarios.

Key words: *Chryseobacterium*, food, spoilage, potential, role, significance, defects, off-flavours, off-odours.

OPSOMMING

Die genus *Chryseobacterium* se voedselbederf potensiaal is die vermoë van ´n rein kultuur (van hierdie genus) om die metaboliete te produseer wat geassosieer is met die bederf van ‘n spesifieke voedselproduk. ‘n Sorgvuldige kombinasie van mikrobiese, sensoriese en chemiese analises word vereis om die voedselbederf potensiaal van die genus *Chryseobacterium* te bepaal. Die rol en betekenis van die genus *Chryseobacterium,* asook hul bewese en potensiële betekenis as voedselbederf bakterieë, is nie so breedvoerig bestudeer soos die taksonomie en nomenklatuur van hierdie genus nie. Die hoofdoel van hierdie studie was dus om die potensiële voedselbederf eienskappe van die genus *Chryseobacterium* te bepaal. Dit was as nodig beskou om ‘n beter begrip van hulle eienskappe en voedselbederf potensiaal te verkry, asook om die voedselwetenskaplike oor die praktiese implikasies van voedsel kontaminasie deur hierdie groep organismes in te lig en om die kennis op hierdie aspekte van lede van die *Chryseobacterium* genus te verbreed.

Die vermoë van *Chryseobacterium* spesies om koolstofbronne te gebruik, soos getoets in hierdie studie, weergee nie direk die waarskynlikheid van voedselbederf defekte nie, maar die BIOLOG sisteem kan as ‘n effektiewe siftingsmetode gebruik word om die koolstofbronne te identifiseer wat verder bestudeer kan word vir hulle potensiaal om voedselbederf defekte te produseer. Fenotipiese toetse op *Chryseobacterium* spesies kan as ‘n alternatiewe metode gebruik word om die hidrolise van voedselkomponente en die produksie van metaboliete te ondersoek wat potensiële voedselbederf gebreke soos verroting en alkalinisasie tot gevolg kan hê. Hierdie gebreke word gewoonlik met onaangename reuke geassosieer. Sommige *Chryseobacterium* spesies het die vermoë om sommige van die voorlopers van biogeniese amines te dekarboksileer by verskillende temperature en in die teenwoordigheid van verskillende natrium chloried konsentrasies. Dit het die vorming van biogeniese amiene, wat amien vergiftiging kan veroorsaak, tot gevolg. Hierdie organismes moet gevolglik as betekenisvolle bederf organismes in voedselprodukte beskou word.

Chryseobacterium spesies was in staat om by 4°C, by ‘n pH van 5 tot 10 en by ‘n natrium chloried konsentrasie van 1 to 3% te groei. Dit is bekend dat *Chryseobacterium* spesies die potensiaal het om melk te kan bederf. Daarom sal ‘n afname in temperatuur (onder 4°C), in pH (onder 5) en ‘n toename in natrium chloried konsentrasie (bo 4%) die groei van hierdie genus inhibeer en het ‘n preserverende werking in produkte met hierdie eienskappe. Optimum groei het by 25°C plaasgevind en dit kan verwag word dat bederf defekte vinnig sal ontwikkel in voedselprodukte wat by hierdie temperatuur gehou word. Die genus *Chryseobacterium* het die potensiaal om bederf defekte te veroorsaak as gevolg van proteolitiese en lipolitiese aktiwiteit. Sulke aktiwiteite kan afgeure en –reuke tot gevolg hê, terwyl die produksie van fosfolipase C lipolise en galsterigheid kan veroorsaak.

Chryseobacterium spesies was in staat om vlugtige komponente in melk te produseer. Die geproduseerde primêre alkohole het waarskynlik nie bygedra tot geure nie, terwyl die karboksielsure verantwoordelik kan wees vir die produksie van verskillende geure (bv. vrugtig). Sensoriese evaluering op geïnokuleerde melkmonsters het die identifikasie van reukbeskrywers soos “vrot” en “stink voete” tot gevolg gehad. Dit is dus voordelig om ‘n tegniek soos gas chromatografie te gebruik om vlugtige komponente te identifiseer wat deur die genus *Chryseobacterium* geproduseer word en om die bederf potensiaal van hierdie genus in ‘n produk soos melk meer akkuraat te evalueer. Die rol en betekenis asook die potensiële voedselbederf defekte van *Chryseobacterium* spesies moet in meer besonderheid in meer praktiese scenarios bestudeer word.

Trefwoorde: *Chryseobacterium*, voedselbederf, potensiaal, rol, betekenis, defekte, afgeure, afreuke.