Growth and Spoilage Characteristics of *Chryseobacterium* Species in Milk

by

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DECLARATION

I declare that the dissertation hereby submitted by me for the M.Sc. 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. Bekker

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
µm	Micrometer
μ_{max}	Maxium growth rate
ANOVA	Analysis of variance
APC	Aerobic plate count
ATCC	American Type Culture Collection, Rockville, Maryland
a_w	Water activity
C.	*Chryseobacterium*
ca.	Approximately
cfu	Colony forming units
cm	Centimeter
DMC	Direct microscopic count
e.g.	For example
et al.	(*et alii*) and others
etc.	Et cetera
F.	*Flavobacterium*
FFAs	Free fatty acids
g	Gram
GC	Gas chromatography
h	Hour(s)
h^{-1}	Per hour
H_2S	Hydrogen sulphide
H_2SO_4	Sulphuric acid
LMG	Laboratory of Microbiology, University of Ghent, Belgium
mg	Milligram
ml	Millilitre
min	Minutes
min^{-1}	Per minute
mm	Millimetre
MS	Mass spectrometry
NaCl	Sodium chloride
nm	Nanometer

OD	Optical density
Ps.	*Pseudomonas*
ppm	Parts per million
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SPC	Standard plate count
SPME	Solid-phase microextraction
Spp.	Species
^{T}	Type strain
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic acid
UFSBC	University of the Free State Bacterial Culture Collection
UHT	Utlra high temperature

CHAPTER 1

INTRODUCTION

Milk is a food product widely consumed by humans and can be considered as the most complete single food available (Porter, 1975). Raw cow's milk typically consists of water (87%), fat (ca. 3.8%), protein (3.3%), lactose (4.7%), calcium (0.12%) and non-fatty solids (8.7%) (Porter, 1975). Milk is also an excellent growth medium for many microorganisms since it is rich in nutrients needed for growth, has a high moisture content and a neutral pH (IFT, 2001). Defects in milk can arise from four sources namely the growth of psychrotolerant bacteria prior to pasteurization, activity of thermo-resistant enzymes, growth of thermo-resistant psychrotolerant bacteria or post-pasteurization contamination (Champagne *et al*., 1994).

Psychrotolerant bacteria are those bacteria that have the ability to grow at temperatures below 7 °C, irrespective of their optimal growth temperature (Champagne *et al*., 1994). They have become an important part of the microbial population of raw milk since the introduction of bulk refrigerated storage (Champagne *et al*., 1994). The growth of psychrotolerant bacteria is primarily responsible for limiting the keeping quality of milk and dairy products held at refrigerated temperatures (Cousin, 1982). The main psychrotolerant bacteria present in milk are Gram-negative rods with *Pseudomonas* spp. comprising at least 50% of the genera (Reinheimer *et al*., 1990). Other genera that are often present in milk include *Achromobacter*, *Aeromonas*, *Alcaligenes*, *Chromobacterium*, and *Flavobacterium* (Mikolajcik, 1979).

Flavobacterial species have been reported to cause spoilage of a variety of food products including fish, meat, poultry and milk and dairy products (Bernardet *et al*., 2006). Many *Flavobacterium* species that were associated with food spoilage have been reclassified as *Chryseobacterium* (Bernardet *et al*., 2006). Some *Chryseobacterium* species associated with food products include *C. bovis* (Hantsis-Zacharov *et al.*, 2008a), *C. haifense* (Hantsis-Zacharov & Halpern, 2007a), *C. joostei* (Hugo *et al*., 2003) and *C. oranimense* (Hantsis-Zacharov *et al*., 2008b) which were

all isolated from raw milk. *Flavobacterium* has been reported to cause bitter and fruity flavours in pasteurized, refrigerated milk as well as discolouration and slime production in cottage cheese (Banwart, 1989). Chapter 2 of this dissertation will review literature on the spoilage potential of *Chryseobacterium* species and discuss the growth kinetics of microorganisms to determine how different growth patterns can affect the spoilage potential of the organism.

Chapter 3 focuses on the growth kinetics and protease production by *Chryseobacterium* species in milk. Lipolytic and proteolytic enzymes produced by psychrotolerant bacteria in milk are the main cause of spoilage of the product (Sørhaug & Stepaniak, 1997). The major cause of bitterness in milk is the formation of bitter peptides due to the action of proteolytic enzymes (Springett, 1996). Proteolysis may also lead to the production of astringent off-flavours. Growth of microorganisms in food is influenced by intrinsic and extrinsic factors (IFT, 2001). To select the proper storage conditions for a food product is crucial in understanding the relationship between time, temperature and other intrinsic and extrinsic factors. Temperature has a dramatic effect on the generation time and the lag period of an organism present in food (IFT, 2001). Uncontrolled growth of bacteria in milk can affect the flavour and appearance as well as the safety of the product (IFT, 2001).

The effect of *C. joostei* on lipids in milk as well as its effect on the sensory properties of the milk was studied in Chapter 4. Lipolytic activity in milk leads to a preferential release of medium- and short-chain fatty acids from milk lipid triglycerides which results in rancid off-flavours (Bengtsson-Olivecrona & Olivecrona, 1991). Both proteolytic and lipolytic activity has been reported for *Chryseobacterium* species found in food products (Vandamme *et al*., 1994; Hantsis-Zacharov *et al*., 2008a). Earlier it had been found that "*Flavobacterium"* together with *Pseudomonas* were amongst the most lipolytic species isolated from raw milk (Muir *et al*., 1979).

Lipid oxidation in food can cause quality deterioration and sensory changes in the products. At the nutritional level, the oxidation of fatty constituents is the major chemical factor resulting in the loss of food wholesomeness due to deterioration of flavour and aroma, as well as in the compromising of nutritional and food safety properties (Kanner & Rosenthal, 1992). At the biological level, the oxidation of lipids

means damage to lipid containing membranes, hormones and vitamins, which are vital components for the normal cell activity. It was found that diets based on food containing oxidised lipids have had far-reaching effects that may even have a bearing on carcinogenesis, premature aging and other diseases (Kanner & Rosenthal, 1992).

Milk is usually characterized by a pleasant and slightly sweet taste, but is very susceptible to flavour defects from a variety of sources including absorption of flavours, bacterial contamination and chemical reactions such as lipolysis and proteolysis (Lemieux & Simard, 1991). Off-flavours in milk caused by microorganisms have been described as acid, bitter, fruity, malty, putrid and unclean (Shipe *et al*., 1978). The volatile compounds that may be responsible for the occurrence of these flavours can be identified by means of gas chromatography–mass spectrometry analysis (Hites, 1997).

Purpose and objectives of this study

Purpose

The main focus of this study was to study the growth patterns and enzyme activity of *Chryseobacterium* species to determine their spoilage potential over a range of circumstances, and compare it to that of *Pseudomonas fluorescens* as a control organism known to be a major food spoilage organism. The growth kinetics of *Chryseobacterium* species have not been studied in much detail before the taxonomy of these organisms had been placed on its present more stable footing. Determination of the growth kinetics of these organisms will provide valuable information on the spoilage potential and patterns of members of this genus, as well as the effect of *Chryseobacterium* on the quality of food products. Understanding the growth patterns of specific *Chryseobacterium* species can also help to prevent and control spoilage of food due to these organisms. Studies on the enzyme production, by these organisms, especially relating to proteases and lipases are also limited. The results obtained from this study will also broaden the current knowledge available on the role of *Chryseobacterium* in the microbial ecology of food spoilage.

Objectives

i. To determine the growth kinetics, including growth rate and cardinal temperatures, in nutrient broth using optical density measurements of *Chryseobacterium joostei*, *C. bovis* and *Ps. fluorescens* over a temperature range of 4 to 50 °C.

ii. To determine the proteolytic activity of *C. joostei*, *C. bovis* and *Ps. fluorescens* in commercial fat-free and full cream UHT milk at temperatures ranging between 4 and 50 °C.

iii. To determine the levels of lipolysis and lipid oxidation caused by *C.joostei* and *Ps. fluorescence* in fat-free and full cream UHT milk at 4 and 25 °C.

iv. To determine the effect of the growth of *C. joostei* and *Ps. fluorescens* on the sensory characteristics of milk and to study the volatile compounds produced by these organisms using headspace gas chromatography-mass spectrometry (SPME-GC/MS).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Chryseobacterium belongs to the family *Flavobacteriaceae* (Bernardet *et al*., 2006). The name *Chryseobacterium* was first proposed by Vandamme *et al.* (1994) to include six species formerly classified as *Flavobacterium*. The following species were included in the genus: *Chryseobacterium balustinum, C. gleum, C. indologenes, C. indolotheticum, C. meningosepticum* and *C. scophthalmum. Chryseobacterium gleum* was selected as the type species since this species was well-characterized and both its genotypic and phenotypic structure have been studied in detail. New species have since been described and the genus currently consists of 52 species with validly published names as well as *C. proteolyticum*, that has not been validly published (Euzéby, 2010). *Chryseobacterium meningosepticum* and *C. miricola* were reclassified in 2005 as *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* respectively (Kim *et al*., 2005a).

Flavobacterium species have been isolated from a variety of clinical and environmental sources (Jooste & Hugo, 1999). Habitats include: soil; freshwater and marine environments; hospitals; diseased mammals, amphibians, reptiles and fish; diseased molluscs, crustaceans, and sea urchins; digestive tract of insects; vacuoles or cytoplasm of amoebae; diseased plants; food and dairy products and their production environments (Jooste & Hugo, 1999; Bernardet & Nakagawa, 2006). Spoilage defects due to flavobacteria have been reported in various products including butter (Wolochow *et al*., 1942; Jooste *et al*., 1986a), creamed rice (Everton *et al*., 1968) and canned vegetables (Bean & Everton, 1969). Other food sources that have been reported to contain *Chryseobacterium spp.* include fish, meat and meat products, poultry and dairy products (Bernardet *et al.*, 2006).

Many of the *Flavobacterium* species that were earlier implicated and associated with the spoilage of food have since been transferred to other genera in the family

Flavobacteriaceae (Hugo & Jooste, 2003). Due to this reclassification and in some cases erroneous classification of the flavobacteria, the information about the incidence and role of flavobacteria in food deterioration is limited. Currently, only 10 of the 76 *Flavobacteriaceae* genera are associated with food, namely *Bergeyella, Chryseobacterium, Empedobacter, Flagellimonas, Flavobacterium, Myroides, Salegentibacter, Tenacibaculum, Vitellibacter* and *Weeksella* (Hugo & Jooste, personal communication). In order to understand the significance of a specific bacterial type in food, it is important to understand its growth patterns. This information in turn, will help in designing methods to control its growth, minimizing possible food spoilage. Information on growth patterns by *Chryseobacterium* in a food product is limited to a M.Sc. study by Fischer (1987) performed at the University of the Free State under supervision of Prof. P.J. Jooste, the founder of flavobacterial research in South Africa.

Bacterial growth can be defined as an increase in the number of cells in a population (Madigan & Martinko, 2006). An increase in the number of cells occurs when one cell divides to become two, two cells divide to become four, etc. During this division all the structural components of the cells are doubled. The time required for a microbial population to double is known as the generation time or doubling time. Exponential growth occurs when the number of cells doubles during a regular time interval (Madigan & Martinko, 2006).

The growth cycle of a microorganism consists of different phases and can be plotted on a graph known as a growth curve (Madigan & Martinko, 2006). The phases include a lag phase, exponential phase, stationary phase and death phase. The stationary phase is the time the cells take to adapt to the new environment before growth occurs. During the exponential phase, rapid cell growth takes place. Growth of the cells is limited by an essential nutrient that is depleted or by waste products of the organisms that accumulate in the media to inhibit growth. When this happens cells enter the stationary phase. During this stage there is no net increase or decrease in the cell number. The death phase follows when cells start to die. Cell death also occurs exponentially, but the rate is much slower than that during exponential growth.

The aims of this literature review will i) be to discuss the genus *Chryseobacterium* and the role of species in this genus in food spoilage; and ii) to discuss the growth kinetics of microorganisms to determine how different growth patterns can affect the spoilage potential of the organism.

2.2 The genus *Chryseobacterium*

2.2.1 History

The genus *Chryseobacterium* was formed to include species previously classified as *Flavobacterium* (Vandamme *et al*., 1994). One of the reasons for this reclassification was that the original description of *Flavobacterium* relied on parameters that are now known to have little importance in taxonomic studies. The first description of *Flavobacterium* included 46 yellow pigmented mainly Gram-negative, rod shaped, non-endospore forming, chemoorganotrophic species (Bergey *et al.,* 1923). In 1939 the polar flagellates were removed from the genus in the fifth edition of *Bergey's Manual of Determinative Bacteriology* (Bergey *et al*., 1939). The genus was further restricted to only Gram-negative species in the seventh edition (Weeks & Breed, 1957). In 1984 *Flavobacterium* was restricted to non-motile and non-gliding species and described as Gram-negative, yellow, non-motile, aerobic rods usually growing at 5 – 30^{o}C (Holmes *et al*., 1984a). Restriction of the genus continued after it was recognized that the type species, *F. aquatile*, did not represent the genus (Holmes, 1993). *Flavobacterium aquatile* was subsequently set aside in Holmes's taxonomic review in the second edition of *The Prokaryotes* (Holmes, 1992) but after a decision by the Judicial Commission of the International Committee of Systematic Bacteriology *F. aquatile* was required to remain the type species (Bernardet *et al*., 1996) and the other bacterial species of the genus *Flavobacterium* had to be relocated to other or new genera.

Flavobacterium species were divided into four natural groups by Holmes (1992) in the second edition of *The Prokaryotes*. The first group (group A) included [*Flavobacterium*] *balustinum*, [*F.*] *breve*, [*F.*] *gleum*, [*F.*] *indologenes*, [*F.*] *indoltheticum* and [*F.*] *meningosepticum*. Phylogenetic studies done by Vandamme

et al. (1994) indicated that these species formed a tight cluster, and therefore *Chryseobacterium* was proposed as a new generic epithet for these organisms. The fish pathogen [*F.*] *scophthalmum* (Mudarris *et al*., 1994) was also renamed since it belonged to the same rRNA cluster (Vandamme *et al*., 1994). The new genus included *Chryseobacterium* [*F.*] *balustinum*, *C.* [*F.*] *gleum*, *C.* [*F.*] *indologenes*, *C.* [*F.*] *indoltheticum* and *C.* [*F.*] *meningosepticum*. Although *Chryseobacterium balustinum* and *C. indoltheticum* were the oldest species, they were not chosen as type species for the genus since they had not been well-characterized and were at that stage represented by only one strain (Vandamme *et al*., 1994). The well-characterized, clinically important *C. meningosepticum* was similarly not chosen as type species since it was the most aberrant member of the genus. It was therefore decided to propose *C. gleum* as the type species because it was well-characterized and both its genotypic and phenotypic structures had been studied in detail by Holmes *et al*. (1984b).

After a study done by Kim *et al*. (2005a), *Chryseobacterium meningosepticum* and *C. miricola* were renamed *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* respectively.

2.2.2 Current taxonomy

Since the first description of *Chryseobacterium* by Vandamme, *et al.* (1994), a number of new species have been added. The genus currently contains 61 species that have been validly published (Euzéby, 2011). *Chryseobacterium proteolyticum* (Yamaguchi & Yokoe, 2000) also forms part of the genus although the name has not yet been validly published. The species and their source of isolation are listed in Table 2.1.

Table 2.1 Species of the genus *Chryseobacterium* and their source of isolation (Euzéby, 2011).

Species	Source	Reference(s)
C. antarcticum	Soil from the Antarctic	Yi *et al*., 2005; Kämpfer *et al*., 2009a
C. anthropi	Human clinical specimens	Kämpfer *et al.,* 2009b
C. aquaticum	Water reservoir	Kim *et al*., 2008
C. aquifrigidense	Water-cooling system in an oxygen-producing plant	Park *et al*., 2008
C. arothri	Pufferfish *Arothron hispidus*	Campbell *et al*., 2008
C. arthrosphaerae	Faeces of pill millipede (*Arthrosphaera magna* Attems)	Kämpfer *et al*., 2010a
C. balustinum	Heart blood of fresh water fish (dace, *Leuciscus leuciscus*)	Harrison, 1929; Vandamme *et al*., 1994
C. bovis	Raw cow's milk	Hantsis-Zacharov *et al*., 2008a
C. caeni	Bioreactor sludge	Quan *et al*., 2007
C. chaponense	Farmed Atlantic salmon (*Salmo salar*)	Kämpfer *et al*., 2011
C. culicis	Midgut of a mosquito	Kämpfer *et al*., 2010b
C. daecheongense	Lake Daecheong sediment	Kim *et al.,* 2005b
C. daeguense	Wastewater of a textile dye works	Yoon *et al*., 2007
C. defluvii	Activated sludge	Kämpfer *et al.,* 2003
C. elymi	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011
C. flavum	Polluted soil	Zhou *et al*., 2007
C. formosense	Rhizosphere of lettuce	Young *et al.,* 2005

C. gambrini	Beer-bottling plant	Hertzog *et al*., 2008
C. ginsenosidimutans	Soil of a *Rhusvernicifera*-cultivated field	Im *et al*., 2011
C. gleum	Human vaginal swab	Holmes *et al.,* 1984b; Vandamme *et al*., 1994
C. greenlandense	Deep Greenland ice core	Loveland-Curtze *et al*., 2010
C. gregarium	Decaying plant material	Behrendt *et al*., 2008
C. hagamense	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011
C. haifense	Raw milk	Hantsis-Zacharov and Halpern 2007a
C. hispanicum	Drinking water distribution system	Gallego *et al*., 2006
C. hominis	Clinical isolate	Vaneechoutte *et al*., 2007
C. humi	Industrially contaminated sediments	Pires *et al*., 2010
C. hungaricum	Hydrocarbon-contaminated soil	Szoboszlay *et al*., 2008
C. indologenes	Human trachea at autopsy	Yabuuchi *et al.,* 1983; Vandamme *et al*., 1994
C. indoltheticum	Marine mud	Campbell and Williams, 1951; Bernadette *et al*., 1994
C. jejuense	Soil	Weon *et al*., 2008
C. jeonii	Moss sample from the Antarctic	Yi *et al*., 2005; Kämpfer *et al*., 2009a
C. joostei	Raw cow's milk	Hugo *et al.,* 2003
C. koreense	Human clinical specimens	Kim *et al*., 2004; Kämpfer *et al.,* 2009b
C. lathyri	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011
C. luteum	Phyllosphere of grasses	Behrendt *et al*., 2007
C. marinum	Antarctic seawater	Lee *et al*., 2007 Kämpfer *et al*., 2009a

C. molle	Beer-bottling plant	Hertzog *et al*., 2008
C. oranimense	Raw cow's milk	Hantsis-Zacharov *et al*., 2008b
C. pallidum	Beer-bottling plant	Hertzog *et al*., 2008
C. palustree	Industrially contaminated sediment	Pires *et al*., 2010
C. piperi	Freshwater creek	Strahan *et al*., 2011
C. piscicola	Diseased salmonid fish	Ilardi *et al*., 2009
C. piscium	Fish	De Beer *et al*., 2006
"C. proteolyticum"	Soil, rice field	Yamaguchi and Yokoe, 2000
C. rhizosphaerae	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011
C. scophthalmum	Gills of diseased turbot (*Scophthalmus maximus*)	Mudarris *et al.,* 1994; Vandamme *et al*., 1994
C. shigense	Lactic acid beverage	Shimomura *et al*., 2005
C. soldanellicola	Roots of sand-dune plants	Park *et al*., 2006
C. soli	Soil samples	Weon *et al*., 2008
C. solincola	Soil	Benmalek *et al*., 2010
C. taeanense	Roots of sand-dune plants	Park *et al*., 2006
C. taichungense	Contaminated soil	Shen *et al.,* 2005
C. taiwanense	Soil	Tai *et al*., 2006
C. treverense	Human clinical source	Yassin *et al*., 2010
C. ureilyticum	Beer-bottling plant	Hertzog *et al*., 2008
C. vrystaatense	Chicken-processing plant	De Beer *et al*., 2005
C. wanjuense	Greenhouse soil	Weon *et al*., 2006
C. xinjiangense	Alpine permafrost	Zhao *et al*., 2011
C. yonginense	Mesotrophic artificial lake	Joung & Joh, 2011

2.3 Description of *Chryseobacterium*

Description as given by Vandamme *et al*. (1994):

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).

The cells of this organism are Gram-negative, non-motile, non-spore-forming rods with parallel sides and rounded ends, typically being 0.5 μm wide and 1 to 3 μm long. Intracellular granules of poly-β-hydroxybutyrate are absent. The organisms are aerobic and chemoorganotrophic. All strains grow at 30 °C while most strains grow at 37 °C. Growth on solid media is typically pigmented (yellow to orange), but non-pigmented strains do occur. Colonies are translucent (occasionally opaque), circular, convex or low convex, smooth, and shiny, with entire edges. In terms of enzyme activity, all species are positive for catalase, oxidase, and phosphatase and strong proteolytic activity occurs. Several carbohydrates, including glycerol and trehalose, are oxidized. Esculin is hydrolyzed while agar is not digested. Chryseobacteria are resistant to a wide range of antimicrobial agents.

Branched-chain fatty acids (i.e., 15:O iso, iso 17:lω9c, 17:O iso 3OH, and summed feature 4 [15:0 iso 2OH or 16:1ω7t or both]) are predominant (Segers *et al*., 1993) and sphingophospholipids are absent. Menaquinone 6 is the only respiratory quinone. Homospermidine and 2-hydroxyputrescine are the major polyamines in *Chryseobacterium indologenes,* whereas putrescine and agmatine are minor components (Hamana & Matsuzaki, 1991).

The type species of the genus is *Chryseobacterium gleum* comb. nov. The DNA base compositions of the species range from 33 to 38 mol% guanine plus cytosine (G+C). *Chryseobacterium* species are widely distributed in soil, water, and clinical sources.

2.4 Ecology of *Chryseobacterium*

As can be seen in Table 2.1, chryseobacteria are present in a wide range of habitats ranging from industrial to natural, clinical and food environments. The significance of *Chryseobacterium* in these environments as far as has been determined to date, will be discussed in the following sections.

2.4.1 Industrial and natural environments

During a study done on the presence of heterotrophic bacteria in drinking water in South Africa, Pavlov *et al*. (2004) reported that *Chryseobacterium* species were amongst the most frequently isolated genera. This study also showed that these *Chryseobacterium* species, produced more than two extracellular enzymes that were associated with pathogenesis toward humans and was consequently found to be one of the most virulent species of this genus isolated from either treated or untreated drinking water in South Africa. Strains of this genus have also been isolated from a groundwater supply in Germany (Ultee *et al*., 2004).

A *Chryseobacterium* strain was isolated from a drain outlet of a sink with an attached waste disposal unit (McBain *et al*., 2003). Two strains of *Chryseobacterium* were also isolated from the slime of paper mills in North America (Oppong *et al*., 2003). The growth and slime production of microorganisms in paper mills can have a negative effect on the papermaking process and quality of the final product.

A *Chryseobacterium indologenes* strain isolated from soil samples in Spain had the ability to degrade various toxic compounds including ferulic acid, 5-hydroxymethylfurfural and furfural (López *et al*., 2003). Another study on soil samples from Indonesia showed similar results with *C. indologenes* being able to degrade toxic compounds such as aniline and 4-chloroaniline (Radianingtyas *et al*., 2003). *Chryseobacterium* were also found among carbohydrate degrading bacteria isolated from biotopes of sub-tropical regions within the Caribbean belt (Rosado & Govind, 2003) and in marigold flowers as part of the normal microbiota (Luis *et al*., 2004).

Isolates from the gut of the American cockroach (*Periplaneta americana*) were shown to phenotypically resemble *C. indologenes* (Dugas *et al*., 2001). It was found that this organism is a permanent symbiont of the mid- and hindgut of the American cockroach. *Chryseobacterium* sp. have also been isolated from other insects including the larvae of the lepidopteran *Acentria ephemerella* (Walenciak *et al*., 2002) and the biting mosquito *Culicoides variipennis* (Campbell *et al*., 2004).

2.4.2 Clinical isolates

Various *Chryseobacterium* strains have been isolated from diseased animals. *Chryseobacterium indologenes* has been found in diseased frogs (Olson *et al.*, 1992) while *C. balustinum* and *C. scophthalmum* have been reported as pathogens of fish (Bernardet & Nakagawa, 2006).

Chryseobacterium spp. are frequently isolated from the hospital environment since water is their natural habitat. The bacteria come in contact with patients through indwelling devices like breathing tubes and catheters, and they are the most frequently isolated flavobacteria in clinical laboratories (Holmes & Owen, 1981). They are however not part of the normal microbiota of humans.

Chryseobacteria can cause infection of the respiratory tract and urinary tract in immunocompromised patients and are resistant to a wide range of antimicrobial agents (Bernardet & Nakagawa, 2006; Bernardet *et al*., 2011). Species that have been reported to cause infection include *C. indologenes* and *C. gleum* (Bernardet *et al*., 2005).

2.5 *Chryseobacterium* and food spoilage

Food spoilage can be considered as any change in a product that makes it unacceptable for human consumption (Hayes, 1985). Although spoiled food is not necessarily unsafe to eat, it is generally regarded as unpalatable by consumers and will not be purchased (Madigan & Martinko, 2006). Spoilage can be due to physical damage (caused by bruising, pressure, freezing, drying and radiation), chemical

damage (oxidation and colour changes), insect damage or the appearance of off-flavours and off-odours from growth and metabolism of microorganisms in the product (Huis in't Veld, 1996; Gram *et al*, 2002). Gram *et al*. (2002) define the spoilage potential of a microorganism as the ability of a pure culture to produce the metabolites that are associated with the spoilage of a particular product.

Chryseobacterium species have been found to cause spoilage in a variety of food products. Since *Chryseobacterium* was previously classified as *Flavobacterium*, earlier literature generally refer to these spoilage organisms as flavobacteria, *Flavobacterium* or CDC group IIb organisms. Reference to these organisms in fact included many of the *Chryseobacterium* species that are currently known (Bernardet *et al*, 2006). The significance of *Chryseobacterium* in different food products will be discussed in more detail in the following section.

2.5.1 Fish

Fish normally have a higher initial bacterial cell count than mammalian meat (Molin, 2000). The microbial population of fish is present on the skin (10^3 – 10^5 cfu/cm^2), gills (10^3 – 10^4 cfu/g) and the contents of the gut (up to 10^9 cfu/g). Fish meat may also contain up to 10^5 cfu/cm^2 after filleting.

Chryseobacterium balustinum (Harrison, 1929; Vandamme *et al*., 1994), *C. piscium* (De Beer *et al*., 2006), *C. piscicola* (Ilardi *et al*., 2009) as well as *C. scophthalmum* (Mudarris *et al.,* 1994; Vandamme *et al*., 1994) were first isolated from diseased fish. *Chryseobacterium balustinum* produced a yellowish slime on the skin of freshly caught halibut (*Hippoglossus hippoglossus*) and was therefore considered a spoilage agent rather than a pathogen (Austin & Austin, 1999). *Chryseobacterium balustinum* was isolated from the skin and muscle of farmed and wild fish again in 2000 (González *et al*., 2000), but it was not regarded as an important spoilage agent because of its prevalence of less than 1% compared to other bacterial isolates.

Some of the bacterial isolates from fresh and ice stored Mediterranean sardines (*Sardina pilchardus*) were identified as flavobacteria (Gennari & Cozzolino, 1989). The isolates could not be identified as any known flavobacterial species, but four

strains did fit in group A of Holmes, of which most species are now included in the genus *Chryseobacterium*. Gennari & Cozzolino (1989) also found that the numbers of flavobacteria decreased during storage on ice. They found that the organisms showed proteolytic activity and that they may be involved in the spoilage of fish during the early stages of cold storage.

Chryseobacterium balustinum, C. gleum and *C. indologenes* strains isolated from Cape marine fish in South Africa showed diverse proteolytic activity as well as H_2S production (Engelbrecht *et al*., 1996). Off-odours that were observed from these organisms included pungent, stale and fruity odours. It was conjectured that these organisms were introduced during the processing of the fish.

2.5.2 Meat

The muscle tissue of live, healthy animals is generally sterile (Banwart, 1989). Bacteria can, however, start to grow and spread through the meat after slaughtering. Spoilage of meat can be noted by the production of off-odours and slime due to the growth of aerobic spoilage organisms on the surface of the meat, bone taint due to the growth of anaerobic or facultative bacteria and discolouration due to alteration of myoglobin. Off-odours generally occur at bacterial counts of 10^7 cfu/cm^2 and slime production becomes visible when the counts reach 10^8 cfu/cm^2 (Molin, 2000).

In unprocessed, refrigerated meat that has undergone spoilage, *Flavobacterium* spp. have been reported to cause off-odours, slime formation as well as discolouration (Banwart, 1989). Olofsson *et al*. (2007) did a study on the bacterial population of refrigerated beef. They used 16S rRNA sequencing to identify the isolates. They found that the microbial flora of freshly cut meat was dominated by *Bacillus*, followed by *Chryseobacterium* and *Staphylococcus*. After the meat was stored at 4 °C, *Pseudomonas* spp. became the dominating bacterial type. Bernardet *et al*. (2005) found that *Chryseobacterium gleum* and *Chryseobacterium indologenes* often form part of the initial bacterial flora of raw meat.

2.5.3 Poultry

Poultry that enter the processing environment can be heavily contaminated with a diverse native microbial population. Microorganisms are present on the feathers, skin, feet and alimentary tract (Kotula & Pandya, 1995). The meat of the animal's body is sterile before slaughtering (Forsythe, 2000). During processing the different steps affect the microbiological status of the chickens. The bacterial count can either be increased or decreased by these steps (Geornaras & von Holy, 2000). Scalding, defeathering, evisceration and chilling generally reduce the microbial count. However, the counts can be increased via cross contamination between carcasses, processing water and equipment (Thomas & McMeekin, 1980; Fries & Graw, 1999). Flavobacteria spoilage occurs more often in poultry than in fresh red meat (Nychas & Drosinos, 1999).

During spoilage of poultry meat, off-odours appear when the bacterial population reaches a level of between 10^6 and 10^8 cfu/cm^2 (Banwart, 1989). Slime formation appears shortly after off-odours are noted. The flavour score of the food decreases as the microbial load increases.

A new species, *Chryseobacterium vrystaatense*, was described after isolation from a chicken processing plant in the Free State (South Africa) by de Beer *et al*. (2005). The latter authors also found that *Chryseobacterium* was present throughout the processing unit. It was suspected that environmental sources, like dust, most likely contributed to the contamination levels of psychotrophic, yellow-pigmented colonies and especially *Chryseobacterium*, in raw broiler carcasses. During a study by Thomas and McMeekin (1980), the *Flavobacterium/Cytophaga* group of bacteria made up 11% and 8% of the microorganisms isolated from the breast- and leg skin of immersion-chilled carcasses respectively, while Mai and Connor (2001) reported an incidence of 17% and 16% of *Pseudomonas* and flavobacteria respectively from chicken carcasses. *Flavobacterium* spp. were also reported to be present in the air and water of two poultry processing plants in Germany (Fries & Graw, 1999).

2.5.4 Dairy products

Milk is subjected to contamination during milking and processing via equipment used for handling, transporting, storage and processing (Banwart, 1989). Microbial spoilage in milk can cause a variety of defects including: off-flavours, lipolysis with development of rancidity, gas production, fermentation of lactic acid with souring as well as discolouration. According to Banwart (1989), *Flavobacterium* has been reported to cause bitter and fruity flavours in pasteurized, refrigerated milk as well as discolouration and slime production in cottage cheese.

Jooste (1985) was one of the first researchers to isolate *Flavobacterium* from milk and butter. *Flavobacterium* strains were isolated from milk during a study by Jooste *et al*. (1986b). Jooste *et al*. (1986b) also found that the practical importance of flavobacteria in dairy 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. *Chryseobacterium gleum* and *Chryseobacterium indologenes*, CDC group IIb as well as *C. joostei* were isolated from milk during the studies done by Hugo and Jooste (1997) and Hugo *et al*. (1999). Fischer *et al*. (1987) found that there was no significant difference between the counts of *Flavobacterium* in raw milk samples during the summer and winter months.

Chryseobacterium bovis (Hantsis-Zacharov *et al*., 2008a), *C. haifense* (Hantsis-Zacharov & Halpern 2007a), *C. joostei* (Hugo *et al.,* 2003) and *C. oranimense* (Hantsis-Zacharov *et al*., 2008b) were all described for the first time after isolation from raw milk. *Chryseobacterium shigense* has been considered as part of the natural microbial community of a lactic acid beverage from Japan (Shimomura *et al*., 2005).

2.5.5 Other products

Chryseobacterium species have also been isolated from other food products. For example *Chryseobacterium ureilyticum, C. gambrini, C. pallidum* and *C. molle* were isolated from beer bottling plants in Germany (Hertzog *et al*., 2008). Although the isolates were not able to grow in the beer, it was found that the *Chryseobacterium*

strains were often found in biofilms on the bottling plant surfaces. The study also showed that *Chryseobacterium* involved in the rapid recolonization of cleaned plant surfaces.

Flavobacterium-like strains were isolated from cauliflowers during a study by Lund (1969). The isolates were not identified, but they showed pectolytic activity which could enable them to cause deterioration of the vegetables. Another *Flavobacterium*-like isolate was thought to be responsible of causing thinning in canned vegetable products (Bean and Everton, 1969). No gas or significant amounts of acid were produced. The isolates were obtained from chlorinated can-cooling water or post-process can-handling equipment. Everton *et al.* (1968) reported 'thinning' of creamed rice due to *Flavobacterium* spoilage.

2.5.6 Spoilage caused by enzymes

Enzymes produced by microorganisms are often responsible for the spoilage of food products. It has been reported that the main cause of spoilage of milk that is kept at refrigerated temperatures, is the production of enzymes by psychrotrophic bacteria (Sørhaug & Stepaniak, 1997). These enzymes may often be heat stable and will therefore not be destroyed by pasteurisation of milk. This can limit the shelf-life of milk and dairy products (Nörnberg *et al*., 2010).

Proteolytic enzymes (proteases) can be present in milk due to the production by microorganisms or it can be of indigenous origin (Kelly *et al*., 2006). The protease that is produced by bacteria can lead to the production of off-flavours and odours, gelation of milk, reduction of cheese yield and coagulation of the milk proteins (Harwalker *et al*., 1993; Tondo *et al*., 2004).

In the first description of *Chryseobacterium* given by Vandamme *et al*. (1994) it was stated that members of the genus show strong proteolytic activity. 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. Hantsis-Zacharov *et al*. (2008a) showed that two *Chryseobacterium* strains isolated from raw

milk showed both proteolytic and lipolytic activity, which make them likely candidates as spoilage organisms in the milk.

Proteolytic activity in milk products can be determined by means of spectrophotometry using the azocasein method (Christen & Marshall, 1984). Other methods including flourimetric and radiometric techniques can also be used (Christen, 1987).

Lipases can be defined as carboxylesterases that hydrolyse acylglycerols (Chen *et al*., 2003). The hydrolysis of as little as 1 – 2% of these triglycerols may lead to the production of rancid off-flavours in food (Huis in't Veld, 2003). Microbial lipases are generally heat stable and can survive heat treatment of raw milk. Indigenous lipase in milk can also contribute to the development of lipolytic rancidity. In addition to the two strains mentioned above, a third strain of *Chryseobacterium* also showed lipolytic activity in the study by Hantsis-Zacharov *et al*. (2008b). The strains were identified as *Chryseobacterium bovis*.

Lipolytic activity in milk can be measured indirectly as changes in the levels of free fatty acids (FFAs) using solvent extraction followed by titration with an alkaline solution (Deeth *et al*., 1975). Other methods include spectrophotometric (Versaw *et al*., 1989; Humbert *et al*., 1997), reflectance colorimetric (Blake *et al*., 1996) and fluorimetric (Stead, 1983) techniques (Huis in't Veld, 2003).

2.6 Factors affecting microbial growth and spoilage in food

There are various factors that will affect the metabolism and multiplication of microorganisms in food (Banwart, 1989). The factors affecting the growth of microorganisms in food can be categorized into *intrinsic* parameters, *extrinsic* parameters, modes of processing and preservation, and *implicit* parameters (Mossel *et al*., 1995). Intrinsic parameters are the physical, chemical and structural properties inherent in the food itself. These include water activity, acidity, redox potential, available nutrients and natural antimicrobial substances. The intrinsic factors are also used to classify foods as perishable (e.g. meat, fish and milk), semi-perishable

(e.g. potatoes and nuts) or stable (e.g. sugar and flour) (Madigan & Martinko, 2006). The environmental factors such as the temperature, humidity and atmospheric conditions during storage make up the extrinsic parameters (Mossel *et al*., 1995). The modes of processing and preservation of food include physical or chemical treatments and can result in changes in the characteristics of a food product and consequently determine the microorganisms that are associated with the food (Madigan & Martinko, 2006). Implicit parameters are mutual influences, synergistic or antagonistic, among the primary selection of organisms resulting from the above mentioned parameters (Mossel *et al*., 1995). The optimum temperature, pH and NaCl concentration for food associated *Chryseobacterium* species are given in Table 2.2.

2.6.1 Temperature

The temperature of the environment in which the microorganisms grow is one of the most important factors that affect their growth and survival (Madigan & Martinko, 2006). All microorganisms have a different range of temperature in which they are able to grow and survive. This temperature range includes a minimum, maximum and optimum growth temperature and these temperatures are known as the *cardinal* temperatures. The organism is not able to grow below the minimum or above the maximum temperatures. The optimum temperature may be optimum for total cell yield, growth rate, rate of metabolism and respiration or the production of some metabolic products. The optimum temperature is, however, usually determined on the basis of the growth rate (Banwart, 1989). The optimum temperature of an organism is always closer to the maximum temperature than to the minimum. This is because the rate of the chemical and enzymatic reactions in the cell increases with a rise in temperature, and will thus become faster until the maximum growth temperature is reached. When an organism is exposed to temperatures higher than its maximum temperature, some proteins in the cell may become denatured and the cells will stop growing and death may occur (Madigan & Martinko, 2006).

Microorganisms can be divided into three classes based on their ability to grow at certain temperatures: psychrophiles that prefer to grow at low temperatures (optimum growth below 20 °C), mesophiles that prefer to grow at intermediate temperatures

(optimum growth between 20 and 42 °C) and thermophiles that prefer to grow at high temperatures (optimum growth between 42 and 70 °C) (Banwart, 1989; Overmann, 2006). Microorganisms that are capable of growing at refrigeration temperatures (<7 °C), but have a higher optimum temperature (25 °C or higher) are usually referred to as psychrotolerant (previously known as psychrotrophs) (Overmann, 2006).

All *Chryseobacterium* strains are able to grow at 30 °C and most strains can grow at 37 °C (Vandamme *et al*., 1994). The ability of some species to grow at 5 °C makes them possible agents for spoilage of food kept at low temperatures. Species that fall into this category include *C. joostei* (Hugo *et al.*, 2003); *C. vrystaatense* (de Beer *et al*., 2005); *C. daecheongense* (Kim *et al*., 2005a); *C. soldanellicola* and *C. taeanense* (Park *et al.,* 2005); *C. shigense* (Shimomura *et al*., 2005); *C. piscium* (de Beer *et al.*, 2006); *C. hispanicum* (Gallego *et al.,* 2006); *C. taiwanense* (Tai *et al*., 2006); *C. wanjuense* (Weon *et al.*, 2006); *C. haifense* (Hantsis-Zacharov & Halpern, 2007a); *C. flavum* (Zhou *et al*., 2007); and *C. caeni* (Quan *et al*., 2007).

2.6.2 pH

As in the case of temperature, microorganisms also have a minimum, optimum and maximum pH value at which growth will occur (Banwart, 1989). For most bacteria the optimum growth is observed at a pH in the region of 7. Acidophiles are organisms that grow best under acidic conditions, thus at a low pH (Madigan & Martinko, 2006). Other microorganisms show optimum growth in substrates with a high pH and are called alkaliphiles. A few species are capable of growth at pH values less than 2 and others at a pH above 9. Moulds can generally grow at lower pH values than yeasts, while yeasts are again more tolerant to low pH values than bacteria (Banwart, 1989).

Most of the *Chryseobacterium* species are able to grow in a wide pH range (from 5 to 10), but the optimum pH for most species are near neutral (pH 7) (for references see Table 2.1.). This makes it possible for these organisms to grow and cause spoilage in a wide range of food products. For example *C. shigense* (Shimomura *et al.*, 2005) isolated from a lactic acid beverage was able to grow in a pH range of 5 – 8 and

C. haifense (Hantsis-Zacharov & Halpern, 2007a) isolated from raw milk was able to grow in the range pH 6.5 – 10.5 with an optimum growth range of pH 7.0 – 9.5.

The pH of food will have an effect on the type of microorganisms that will be able to grow and cause spoilage of the food (Banwart, 1989). pH of food in turn is determined by the balance between the buffer capacity and the acidic or alkaline substances in the food. Protein rich foods generally have a greater buffering capacity than foods that contain little protein, because of the strong buffering capacity of proteins. Fruits, soft drinks, vinegar, and wines have very low pH values and are not likely to be spoiled by bacteria since the pH values are below the level at which bacteria normally grow (Jay *et al*., 2005). The pH of meat and seafood are usually close to neutral and this make them much more susceptible to microbial spoilage. The pH of the food can be altered by the microorganisms themselves or it can be influenced by other environmental factors. When the temperature of the environment is increased it causes the pH to decrease. The salt concentration also has an effect on the pH. When organisms are grown above or below their optimum pH, an extended lag phase will result.

2.6.3 Water activity

Microorganisms also have a minimum, optimum and maximum water activity (a_w) for growth (Banwart, 1989). The water activity of food ranges from 0 to 1.00, with pure water having an a_w of 1.00. Thus, the maximum a_w at which microorganisms can grow is somewhat less than 1.00. Moulds can grow at a lower a_w than yeasts, and yeasts can again grow at a lower a_w than bacteria. Bacteria usually require a minimum a_w of 0.9 to 0.91. Halotolerant bacteria and *Staphylococcus aureus* can grow at a lower minimum a_w of approximately 0.75.

The water activity of most fresh food lies between 0.98 to above 0.99 (Banwart, 1989; Jay *et al*., 2005). Foods like sugar, cereals and biscuits have very low a_w values and are not likely to be spoiled by bacteria (Banwart, 1989). The a_w of food can be lowered by dehydration, freezing or by adding solutes (such as NaCl). Water activity of food is also influenced by environmental factors such as pH, temperature and oxidation-reduction potential (Jay *et al*., 2005). If the temperature of a food is

changed from the optimum, the a_w at which growth and spore germination occurs is reduced (Banwart, 1989). Also, when the pH is increased or decreased from the optimum, the minimum a_w needed for growth is reduced. When the water activity of a food is lowered it will cause an increase in the lag phase of the bacteria (Jay *et al*., 2005).

Most *Chryseobacterium* species listed in Table 2.1 are halotolerant and can tolerate a sodium chloride (NaCl) concentration of up to 5%. Examples are *C. haifense* (Hantsis-Zacharov & Halpern, 2007a) that is able to grow in the presence of 0 – 2.5% NaCl with optimum growth at 0 – 1.5%; *C. vrystaatense* (de Beer *et al*., 2005) isolated from a chicken processing plant is capable of growth at NaCl concentrations of 1 and 2%. Some strains of this species were also able to grow at 3% NaCl. *Chryseobacterium ureilyticum* and *C. molle* (Hertzog *et al*., 2008) that were isolated from a beer bottling plant were both able to grow at 2% NaCl.

2.6.4 Nutrients

For microorganisms to be able to grow and survive in food they require water, a source of energy and nitrogen, vitamins and other growth factors as well as minerals (Jay *et al*., 2005). The organisms that are found in food can make use of sugars, alcohols or amino acids as a source of energy, while some may even utilize complex carbohydrates such as starches and amino acids by first degrading it to simple sugars. Some organisms also have the ability to use fats as an energy source. *Chryseobacterium* species are known to be chemoorganotrophs (Vandamme *et al*., 1994). They consequently use organic compounds as energy source.

Heterotrophic organisms primarily utilize amino acids as nitrogen source (Jay *et al.,* 2005). Amino acids are also needed to produce cellular proteins, including enzymes (Banwart, 1989). Some organisms can also utilize nitrogen in the form of nitrates or ammonia to produce amino acids, while others need to be supplied with amino acids in the substrate. There are also organisms that can utilize peptides and proteins as a source of amino acids (Jay *et al*., 2005).

Some microbes can synthesize their own B vitamins that are essential for their growth and survival, while others obtain these vitamins from the food environment (Jay *et al*., 2005). Gram-positive bacteria must be supplied with the B vitamins, while Gram-negative bacteria and moulds are able to synthesize most of the required B vitamins. Some organisms also need trace amounts of elements or minerals that are found in cellular components (Banwart, 1989). Sodium, potassium, calcium and magnesium are needed as well as, in smaller amounts, iron, copper, manganese, zinc, cobalt and molybdenum. Phosphorus and sulphur are also needed. These trace elements help to enhance the enzyme activity and are used for production of toxins and other secondary metabolites.

Table 2.2 Growth conditions of food-related chryseobacteria (Hugo & Jooste, 1997).

Species	**Habitat**	**Optimum temperature (°C)**	**pH Growth range**	**NaCl (%) Growth range**	**Reference**
C. aquaticum	Water reservoir	25-30	6-7	1-3	Kim *et al.*, 2008
C. arothri	Pufferfish kidneys	20-37	ND	2	Campbell *et al.*, 2008
C. balustinum	Marine fish	20-25	5-10[a]	0[a]	Engelbrecht *et al.*, 1996
C. bovis	Raw cow milk	30-32	6.5-8.5	0-1.75	Hantsis-Zacharov *et al.*, 2008
C. formosense	Lettuce rhizosphere	25-32	ND	ND	Young *et al.*, 2005
C. gambrini	Beer-bottling plant	25, 37, 42	ND	0-1	Hertzog *et al.*, 2008
C. gleum	Clinical, soil, water	25-30	5-10[a]	0-2 [a]	Holmes *et al.*, 1984
C. haifense	Raw milk	32	7.0-9.5	0-1.5	Hantsis-Zacharov & Halpern, 2007a
C. hispanicum	Drinking water	25-28	7	0	Gallego *et al.*, 2006
C. indologenes	Clinical, soil, water	25-30	5-10[a]	0-1[a]	Yabuuchi et al., 1983

C. joostei	Raw cow milk	25	5-10[a]	0-2[a]	Hugo *et al.*, 2003
C. molle	Beer-bottling plant	25, 37	ND	0-1	Hertzog *et al.*, 2008
C. pallidum	Beer-bottling plant	25, 37	ND	0-2	Hertzog *et al.*, 2008
C. piscium	Fresh marine fish	25	ND	0-5	de Beer *et al.*, 2006
'C. proteolyticum'	Rice field soil	30	6-8	ND	Yamaguchi & Yokoe, 2000
C. scophthalmum	Diseased turbot	15-25	5-10[a]	0-4	Mudarris *et al.*, 1994
C. shigense	Lactic acid beverage	20-30	5-8	ND	Shimomura *et al.*, 2005
C. taiwanense	Farmland soil	30	6-8	0-4	Tai *et al.*, 2006
C. ureilyticum	Beer-bottling plant	25	ND	0-2	Herzog *et al.*, 2008
C. vrystaatense	Raw chicken portions	25	ND	0-2	de Beer *et al.*, 2005
C. wanjuense	Greenhouse soil	38	7	0-1	Weon *et al.*, 2006

[a] Data obtained from Mielmann (2006). Values for pH and NaCl concentration depict the growth range and not the optimum conditions.

2.7 Bacterial growth kinetics

Bacterial cells multiply by means of cell division (Madigan & Martinko, 2006). During this division duplication (doubling) of all the structural components of the cell takes place. The time required for the cells to divide and to duplicate is known as the generation time or doubling time. The generation time is influenced by the growth medium and the incubation conditions. When bacterial cells are cultured in culture media, the doubling time is shorter than when the cells grow in natural environments. This is due to the fact that growth conditions (e.g. temperature, pH, moisture availability) in nature may be constantly changing and the cells need time to adjust to the new conditions before a new generation can be formed.

Bacterial growth can be divided into different phases that can be plotted onto a graph to form a growth curve. Buchanan (1918) divided the growth into seven phases (Figure 2.1) namely: initial stationary phase (indicated by 1-a on the curve), lag phase (or positive growth acceleration phase; a-b on the curve), logarithmic growth phase (b-c on the curve), phase of negative growth acceleration (c-d on the curve), maximum stationary phase (d-e on the curve), phase of accelerated death (e-f on curve) and logarithmic death phase (f-g on curve). The phases can be simplified and most often the phases are only referred to as lag phase, exponential phase, stationary phase and death phase (Madigan & Martinko, 2006).

During the initial stationary phase, the bacterial count remains constant and the graphic plot is a straight line (Buchanan, 1918). The number of bacterial cells starts to increase with time and this gives rise to the lag phase. During this lag phase the bacterial cells adapt to the environment and damaged cells are repaired (Madigan & Martinko, 2006). This increase in growth rate per organism does not continue indefinitely, but only to a certain point that is determined by the average minimal generation time per organisms under the conditions of the test.

During the logarithmic growth phase (or the exponential phase) the rate of increase per organisms remains constant (Buchanan, 1918). Each cell thus divides to form two cells. Each of these two cells divides again to give two more cells and so on (Madigan & Martinko, 2006). This continues for a brief period of time, depending on the available resources and other factors. It is considered that cells in this stage are in their healthiest state and that prokaryotic cells generally grow faster than eukaryotic cells. The maximum specific growth rate can be determined by the slope of the line observed during exponential growth (Zwietering *et al*., 1990). Factors that can affect the rate of the exponential phase include: the temperature of the surrounding environment, the composition of the culture medium as well as the genetic characteristic of the organism itself.

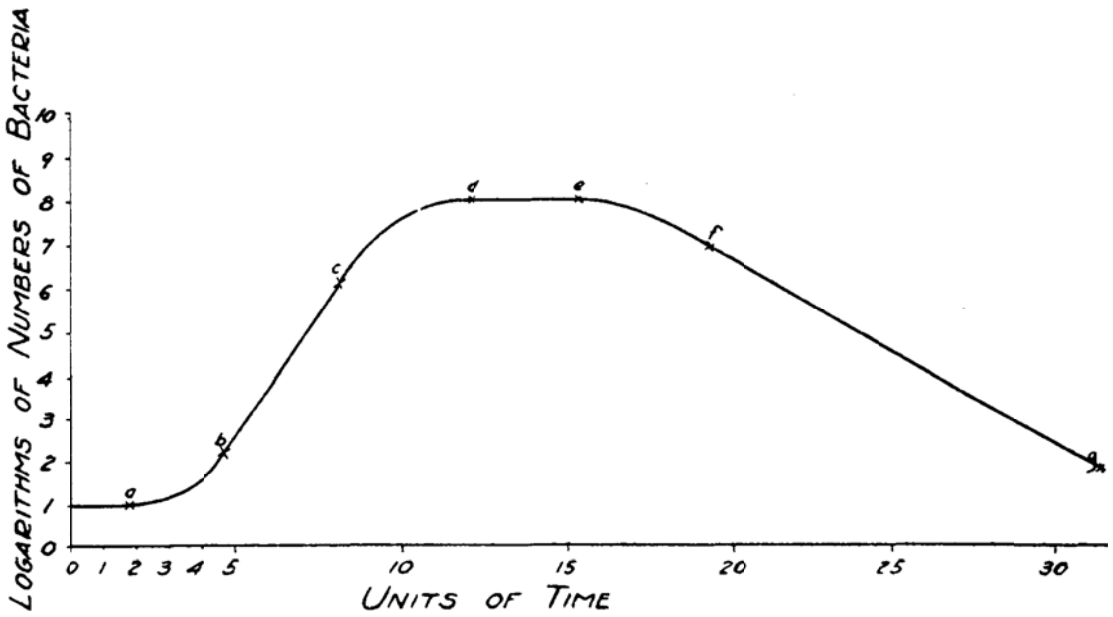

Figure 2.1 Diagrammatic plot of logarithms of numbers of bacteria present in a culture (Buchanan, 1918)

Before the cells enter the stationary phase, the rate of growth per organism decreases (Buchanan, 1918). This phase is known as negative growth acceleration. The bacteria continue to increase in numbers, but less rapidly than during the exponential phase. The cells enter the stationary phase either when an essential nutrient becomes depleted, or when some waste products of the organisms accumulate in the medium which then inhibits the growth of the organism (Madigan & Martinko, 2006). During the stationary phase the rate of increase in cell numbers is zero (Buchanan, 1918) although many of the cell functions, including energy metabolism and some biosynthetic processes, may continue (Madigan & Martinko, 2006). Slow growth may be observed in some organisms during this phase, but no net increase in cell numbers occurs. The reason that the number of cells stays constant is that although some cells will grow, others will die and the two processes balance each other out. This is known as *cryptic* growth.

During the accelerated death phase the cells start to die and the number of cells starts to decrease slowly (Buchanan, 1918). The death rate increases with time until the logarithmic death phase is reached during which the death rate remains constant.

Death may in some instances be accompanied by actual cell lysis (Madigan & Martinko, 2006).

In a study by Fischer (1987) the growth rates of *Flavobacterium, Pseudomonas fluorescens and Acinetobacter*, isolated from raw milk, were measured. It was found that both *Pseudomonas* and *Flavobacterium* had maximum growth rates at 30 °C. The doubling time as well as the lag phase of *Pseudomonas* decreased with an increase in temperature. A *Flavobacterium* strain isolated during the winter months had a longer doubling time at 8 and 25 °C than the rest of the *Flavobacterium* strains. This strain also had a shorter doubling time at temperatures below 8 °C. The specific growth rate and doubling time for the organisms were calculated. *Pseudomonas* had a specific growth rate of 0.483 h^{-1} while *Flavobacterium* had a specific growth rate of 0.40 h^{-1}. The doubling time of *Pseudomonas* was 1.42 h while *Flavobacterium* had a doubling time of 1.63 h.

2.8 Methods to measure microbial growth

Various methods exist to measure the number of microbial cells in food. The four basic methods used for determining the total count of the organisms are: standard plate counts (SPC) or aerobic plate counts (APC) for viable cells or colony forming units (cfu); the most probable number (MPN) method as a statistical determination of viable cells; the dye reduction techniques to estimate numbers of viable cells that possess reducing capacities and the direct microscopic counts (DMC) for both viable and non-viable cells (Jay *et al*., 2005). Another method that can be used is the indirect measurement of cell numbers by turbidity (Madigan & Martinko, 2006). Standard plate count, DMC, and turbidity will be discussed.

2.8.1 Standard plate count

Standard plate counts (SPC) are used to determine the amount of viable cells in a product (Madigan & Martinko, 2006). Two ways of performing SPC exists, namely the spread plate method and the pour plate method. In both cases the food product is blended or homogenized, a serial dilution is made and the desired solution is plated (Jay *et al*., 2005). When the spread plate method is performed, a volume of

0.1 ml or less of the desired dilution is spread on a previously prepared agar plate using a sterile glass spreader (Madigan & Martinko, 2006). During incubation the colonies will form on top of the agar and can be counted after the desired time, usually 48 – 72 hours at 32 °C. In the pour plate method, a volume of 0.1 to 1.0 ml is poured into an empty sterile Petri dish. Melted agar is poured into the plate and the two liquids are mixed by swirling the plate. The colonies will form throughout the agar and not just on the surface of the agar. In the spread plate method, the agar is first poured into the Petri dish and allowed to cool and set. Usually an inoculum volume of 0.1 ml is dropped onto the surface of the agar and spread with a sterile, bent glass rod ("hockey stick") until the superfluous fluid has been absorbed into the agar. The spread plate method is favoured in the case of heat-sensitive organisms, since the organisms do not come in contact with the hot agar.

2.8.2 Direct microscopic count (total cell count)

The direct microscopic count (DMC) is a total cell count that can be done on liquid samples or samples that have been dried on microscope slides (Madigan & Martinko, 2006). Counting chambers are used for liquid samples. The chamber has a marked grid on the surface with squares of known area. Each square on the grid contains a known volume of inoculum. The number of cells per unit on the grid is counted using phase contrast microscopy (Madigan & Martinko, 2006). The total number of cells per milliliter of the inoculum can be determined by converting the number of cells per small chamber by multiplying this number with a conversion factor based on the volume of the chamber sample. Samples can also be viewed by making a smear on a microscope plate and staining with an appropriate dye (Jay *et al*., 2005).

2.8.3 Turbidity

When measuring cell numbers by turbidity, a dilution series of the cells is prepared and the turbidity of each dilution is observed (Banwart, 1989). The turbidity of a cell suspension is caused by the scattering of light that passes through the suspension (Madigan & Martinko, 2006). More cells in the suspension causes more light scattering and hence more turbidity. There are two ways to measure turbidity – with a photometer or a spectrophotometer. Both of these devices pass light through the

cell suspension and detect the amount of unscattered light that emerges. An increase in cells will thus cause a decrease in the amount of unscatterd light. The difference between these instruments is that a photometer makes use of a single broad bandpass filter to generate incident light, where a spectrophotometer uses a more precise prism or diffraction grating to generate incident light. The wavelengths that are generally used to measure the turbidity of bacterial cell suspension are 540 nm (green), 600 nm (orange) or 660 nm (red). Photometer units, like Klett units (for the Klett-Summerson photometer) or optical density (OD) for a spectrophotometer, are used to measure the unscattered light.

When measuring unicellular organisms, the OD is proportional to the cell number (Madigan & Martinko, 2006). A standard curve, that relates some direct measurement of cell number or mass to indirect measurement obtained by turbidity, needs to be prepared before the number of cells can be determined using turbidity measurements. A disadvantage of this method is that when the concentration of cells becomes too high, the light that is scattered away from the photocell by one cell can be scattered back by another cell. This will cause the illusion that the light was not scatterered at all and will be measured by the photocell. This in return will cause the correspondence between cell number and turbidity to drift from linearity. The measurements done by turbidity can however be accurate and it is quick and easy to perform. Another advantage is that the sample is not destroyed or significantly disturbed and can thus be measured repeatedly.

2.8.4 Arrhenius plots

The Arrhenius equation is derived from thermodynamics and it is used to describe the influence of temperature on the rate of chemical reactions (Gudmundsson & Kristbergsson, 2009):

$$k = Ae^{-E/RT}$$

Where k is the rate constant (k can be replaced by μ_{max} for specific growth rate), A is an entropy constant, E is the activation energy (kJ/mol), R is the universal gas constant (R = 8.314 J/mol/K), and T is the absolute temperature (K).

It can also be used to describe the effect of temperature on microbial growth and the kinetics of quality deterioration (Gudmundsson & Kristbergsson, 2009). It can be used to set up a model to predict the microbial behaviour in a food product (Devlieghere *et al*., 2009). The activation energy, *E*, is a measure of the temperature sensitivity of the reaction and its value can be estimated by plotting experimental data for rate constants at different temperatures. This sensitivity to a change in temperature can then be used to predict the spoilage potential of different organisms under changing environmental conditions (Gudmundsson & Kristbergsson, 2009).

2.9 Measurement of spoilage by sensory analysis

In the description of quality given by Molnar (1995) it is mentioned that the quality of food products is determined by, among others, their sensory attributes. It is not easy to give a clear definition for sensory quality because it is linked to the food properties or characteristics as well as the interaction between the food and the consumer (Costell, 2002). However, Kramer (1959) defined sensory quality as ‘‘the composite of those characteristics that differentiate among individual units of a product and have significance in determining the degree of acceptability of that unit by the user’’.

The growth of microorganisms and their metabolic products can cause the sensory qualities of a food product to deteriorate. During food spoilage organoleptic deterioration can occur before there are any signs of chemical changes (Mossel *et al*., 1995). The reason for this is due to the fact that some odiferous metabolites can be detected organoleptically at very low levels. Dimethyl sulphide or methyl mercaptan at levels lower than 1 ppm is sufficient to cause off-odours (Fields *et al*., 1968). The production of proteolytic and lipolytic enzymes by *Chryseobacterium* in milk may also be responsible for the development of off-odours (Bernardet *et al*., 2002).

Sensory analysis on milk samples inoculated with *Chryseobacterium* species was done during a master’s study at the University of the Free State (Mielmann, 2006). Samples of both fat-free and full cream UHT processed milk were inoculated with *C. joostei, C. gleum* and *C. indologenes* respectively. Panellists were asked to smell

the samples, together with uninoculated control samples, and indicate the level of spoilage and also describe the type of odour. The results indicated that fat-free milk inoculated with *C. gleum* as well as *C. joostei* were strongly spoiled while the same medium inoculated with *C. indologenes* was only weakly spoiled. The odour caused by *C. gleum* was described as fruity, flowery, smelly feet and blue cheese. In the case of *C. indologenes* it was described as sour, agar and putrid, while *C. joostei* produced odours characterised as sour, flowery, putrid and smelly feet. The results for the full cream milk indicated that growth of both *C. indologenes* and *C. joostei* in this medium resulted in only weak spoilage while growth of *C. gleum* resulted in samples that were strongly spoiled. The description of the odours were as follows: for *C. gleum* it was sour, fruity, rancid, flowery, putrid, butter and blue cheese; for *C. indologenes* it was sour, fruity and putrid; and for *C. joostei* it was sour, "agar" and putrid.

2.9.1 Gas chromatography-mass spectrometry

Gas chromatography (GC) and mass spectrometry (MS) can also be used to detect spoilage in food and identify the volatile compounds responsible for the production of off-odours in milk. The GC separates the volatile and semi-volatile compounds, while the MS provides detailed structural information on them and thus accurately identifies the compounds (Hites, 1997).

Monomeric microbial chemicals, known as chemical markers, can be used to detect and identify microorganisms in complex samples by means of GC-MS analysis (Larsson & Saraf, 1997). These markers can be bacteria-specific or species specific and each one may constitute either an integral part of a microbial cell or a cellular excretion product.

The free fatty acids (FFAs) that are produced during lipolysis, and which are responsible for the production of off-flavours and odours, are measured using the GC-MS technique (Wang & Xu, 2009). Wang & Xu (2009) used the result of the GC-MS analysis to identify the FFAs in milk responsible for the odours that were described during sensory analysis of the same samples. This technique was also

used to identify *Pseudomonas* species in raw milk by detecting ethyl esters, ethyl butanoate and ethyl hexanoate (Hettinga *et al*., 2008).

2.10 Conclusions

Chryseobacterium species are present in a wide range of environments. They are especially important in the food environment where they are able to cause spoilage. *Chryseobacterium* spp. have been isolated from a variety of food products including fish, meat, poultry, milk and dairy products as well as vegetables and canned food products. Some species in this genus have the ability to grow at 5 °C, in the presence of NaCl (0 – 5%) and over a wide pH range. These characteristics lead to the conclusion that these organisms are able to grow in, and cause spoilage of, different foods, including refrigerated food.

The growth kinetics of bacteria can be studied by making use of different methods. The spoilage patterns of microorganisms on or in food can be studied by looking at their growth characteristics and patterns in different food environments. The growth of bacteria in food can be influenced by factors that include water activity, acidity, redox potential, available nutrients, natural antimicrobial substances, temperature, humidity and atmosphere conditions. When these factors are manipulated, the growth of these spoilage organisms can be controlled and spoilage of the food prevented. The growth patterns and factors that influence these patterns of *Chryseobacterium* have not been studied before.

Sensory analysis can be used as a method to detect spoilage in food. Spoilage can often be detected by organoleptic changes before any measurable chemical changes take place. Proteolytic and lypolytic enzymes produced by *Chryseobacteirum* are usually responsible for the production of off-odours and flavours when they are present and multiply in milk. The level of spoilage of milk contaminated with *Chryseobacterium* spp. can be determined organoleptically by means of a sniffing test. The type of odour produced by these organisms can also be described using this method. GC-MS can be used in conjunction with sensory analysis to identify the compounds responsible for the production of off-odours.

CHAPTER 3

EFFECT OF TEMPERATURE ON THE GROWTH KINETICS AND PROTEOLYTIC ACTIVITIES OF *Chryseobacterium* SPECIES

Abstract

The effect of temperature on the growth kinetics and proteolytic activity of Chryseobacterium joostei *and* Chryseobacterium bovis *was determined during this study. The results were compared to the activities of* Pseudomonas fluorescens. *For the growth studies, cultures were incubated in nutrient broth in a Temperature Gradient Incubator (from 4 – 50 °C) and optical density was measured at different time intervals. Temperature profiles for each organism were constructed. For protease determination the cultures were incubated in fat-free UHT processed milk in the Temperature Gradient Incubator for 72 hours. Cell free extracts were used to determine the proteolytic activity using the azocasein method. Results of the growth studies showed that* C. joostei *had the fastest growth rate between 7 and 36 °C, followed by* Ps. fluorescens *and* C. bovis*. Similar results were obtained for the proteolytic activity with* C. joostei *having the highest activity per mg protein between 4 and 50 °C, followed by* C. bovis *and* Ps. fluorescens*. The results showed that* Chryseobacterium *species potentially have the ability to cause even more spoilage in milk on the basis of growth rate and proteolytic activity than* Ps. fluorescens *that is deemed to be the major spoilage psychrotolerant microorganism in milk.*

3.1 Introduction

Chryseobacterium species have been isolated from a wide variety of habitats including soil, freshwater, marine environments, humans, hospital equipment, and diseased animals (Bernardet & Nakagawa, 2006). The psychrotolerant ability of some of these species gives them the ability to grow and cause spoilage of

refrigerated food products, especially milk and dairy products (Cousin, 1982). Species that have been isolated from milk include: *C. bovis* (Hantsis-Zacharov *et al.*, 2008a), *C. haifense* (Hantsis-Zacharov & Halpern, 2007a), *C. joostei* (Hugo *et al*., 2003) and *C. oranimense* (Hantsis-Zacharov *et al*., 2008b).

The production of proteolytic and lipolytic enzymes by *Chryseobacterium* contributes to the spoilage potential of these organisms (Bernardet *et al*., 2006). The production of proteolytic enzymes can cause off-odours and off-flavours in milk as well as gelation of milk and coagulation of milk proteins (Harwalker *et al*., 1993; Tondo *et al*., 2004). It has also been reported that the major cause of bitterness in milk is the formation of bitter peptides due to the action of these enzymes (Huis in't Veld, 1996). The production of these proteolytic enzymes by *Chryseobacterium* has also been reported to cause spoilage in butter (Wolochow *et al*., 1942; Jooste *et al*., 1986a), creamed rice (Everton *et al*., 1968) and canned vegetables (Bean and Everton, 1969).

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, a_w and atmosphere (Gram *et al*., 2002). It generally requires a careful combination of microbiology, sensory analysis and chemistry to determine which microorganisms are the specific spoilage organisms of a particular food product.

The aim of this study was to determine the effect of temperature on the growth rate of *Chryseobacterium* species grown in a broth medium and to determine the minimum, maximum and optimum growth temperatures of these organisms. The proteolytic activities of these species were determined over a range of temperatures and the results of both growth and proteolytic activity was compared to that of *Pseudomonas fluorescens.* This organism was used for comparison because it is generally regarded as predominant spoilage organisms of refrigerated milk (Champagne *et al*., 1994).

3.2 Materials and Methods

3.2.1 Effect of temperature on the growth kinetics of *Chryseobacterium* species in broth

Strains used

The strains used in this study are listed in Table 3.1. Both *Chryseobacterium bovis* (Hantsis-Zacharov *et al.*, 2008a) and *C. joostei* (Hugo *et al.*, 2003) was isolated from raw cow's milk. *Pseudomonas fluorescens* was used as the control organism. Master cultures were maintained in freeze-dried form in the Culture Collection (UFSBC) of the University of the Free State's Food Science division. Reactivation was done in 10 ml Nutrient Broth (Oxoid CM0001) and purity was checked by streaking on Nutrient Agar (Oxoid CM0003) plates. Reactivation incubation was at 25 °C for 24-48 h. Working cultures were maintained on Nutrient Agar slants at 4 °C and were sub-cultured every 2 months.

Table 3.1 Strains used in this study.

Strain	Code
Chryseobacterium bovis	LMG 34227^T
Chryseobacterium joostei	UFSBC 256^T
Pseudomonas fluorescens	ATCC 13525^T

LMG, Laboratory of Microbiology, University of Ghent, Belgium; UFSBC, University of the Free State Bacterial Culture Collection, Bloemfontein, South Africa; ATCC, American Type Culture Collection, Rockville, Maryland.

Inoculation of broth for the determination of growth characteristics

Isolates were incubated in 10 ml nutrient broth at 25 °C for 24 hours. The 10 ml broth culture was transferred to 100 ml nutrient broth. The inoculated broth was incubated at 25 °C for 24 to 48 h. Depending on the density of growth, 20 to 50 ml of the 100 ml broth culture was used to inoculate a sterile 400 ml nutrient broth. Ten millilitres of this broth was transferred immediately after inoculation to sterile Temperature Gradient Incubator tubes. The tubes were put in the Temperature

Gradient Incubator (Scientific Industries Inc., New York, USA) consisting of an aluminium bar that was cooled at one end and heated at the other to obtain a stable temperature gradient, following a procedure described previously (du Preez & Toerien, 1978). The bar contained thirty equidistant sample wells on both sides into which L-shaped tubes of optically selected glass (40 ml total volume and 17 mm in diameter) were inserted and capped with loose-fitting metal caps. Whereas tubes on the one side contained bacterial cultures, the corresponding tubes on the other side contained water to allow frequent measurement of the temperature. The bar was rocked through a 30° arc at 60 oscillations min^{-1}, provided mixing and aeration. Incubated was done at temperatures ranging between ca. 9 to 50 °C. For the determination of growth at 4 °C the tubes were put in a roller drum in a walk-in temperature controlled refrigerator at a constant temperature of 4 °C.

Measurement of growth in broth

The growth of the isolates was measured by means of optical density (OD) measurements in a Biowave C0800 cell density meter (Walden Precision Apparatus Ltd., Cambridgeshire, United Kingdom) at 600 nm. The first measurement was done after 30 minutes of incubation at the various temperatures to give the tubes time to reach their incubation temperatures. The OD was measured at intervals ranging between 30 minutes and 1 h. Longer time intervals were used for measurements at lower temperatures after the OD of the tubes that were incubated at the optimum temperature of each organism reached an OD value of two. For measurement of growth at 4 °C the tubes were placed in a roller drum in a walk-in refrigerator at a constant temperature of 4 °C.

Data analysis

The maximum specific growth rate at each temperature was determined by linear regression analysis. An Arrhenius model was used to describe the linear relationship between the specific growth rate and temperature according to the following equation (Pirt, 1975):

$$\mu_{max} = Ae^{-\left[\frac{E_a}{RT}\right]}$$

where μ is the specific growth rate, A an entropy constant, E_a the activation energy (temperature coefficient), R the universal gas constant (8.314 J mol^{-1} K^{-1}), and T the absolute temperature in K.

3.2.2 Proteolytic activity in milk

The proteolytic activity was assayed by the azocasein method according to Christen and Marshall (1984) and Deeth *et al*. (2002), with some modification. Microbial cultures, *C. joostei*, *C. bovis* and *Ps. fluorescens* respectively, were reactivated in 10 ml nutrient broth for 24 h at 25 °C. Six millilitres of each broth culture was then transferred to 60 ml commercial UHT fat-free milk. The inoculated milk was incubated at 25 °C for 24 h. After incubation, 40 ml of the latter milk culture was used to inoculate 150 ml commercial UHT fat-free milk, of which 10 ml was transferred to sterile test tubes immediately after inoculation. The tubes were incubated in a Temperature Gradient Incubator for 72 h. Activity at 4 °C was measured by incubating the tubes in a roller drum in a walk-in refrigerator at a constant temperature of 4 °C. Samples were analysed at 0 and 72 h. An uninoculated milk sample was also included as control.

Preparation of cell free extract

Cell free extracts were prepared by removing 1 ml of milk from each tube and centrifuging for 15 min at 14 000 rpm. The supernatant of each sample was used for determination of protease activity.

Protease assay

For determination of protease activity, 250 μl of the cell free extract was added to 1 ml 1% azocasein (Sigma-Aldrich A2765-10G) (10 g/l, dissolved in phosphate buffer, pH 7.2). For the blank sample, 250 μl of phosphate buffer was added instead

of the cell free milk extract. The mixtures were incubated for 1 h at 37 °C. After incubation the reaction was stopped by adding 1 ml of 5% trichloroacetic acid (TCA). The mixture was centrifuged for 15 min at 14 000 rpm and the absorbance of the supernatant was read at 345 nm. Protein determination was done according to the Biuret method.

The maximum protease per mg protein was calculated and the cardinal temperatures for protease production were determined for each organism.

3.3 Results and discussion

3.3.1 Growth kinetics

Growth rates and cardinal temperatures for all three organisms are shown in Figure 3.1 and Table 3.2, respectively. *Chryseobacterium joostei* had the highest maximum specific growth rate (μ_{max}) followed by *Ps. fluorescens* and *C. bovis*. All three species were able to grow at 4 °C.

Chryseobacterium joostei had a μ_{max} of 0.98 h^{-1} at an optimum temperature of 28.9 to 31.3 °C. The organism was still able to grow at 4 °C and had a maximum growth temperature of 38.7 °C. When this species was first described by Hugo *et al*. (2003), it was found that the optimum growth temperature was 25 °C. This difference in optimum temperatures could be ascribed to the different methods used for determining growth. Hugo *et al.* (2003) also found that *C. joostei* had the ability to grow at 5 °C, but not at 37 or 42 °C. In the first description of *Chryseobacterium*, Vandamme *et al*. (1994) stated that all strains were able to grow at 30 °C, while most strains also had the ability to grow at 37 °C.

No previous literature is available on the specific growth rate of *Chryseobacterium*, except for a master's study done by Fischer (1987). The study was done on the growth rate of *Flavobacterium* strains, namely *F. breve* strain SPB 17, *Flavobacterium* sp. "Cluster 5" (strain SPB 815) and *Flavobacterium* sp. Group IIb (Strain SPA 39), of which many species was later reclassified as *Chryseobacterium*.

It was found that the organism had a μ_{max} of 0.40 h^{-1}. This rate is significantly lower than the 0.98 h^{-1} observed for *C. joostei* during this study.

Chryseobacterium bovis had the lowest μ_{max}, 0.62 h^{-1} at an optimum temperature of 30.7 °C with growth still occurring at 4 °C as well as 40.2 °C. It was reported during the first description of this species that growth occurs between 7 and 37 °C, with one strain still showing growth at 41 °C (Hantsis-Zacharov *et al*., 2008a). Optimum growth during that study was observed at 30 to 32 °C.

The maximum specific growth rate of 0.85 h^{-1} for *Pseudomonas fluorescens* was observed at a temperature of 27.7 °C. Growth still occurred at 4 °C and this species had the lowest maximum temperature of 36.6 °C. According to the description given by Garrity *et al*. (Palleroni, 2005), *Ps. fluorescens* has the ability to grow at 4 °C but not at 41 °C. The optimum temperature given for growth is between 25 and 30 °C.

Studies done on the growth rate of *Ps. fluorescens* species have found the maximum growth rate to differ when grown in different media. Makhzoum *et al*. (1995) reported a growth rate of 0.48 h^{-1} in peptone water and 0.41 h^{-1} in minimal medium. Fisher (1987) reported a growth rate of 0.483 h^{-1} when grown in skim milk. A rate of 0.56 h^{-1} was reported by Gügi *et al*. (1991) in citrate mineral salts medium supplemented with 10% liquid skim milk.

Although *Ps. fluorescens* was not evaluated at a lower temperature than 4°C, the higher growth rate value of 0.04 h^{-1} compared to that of *C. joostei* and *C. bovis*, 0.02 and 0.01 h^{-1} respectively, at the same temperature, indicates that it might be more psychrotolerant.

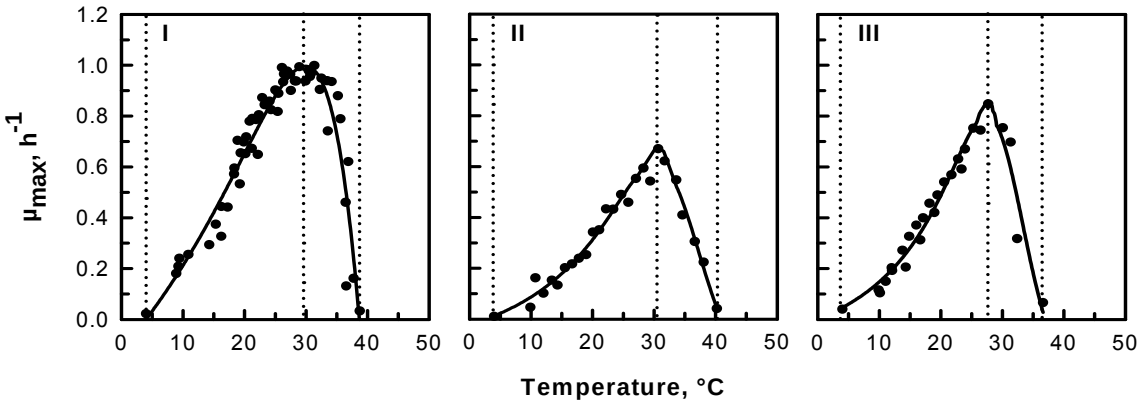

Figure 3.1 The maximum specific growth rates (μ_{max}) of *C. joostei* UFS BC256^T (I), *C. bovis* LMG 34227^T (II) and *Ps. fluorescens* ATCC 13525^T (III) at different temperatures. The broken vertical lines indicate the cardinal temperatures (Table 3.2).

Table 3.2 The cardinal temperatures (°C) and maximum specific growth rates (μ_{max}) of *C. joostei* UFS BC256^T, *C. bovis* LMG 34227^T and *Ps. fluorescens* ATCC 13525^T.

Bacterium	Temperature (°C)			Maximum specific growth rate (μ_{max})
	Minimum[1]	Optimum	Maximum[2]	
C. joostei	4	28.9 – 31.3	38.7	0.98
C. bovis	4	30.7	40.2	0.62
Ps. fluorescens	4	27.7	36.6	0.85

[1] Minimum temperature evaluated for growth
[2] Maximum temperature were growth still occurred

3.3.2 Arrhenius plots

Arrhenius plots of the three organisms are shown in Figure 3.2. The low activation energy values (Table 3.3) of *C. joostei* in the temperature range of 22.3 to 38.9 °C, that of *C. bovis* in the range of 17.8 to 30.7 °C and that of *Ps. fluorescens* in the range of 19.0 to 27.7 °C, indicate that the cells were least sensitive to temperature changes in these respective temperature ranges.

Chryseobacterium joostei had the lowest overall activation energy indicating that this organism is the least sensitive to changes in temperature. *Chryseobacterium bovis* showed the greatest response to temperature change in the range of 9.9 to 16.7 °C. The high overall activation energy also indicate that *C. bovis* was the most sensitive to a change in temperature. The results show that *C. joostei* have the ability to grow over a wider temperature range without being affected by changes in the temperature. This also means that *C. joostei* can possibly cause spoilage over a wider range of temperatures than *Ps. fluorescens*.

3.3.3 Proteolytic activity

The protease activity per mg protein for each organism is given in Table 3.4. *Chryseobacterium joostei* had the highest activity followed by *C. bovis*. *Pseudomonas fluorescens* showed significantly lower activity than the *Chryseobacterium* species. The *Chryseobacterium joostei* protease also had the highest optimum temperature at 41.9 °C, followed by that of *Ps. fluorescens* at 31.2 °C and *C. bovis* at 28.0 °C. All three organisms still showed some protease activity at 4 °C.

In the only literature available on protease production by *Flavobacterium* (*Chryseobacterium*), Roussis *et al*. (1999) reported that *Flavobacterium* MTR3 proteinases were active at 32 – 45 °C, and exhibited considerable activity at 7 °C. Venter *et al*. (1999) found that *C. indologenes* Ix9a exhibited optimum protease production at a temperature of 50 °C at pH 7.5. The activity decreased rapidly as the temperature increased.

Table 3.3 Activation energies for *C. joostei* UFS BC256^T, *C. bovis* LMG 34227^T and *Ps. fluorescens* ATCC 13525^T (C) calculated from Arrhenius plots in Figure 3.2.

Bacterium	Temperature range (°C)	Zone	Slope	Activation energy (kJ mol^{-1})
C. joostei	8.9 – 22.3	A – B	-8724	72.53
	22.3 – 28.9	B – C	-2424	20.15
C. bovis	9.9 – 16.7	D – E	-18177	151.12
	17.8 – 30.7	E – F	-6787	56.43
Ps. fluorescens	9.9 – 18.2	G – H	-13603	113.10
	19.0 – 27.7	H – I	-6228	51.78

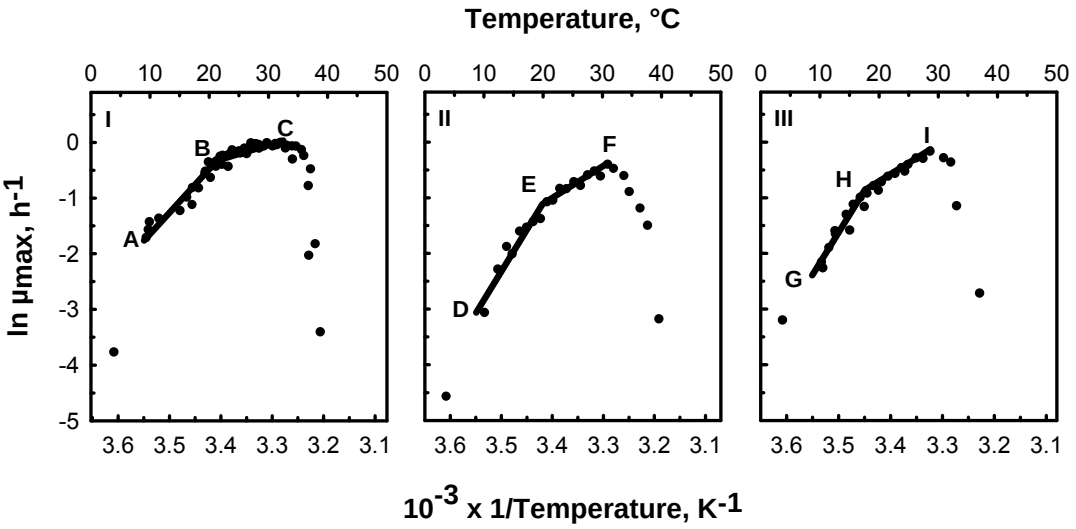

Figure 3.2 Arrhenius plots of the maximum specific growth rates (μ_{max}) of *C. joostei* UFS BC256^T (I), *C. bovis* LMG 34227^T (II) and *Ps. fluorescens* ATCC 13525^T (III) as a function of cultivation temperature in Kelvin (K). A to I indicating the activation energy zones (Table 3.3).

Table 3.4 The cardinal temperatures (°C) for protease production and the maximum protease (mg^{-1} protein) of *C. joostei* UFS BC256^T, *C. bovis* LMG 34227^T and *Ps. fluorescens* ATCC 13525^T.

Bacterium	Temperature (°C)			Maximum protease production (mg^{-1} protein)
	Minimum[1]	Optimum	Maximum	
C. joostei	4	41.9	53.4	1.587
C. bovis	4	28.0	49.7	1.321
Ps. fluorescens	4	31.2	49.7	0.208

[1] Minimum temperature evaluated for protease production

Optimum protease production by *C. joostei* occurs at a temperature where no growth was observed any more. This is possibly due to the fact that the protease enzyme is secreted extracellularly (Hantsis-Zacharov & Halpern, 2007b). Extracellular enzymes are those enzymes that are completely dissociated from the cell, in the absence of cell lysis, and found free in the surrounding medium (Priest, 1977). Optimum protease production by *Pseudomonas* usually occurs at 17.5 – 30 °C (Braun & Sutherland, 2003).

Datta & Deeth (2003) reported that proteolysis of UHT milk may lead to the development of bitter flavours and an increase in viscosity, with eventual formation of a gel. They also stated that only trace levels of proteinase are required to cause gelation of UHT milk during storage.

3.4 Conclusions

The growth rates and level of protease production by bacteria can be an indication of the extent and rate of spoilage that it can lead to. During the study on the growth characteristics of *Chryseobacterium joostei*, *C. bovis* and *Ps. fluorescens* it was found that *C. joostei* had the highest growth rate at temperatures above 7 °C. At lower temperatures, *Ps. fluorescens* showed higher growth rates than the *Chryseobacterium* species. All three organisms showed growth at 4 °C. These results showed that all three bacteria are psychrotolerant mesophiles. The Arrhenius

plots showed that *C. joostei* were the least sensitive to temperature changes while *C. bovis* were the most sensitive. For protease activity, *Chryseobacterium joostei* showed the highest activity followed by *C. bovis* while *Ps. fluorescens* showed significantly lower activity than the *Chryseobacterium* species. All three species still showed some protease activity at 4 °C. When considering the growth rate and protease production, the results of this study clearly indicate that *C. joostei* may have the ability to cause more spoilage than *Ps. fluorescens* at temperatures above 7 °C. When only growth is taken into consideration, *Ps. fluorescens* will cause more spoilage at 4 °C.

CHAPTER 4

LIPID BREAKDOWN AND SENSORY ANALYSIS OF MILK INOCULATED WITH *Chryseobacterium joostei*

Abstract

This study investigated the lipolytic activity, lipid oxidation, sensory effect and production of volatile compounds by C. joostei *and* Ps. fluorescens *in fat-free and full cream milk incubated at 4 and 25 °C. Lipolytic acitivity were studied by measuring the amount of free fatty acids (FFA). High levels of activity were observed for both organisms.* Ps. fluorescens *incubated at 4 °C and* C. joostei *at 25 °C showed significantly higher amounts of FFA's than the uninoculated control sample in the fat-free milk. In the full cream milk,* C. joostei *grown at 25 °C showed levels of lipolysis that was significantly higher than the control and 4 °C samples.* Pseudomonas fluorescens *incubated at 25 °C also showed significantly higher FFA levels than the control sample. Lipid oxidation was measured with TBA analysis. Although inoculated milk samples demonstrated higher levels of oxidation compared to non-inoculated samples, there were no significant differences between any of the inoculated milk samples. Sensory analysis showed that* C. joostei *produced stronger odours than* Ps. fluorescens*. Different odours were also described for the two species. Volatile compounds identified with headspace SPME GC/MS analysis included ketones, fatty acids and alcohols.*

4.1 Introduction

Sensory changes in milk can be caused by lipolysis, lipid oxidation and proteolysis. Rancid flavours can be caused by both lipolysis and lipid oxidation, but the two types are distinctively different in origin and flavour (Deeth, 2006). Bitter flavours caused by protease have also been reported (Lemieux & Simard, 1991). The oxidation of dairy products leads to flavours termed oxidized, cardboardy, metallic, tallowy, oily,

and fishy (Shipe *et al.*, 1978). Volatile compounds, responsible for the formation of off-odours in milk can be detected by gas chromatography–mass spectrometry (GC-MS) (Wang & Xu, 2009).

The hydrolysis of triglycerides by lipases in milk is responsible for spoilage as a result of the production of short and medium chain fatty acids (Deeth, 2006). Lipases can be produced by microorganisms growing in milk or such enzymes can be indigenous in the milk (Huis in't Veld, 1996). During a study by Muir *et al*. (1979) it was reported that *Pseudomonas, Flavobacterium* and *Alcaligenes* species were the most lipolytic bacterial species isolated from raw milk. Off-flavours produced by lipase enzymes have been described as rancid, butyric, unclean, soapy astringent and bitter (Deeth & Fitz-Gerald, 1994; Deeth, 2006).

Oxidation of unsaturated lipids can also be responsible for spoilage of milk and milk products. This oxidation can produce off-odours and -flavours and decrease the nutritional quality and safety of products by the formation of secondary products in foods after cooking and processing (Frankel, 1980). The changes in the aroma of foods affected by lipid oxidation are due to the volatile odorous compounds that are formed in the process (Kanner & Rosenthal, 1992). Other sensory changes include flavour modifications caused by hydroxylic acids; darkening of colour as a result of condensation reactions between oxidation products and proteins; and texture changes that might occur as a result of the oxidative induction of protein crosslinkages (Kenner & Rosenthal, 1992). Nutritive value and safety of the foods are also impaired by lipid oxidation.

Chryseobacterium joostei and *Ps. fluorescens* were chosen for this study due to their high growth rates. The main aim of this study was to determine the lipolytic activity of and lipid oxidation caused by *C. joostei* and *Ps. fluorescens* in milk at 4 and 25 °C. More specific objectives were to determine the level of spoilage and types of odour produced by means of sensory analysis and to identify the volatile compounds produced by these two organisms in milk by means of GC-MS analysis.

4.2 Materials and Methods

4.2.1 Determination of lipid oxidation and lipolysis

Sample preparation

Chryseobacterium joostei (UFSBC 256^T) and *Ps. fluorescens* (ATCC 13525^T) cultures were grown in Nutrient Broth at 25 °C until turbidity similar to a McFarland standard no. 2 was obtained. One millilitre of the broth was then used to inoculate 15 ml amounts of commercial UHT fat-free and full cream milks respectively. The inoculated milk portions were then incubated for 72 h at 25 °C and for 10 days at 4 °C. After incubation, samples were stored in 4 to 5 ml aliquots in cryotubes at –40 °C until lipolysis and lipid oxidation analyses could be performed.

Determination of lipolytic activity

Lypolytic activities of the organisms were determined by measuring the free fatty acid content in the treated milk. Analysis was done according to the method described by Deeth *et al*. (1975) with some modification. Frozen milk was placed in a water bath at 30 °C until completely thawed. Two millilitres of defrosted milk was mixed with 10 ml extraction mixture (isopropanol:petroleum ether:4N H_2SO_4, 40:10:1). Six millilitres of petroleum ether and 4 ml water was then added. The tubes were shaken 10 times before and after they were vortexed for 30 seconds. The tubes were subsequently left for 20 min to allow separation of the two phases. The top layer was transferred to a 25 ml measuring cylinder and the volume was noted. Five millilitres of the upper layer was then transferred to a small Erlenmeyer flask and 6 drops 1% phenolphthalein were added. The solution was titrated with 0.002 N KOH until a light pink colour could be observed. A blank sample was prepared by replacing the milk with dH_2O to obtain the background titration. The concentration of free fatty acids was calculated using the following formula and the results were statistically analysed:

$$\mu \text{ equiv. FFA ml}^{-1} = \frac{T \times N}{P \times V} \times 10^3$$

where $P = \frac{\text{5 ml top layer}}{\text{Total volume of top layer}}$

T = net titration value

N = normality of the KOH

V = volume of milk

TBA analysis

Lipid oxidation, caused by *C. joostei* and *Ps. fluorescens* growing in the milk samples, was measured by means of the thiobarbituric acid (TBA) technique (Raharjo *et al*., 1992). One set of frozen samples was thawed and weighed and 20 ml of 5% TCA was added. The mixture was homogenized for 1 min. Centrifuge tubes with sample were weighed to balance the centrifuge and 5% TCA was added to obtain the correct weight. Samples were centrifuged at 10 000g for 5 min at 4 °C. The supernatant was filtered through Whatman no. 1 filter papers into 25 ml volumetric flasks. After filtration the flasks were filled up to the mark with 5% TCA and 4 ml quantities of the samples were each mixed with 4 ml TBA by vortexing in stoppered tubes. For preparation of a standard curve, 4 ml 1,1,3,3-tetraethoxypropane (TEP) standards were mixed with 4 ml TBA in stoppered tubes. The tubes were placed in a boiling water bath for 5 min and then cooled to room temperature. Absorbance was read at 532 nm. Results of the TBA determination were analysed statistically.

4.2.2 Sensory Analysis

The milk samples inoculated with potential spoilage organisms were subjected to sensory analysis to detect signs of spoilage. The sensory panel consisted of ten semi-trained staff and students from the Agricultural Faculty of the University of the Free State. The age of the panellists ranged from 21 to 65 years with three male and seven female panellists. During training, the panellists were asked to differentiate between a spoiled and an unspoiled sample by performing a sniffing test. The spoiled sample was prepared by inoculating 10 ml of fat-free milk with

Chryseobacterium joostei and incubating at 25 °C for 72 h. The control sample was uninoculated fat-free milk. Panellists were also requested not to wear any perfumed personal care products on the day of analysis.

For the sniffing test, 10 ml volumes of commercial, UHT processed, fat-free and full cream milk were transferred aseptically to sterile McCartney bottles. The milk samples were inoculated with 0.1 ml of a 24 h old Nutrient Broth culture of *C. joostei* and *Ps. fluorescens* respectively. One set of fat-free and full cream milk samples was incubated at 25 °C for 72 h while the other set of samples was incubated at 4 °C for 10 days. An uninoculated sample of each milk type was used as control. All samples were heated at 50 °C for 30 min immediately before sensory evaluations were performed. This was done to allow better assessment of the volatile fractions in the milk. The milk samples were presented to the panel members in the same McCartney bottles in which the inoculation had been done. The bottles had tight fitting caps to prevent any loss of volatile compounds. Samples were coded with three digit numbers and served randomly on white trays.

The inoculated and control samples were presented to panellists in individual booths, and under red lights to prevent colour changes in the milk from influencing the panellists. The sample bottles were uncapped, sniffed and closed immediately to prevent sensory exhaustion. When exhaustion did occur, panellists were asked to sniff their own skin.

Three levels of spoilage were chosen to define the intensity of spoilage namely: level 1 = no spoilage; level 2 = weak spoilage; and level 3 = strong spoilage. Panellists were also asked to describe the type of odour, if any, produced by the different organisms. Samples were considered weakly and strongly spoiled when more than 50% of the panellists rated it as level 2 and 3 respectively. The analysis was done in duplicate and the results were statistically analysed.

4.2.3 Determination of volatile compounds

Sample preparation

Chryseobacterium joostei and *Ps. fluorescens* samples were inoculated into 10 ml nutrient broth and incubated for 24 h at 25 °C. Two millilitres of the broth culture was used to inoculate 20 ml of fat-free and full cream UHT milk, respectively. The samples were then incubated either at 4 °C for 14 days or 25 °C for 7 days in order for spoilage to set in. Samples were frozen until solid-phase microextraction-gas chromatographic-mass spectrometric (SPME-GC/MS) analysis could be performed.

SPME-GC/MS analysis

The analysis was done according to the method described by Marsili (1999) with some modifications as described in the next paragraph. Volatile analysis was carried out by headspace (HS) solid phase micro-extraction (SPME) gas chromatography / mass spectrometry.

A manual SPME fibre holder and Carboxen fibre assembly with 75 µm sorbent thickness was used (Supelco, Belafonte). The depth setting on the holder was 1.5 for sorption and 3.5 for desorption. Ten gram of sample was weighed into a 20 ml headspace vial and equilibrated for 30 min at 60 °C. An SPME fibre was exposed to the headspace for 20 min after which desorption was carried out in the injection port of the gas chromatograph for 2 min. Fibres were reconditioned in the injection port of another gas chromatograph for 10 min at 250 °C before exposure to the next sample.

A Finnigan Trace GC Ultra gas chromatograph was employed for analysis. Desorption was carried out in split mode for 2.5 min followed by a split ratio of 20:1. The temperature was set at 200 °C and the port was equipped with a 0.75 mm glass injection liner. A SolGel column of 30 m length x 0.25 mm inner diameter x 0.25 µm film thickness (Phenomenex) was initially held a 40 °C for 5 min after which the temperature was raised at 5 °C per min to 200 °C and held for 2 min. The carrier gas was helium at a constant flow rate of 1 ml/min. The MS transfer line temperature was

280 °C. Mass analysis was performed with a Finnigan DSQ mass spectrometer with electron impact ionization at 70 eV and a source temperature of 200 °C. The scan range was 40 to 350 m/z. Instruments were controlled and results analysed with Thermo Xcalibur 1.4 software and the NIST 02 spectral library.

4.2.4 Statistical analysis

Differences in parameters for lipolysis, TBA and sensory analysis between different treatment groups were determined by using a one way analysis of variance (ANOVA) procedure. The Tukey-Kramer multiple comparison test (α=0.05) was used to identify differences between treatment means (NCSS, 2007).

4.3 Results and Discussion

4.3.1 Lipolytic activity

Results for the free fatty acid analysis are shown in Figure 4.1. When considering the suggested standard of Deeth *et al*. (1975) that a FFA content of more than 1 µ equiv/ml can be considered as a high level of lipolysis, it can be seen that all inoculated milk samples produced high levels of lipolysis.

In the fat-free milk, *Ps. fluorescens* incubated at 4 °C and *C. joostei* at 25 °C showed significantly higher amounts of FFAs than the control sample as well as the *Ps. fluorescens* at 25 °C. In the full cream milk, *C. joostei* grown at 25 °C showed levels of lipolysis that was significantly higher than the control and 4 °C samples. *Pseudomonas fluorescens* incubated at 25 °C also showed significantly higher FFA levels than the control sample. No significant differences in FFA level were observed between the milk types (fat-free and full cream milk) in which the organisms had been cultured. These results are similar to those observed by Griffiths (1986), who reported that the levels of lipase produced by a *Ps*. *fluorescens* strain in skim and whole milks were similar.

Flavobacteria are generally less well-known for lipase production. However, significant lipase production by some strains has been reported by some researchers

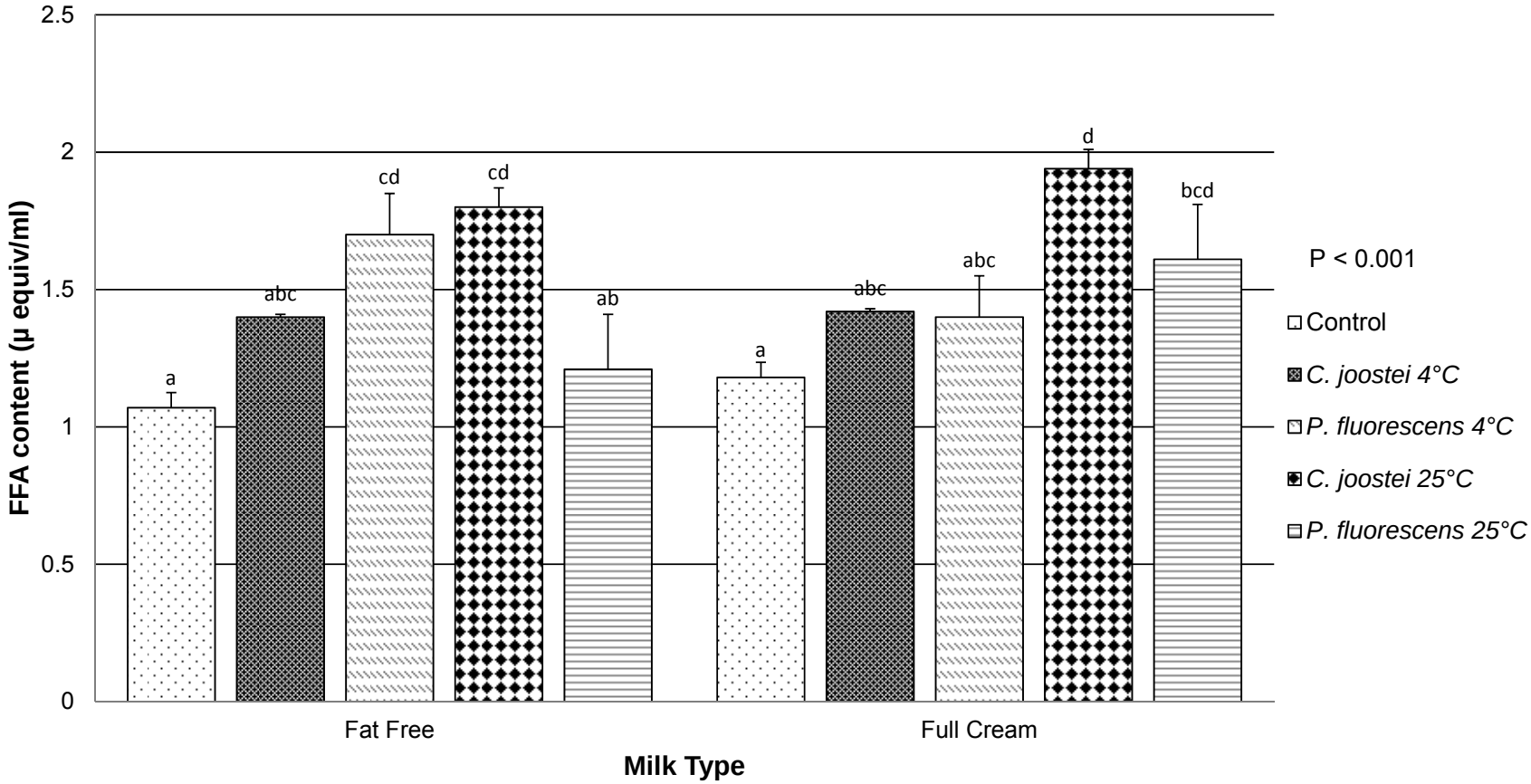

Figure 4.1 Analysis of variance (ANOVA) of lipolytic activity in the samples. Means with different subscripts differed significantly

in the past (Roussis *et al*., 1999). Research on the levels of lipase production by *C. joostei* has not been done before.

During a study done by Makhzoum *et al*. (1995) on the factors affecting lipase production by *Ps. fluorescens*, it was found that lipase production was strongly influenced by the incubation temperature. Hantsis-Zacharov & Halpern (2007b) found that *Pseudomonas* strains isolated from raw milk exhibited higher lipolytic activity than the *Chryseobacterium* strains that were isolated in their study.

4.3.2 Lipid oxidation

The results of the TBA analysis for determination of secondary lipid oxidation are shown in Figure 4.2. Very low levels of oxidation were observed in all samples. Levels are only considered as being high when values higher than 1 mg malonaldehyde/kg are observed. In the fat-free milk samples there was a significant difference between the values of the control and the inoculated samples, except for *Ps. fluorescens* at 25 °C. There were, however, no significant differences between the different treatments of the fat-free milk. In the full cream milk, no significant differences were observed between the samples, although *Ps. fluorescens* at 4 °C showed slightly lower TBA values. There were also no significant differences between the samples of fat-free and full cream milk.

Primary oxidation takes place when unsaturated fatty acids are oxidized to form odourless, tasteless hydroperoxides (Kanner & Rosenthal, 1992). These hydroperoxides are then further degraded to yield flavourful carbonyls and other compounds that are responsible for off-odour and -flavour production (O'Connor & O'Brian, 1994). TBA analysis is a measurement of secondary oxidation products. Since there were high levels of FFA production during lipolysis, it can be suggested that oxidation was still taking place at primary level and had not yet progressed to a secondary level. This could be the reason for the very low levels of TBARS observed as well as the lack of significant differences between the samples.

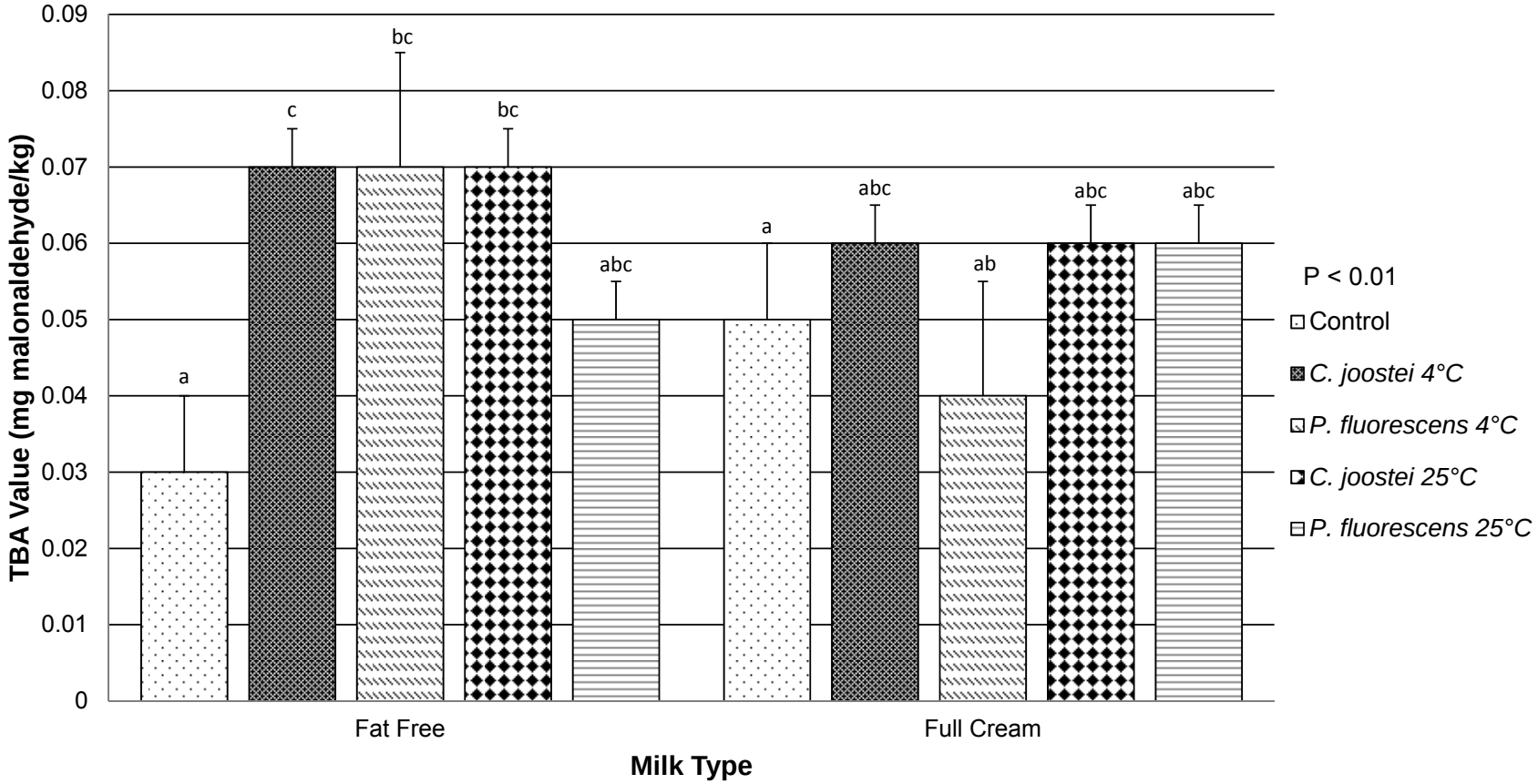

Figure 4.2 Analysis of variance (ANOVA) of lipid oxidation values of samples. Means with different subscripts differed significantly

4.3.3 Sensory analysis

Results for the spoilage scores are given in Figure 4.3. It can be seen that *C. joostei* produced stronger odours after incubation, at 4 and 25 °C, than *Ps. fluorescens*. Scores above 2 indicate strong spoilage of the samples. No significant differences were observed between the control samples and those inoculated with *Ps. fluorescens.* All the samples that were inoculated with *C. joostei* differed significantly from the controls as well as the *Ps. fluorescens* samples.

The fat-free milk samples gave slightly higher scores than the full cream samples. This can possibly be the result of the protease activity that was observed for *C. joostei* samples. Deeth *et al*. (2002) also reported that fat-free milk reached unacceptable sensory scores before the corresponding full cream milks during cold storage and concluded that this might be due to the greater proteolysis in the skim milk.

The odours that were observed for the different samples are given in Tables 4.1 and 4.2. All the fat-free samples produced odours described as “smelly feet”, “blue cheese” and “agar”. *Chryseobacterium joostei* at 25 °C produced a “putrid” odour as well. *Pseudomonas fluorescens* at 4 °C also produced “fruity” and “nutty” odours while “flowery” and “sour” odours were described at 25 °C. In the full cream milk “smelly feet”, “sour” and “agar” odours were observed in all the samples presented to the panel. “Putrid” odours were also observed for both *C. joostei* samples while an odour resembling “blue cheese” was observed at 4 °C. The *Ps. fluorescens* sample at 4 °C produced “fruity” and “bitter” odours.

Strong odours caused by *Chryseobacterium* strains have been reported (Bernardet *et al*., 2006). These odours have been described as “fruity” and “cheesy” in solid and liquid media. Hayes *et al.* (2002) reported “cheesy” (aromas associated with short-chain free fatty acids; reference - feta cheese butyric acid), “barn” (aromas associated with barn animal manure, urine, and sweat; reference - stock trailer recently used to haul cattle), “rotten” (aromas associated with decomposed meats; reference - old hamburger meat) in milk spoiled by *Ps. fluorescens* strains. No “fruity” odours were observed in these samples.

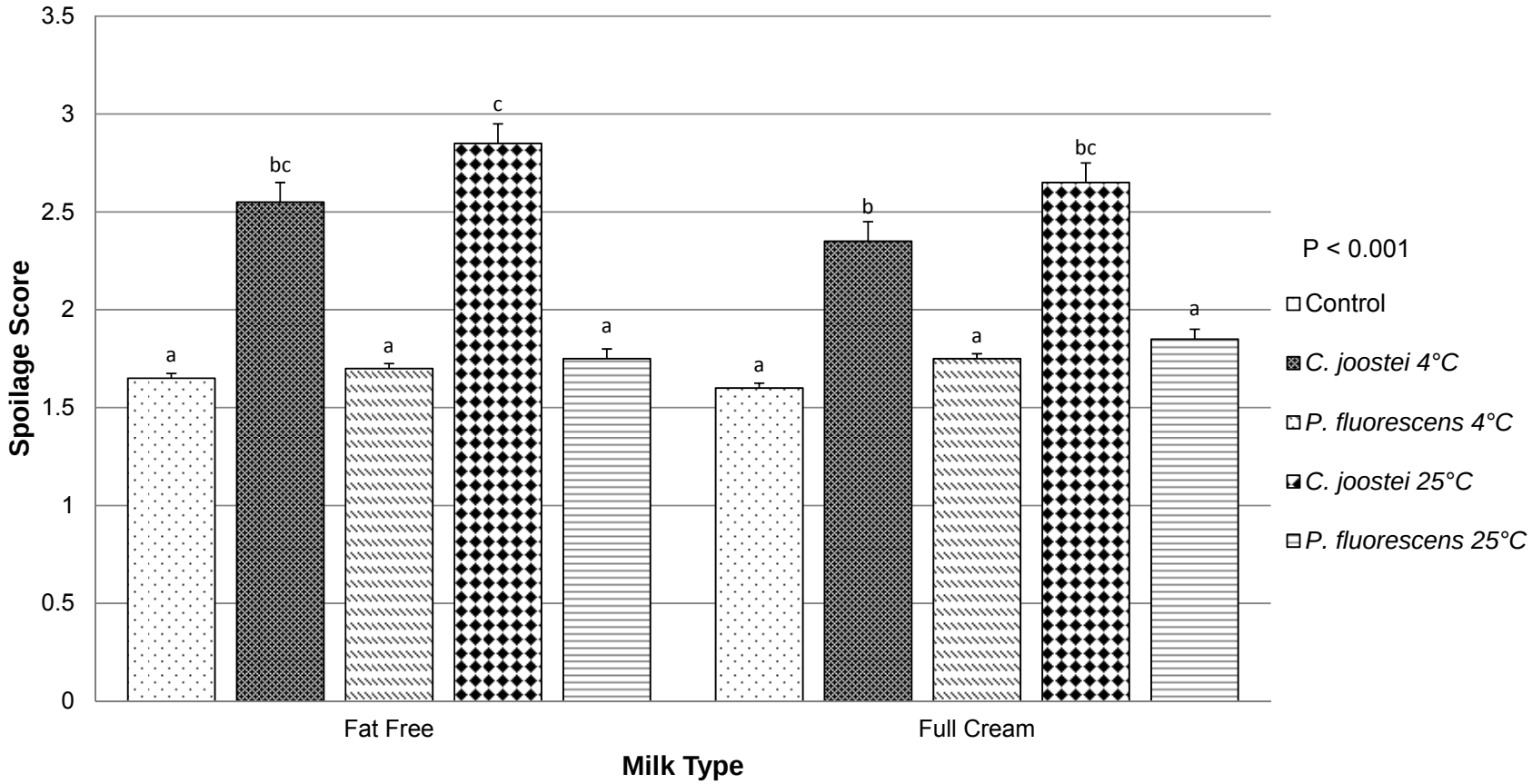

Figure 4.3 Analysis of variance (ANOVA) of the sensory scores. Means with different subscripts differed significantly

Table 4.1 Odour description in fat-free milk

Temperature	*C. joostei*	*Ps. fluorescens*
4 °C	Smelly feet; blue cheese; agar	Smelly feet; blue cheese; agar; fruity; nutty
25 °C	Smelly feet; blue cheese; agar; putrid	Smelly feet; blue cheese; agar; flowery; sour

Table 4.2 Odour description in full cream milk

Temperature	*C. joostei*	*Ps. fluorescens*
4 °C	Smelly feet; blue cheese; agar; sour; putrid	Smelly feet; sour; agar; fruity; bitter
25 °C	Smelly feet; sour; agar; putrid	Smelly feet; sour; agar

It has been reported that “rancid” and “fruity” aromas may be caused by free fatty acids, as a result of bacterial lipases, and their ethyl esters, respectively (Forss, 1971; Hayes *et al*., 2002). “Unclean” and “putrid” flavours are caused by products of protease activity (Mayerhofer *et al*., 1973; Shipe *et al.*, 1978). The occurrence of bitter flavours in UHT milk are caused by heat-stable proteases and llpolytic enzymes produced by psychrotolerant bacteria (Lemieux & Simard, 1991).

The limited breakdown of the milk proteins results in the formation of bitter peptides and decomposition of the amino acids to produce “putrid” flavours (Shipe *et al.*, 1987). Results from this study confirm these findings since “putrid” odours were only observed for the samples treated with *C. joostei* and this organism showed much higher protease activity than *Ps. fluorescens*.

It was reported that isovaleric acid has a sweat-like odour and it was described as “sweaty feet” (Amoore, 1977). These results are consistent with those of Jooste *et al*. (1986a) who found that isovaleric acid was responsible for the production of “sweaty feet” odour caused by flavobacteria in milk. It is thus possible that isovaleric acid was also responsible for the “smelly feet” odour that was observed in all the samples.

4.3.4 Determination of volatile compounds

The results of the determination of volatile compounds in milk samples are given in Tables 4.3 and 4.4. Tthe chromatograms for the fat-free control milk sample as well as the fat-free samples inoculated with *C. joostei* and *Ps. fluorescens* incubated at 25 °C are shown in Figure 4.4, 4.5 and 4.6 respectively. Volatiles are expressed in the tables as a percentage of the total volatiles present in the sample. Uninoculated fat-free and full cream control samples were also analysed.

In the fat-free milk (Table 4.3), acetone, butanoic acid, dichloromethane and hexanoic acid were present in the control as well as all the inoculated samples, while β-pinene, 2-heptanone and 2-pentanone were present in the inoculated samples, but not in the control. 3-Hydroxy-2-butanone, 2-butanone, heptanone and trichloroethane were only present in the control sample and not in any of the inoculated samples. Very high levels of acetone were present in the control samples while the *C. joostei* samples incubated at 4 and 25 °C also showed high values compared to that of the *Ps. fluorescens* samples. Isovaleric acid (3-methylbutanoic acid), hexane and an unknown sulphur compound were only present in the *C. joostei* samples while furfuryl alcohol and hexanal were only present in the *Ps. fluorescens* samples. *Chryseobacterium joostei* samples incubated at 25 °C produced the highest concentration of dimethyl sulphide. The *Ps. fluorescens* sample incubated at 4 and 25 °C produced high levels of dichloromethane when compared to the control and *C. joostei* samples. *Pseudomonas fluorescens* incubated at 4 °C produced higher levels of 2-heptanone while *Ps. fluorescens* incubated at 25 °C produced higher levels of 2-pentanone.

In the full cream milk (Table 4.4) butanoic acid, dichloromethane, hexanoic acid, 2-nonanone and 2-pentanone were present in the control as well as all the inoculated samples. Isovaleric acid and 2-heptanone were present in all the inoculated samples but not in the control, while butyrolacetone and 1-pentanone were only present in the control sample. β-Pinene, dimethyl disulphide and an unknown sulphur compound were only produced by *Ps. fluorescens* at 4 and 25 °C, while dimethyl sulphide, furfuryl alcohol and hexanal were only produced by *C. joostei* incubated at 4 and 25 °C. Acetone again showed a much higher concentration in the control sample.

Table 4.3 Concentration (relative percentage) of compounds detected in fat-free milk by GC/MS

	Fat-Free				
	Control	*Ps. fluorescens* 4 °C	*C. joostei* 4 °C	*Ps. fluorescens* 25 °C	*C. joostei* 25 °C
β-Pinene	ND	6.11	3.09	5.30	3.69
3-Hydroxybutanone	ND	0.12	0.62	0.28	ND
3-Hydroxy-2-butanone	0.40	ND	ND	ND	ND
3-Methyl-2-butanol	ND	ND	9.89	6.87	ND
Acetic acid	0.97	7.06	1.98	ND	0.08
Acetone	67.32	0.80	34.26	10.39	41.48
Butan-2-one	13.52	ND	ND	ND	ND
Butanoic acid	2.96	1.56	1.05	0.96	1.31
Dichloromethane	3.40	13.98	3.73	25.57	6.68
Dimethyl disulfide	ND	0.36	2.16	1.03	12.29
Dimethyl sulfide	ND	ND	ND	ND	ND
Ethyl acetate	ND	ND	ND	ND	ND
Furfuryl alcohol	ND	5.51	ND	1.08	ND
2-Heptanol	ND	1.85	1.84	5.94	ND
Heptanone	1.93	ND	ND	ND	ND
2-Heptanone	ND	35.30	8.81	19.21	14.56
Hexanal	ND	4.12	ND	1.32	ND
Hexane	1.80	ND	10.83	ND	ND
Hexanoic acid	1.59	9.63	1.71	2.22	1.51
Isovaleric acid	ND	ND	10.36	ND	5.08
Isoveraldehyde	2.61	ND	ND	ND	ND
2-Nonanone	ND	2.07	1.64	1.81	ND
2-Pentanone	ND	11.51	6.37	18.02	9.24
Trichloroethane	3.49	ND	ND	ND	ND
Ukn. sulphur comp.	ND	ND	1.66	ND	4.08

ND: Not detected

Table 4.4 Concentration (relative percentage) of compounds detected in full cream milk by GC/MS

	Full Cream				
	Control	*Ps. fluorescens* 4 °C	*C. joostei* 4 °C	*Ps. fluorescens* 25 °C	*C. joostei* 25 °C
β-Pinene	ND	0.30	ND	0.15	ND
3-Hydroxybutanone	ND	0.62	0.36	ND	0.23
3-Methyl-2-butanol	ND	19.35	ND	ND	ND
Isovaleric acid	ND	3.29	0.40	7.27	0.18
Acetic acid	1.10	1.13	1.97	1.20	0.83
Acetone	59.13	41.17	15.82	28.82	ND
2-Butanone	6.21	ND	ND	12.92	17.51
Butanoic acid	0.76	0.71	2.53	0.92	0.93
Butyrolacetone	0.20	ND	ND	ND	ND
Dichloromethane	2.80	7.15	55.25	9.71	44.48
Dimethyl disulfide	ND	6.12	ND	18.69	ND
Dimethyl sulfide	1.83	ND	5.34	ND	3.75
Ethyl acetate	ND	ND	ND	ND	17.51
Furfuryl alcohol	ND	ND	1.14	ND	0.50
2-Heptanol	9.40	ND	0.59	0.63	ND
2-Heptanone	ND	9.95	4.95	4.68	4.05
Hexanal	ND	ND	0.29	ND	0.15
Hexane	ND	ND	ND	ND	ND
Hexanoic acid	1.16	1.18	1.43	1.51	0.75
2-Nonanone	1.02	1.34	1.18	1.85	0.79
1-Pentanone	1.60	ND	ND	ND	ND
2-Pentanone	14.85	4.81	8.73	5.03	8.34
Ukn. sulphur comp.	ND	2.89	ND	6.61	ND

ND: Not detected

Dichloromethane concentrations were higher in the *C. joostei* samples than in the *Ps. fluorescens* samples.

When comparing the fat-free and full cream samples, it can be seen that acetic acid, butanoic acid, dichloromethane, 2-heptanone, hexanoic acid and 2-pentanone were present in all the inoculated samples. Isovaleric acid were present in all the inoculated samples, except in the fat-free *Ps. fluorescens* samples incubated at both 4 and 25 °C. The only sample that did not contain acetone, was the full cream milk sample inoculated with *C. joostei* at 25 °C. In both the fat-free and full cream samples the acetone concentration was lower in the inoculated samples. It has been reported that acetone could be used a variety of bacteria as a source of carbon and energy for growth (Sluis *et al*., 1996). This could explain the lower levels of acetone in the inoculated samples than in the control samples. Ethyl acetate was only present in the full cream milk sample inoculated with *C. joostei* at 25 °C. Dimethyl sulphide was also found only in the full cream milk in the samples inoculated with *C. joostei*. Hexane was the only compound that was solely observed in the fat-free milk and it was present in the control as well as in the 4 °C *C. joostei* sample. There were no significant differences that could be used to differentiate between the two organisms in the different samples.

In a study on the volatile compounds in cow's milk it was found that 2-butanone, 2-pentanone, 2-heptanone and hexanal were normally present in raw cow's milk (Toso *et al*., 2002). Ketones were the most abundant compounds in the milk samples, with acetone being the main constituent. Hettinga *et al*. (2008) found similar results for raw cow's milk using headspace analysis.

A list of the possible odours that can be produced by each volatile that was observed are presented in Table 4.5. Fatty acids are responsible for the production of cheesy and sour odours in milk (Wang & Xu, 2009). The presence of isovaleric acid has been reported to cause a sweat-like odour (Amoore, 1977; Jooste *et al*., 1986a) and it can thus be confirmed that this volatile could be responsible for the "smelly feet" odour that was detected in all the samples during the sensory evaluation.

Table 4.5 Possible odour production by observed volatiles

Volatile Compound	Descriptive Odour	Reference
3-Hydroxy-2-butanone	Buttery, milky, mushroom	Arora, 1995; Ranau & Steinhart, 2005
3-Hydroxybutanone	Buttery	Lu *et al*., 2011
3-Methyl-2-butanol	Fruity	Chatterjee *et al*., 2009
β-Pinene	Fragrant, fresh, conifer-like	Mariaca *et al*., 1997
Acetic acid	Acid (sour), spicy	Ranau & Steinhart, 2005; Wang & Xu, 2009
Acetone	Milk, ethereal, fruity	Nursten, 1997; Lecanu *et al*., 2002
2-Butanone	Rancid	Bravo *et al*., 1992
Butanoic acid	Cheesy, sour cream, sweaty, sour	Ranau & Steinhart, 2005; Wang & Xu, 2009
Butyrolacetone	Sweet, aromatic, slightly buttery	Li *et al*., 2011
Dichloromethane	Sweet, aromatic	Winneke, 1981
Dimethyl disulfide	Cabbage like, garlic	Lecanu *et al*., 2002; Ranau & Steinhart, 2005
Dimethyl sulfide	Cabbage like, rotten	Ranau & Steinhart, 2005
Ethyl acetate	Ethereal-fruity like	Li *et al*., 2011
Furfuryl alcohol	Vitamin, rubber	Karagül-Yüceer *et al*., 2002
2-Heptanol	Fresh lemongrass, herbal, sweet, floral fruity, green	Chatterjee *et al.*, 2009
2-Heptanone	Blue cheese, spicy, Roquefort cheese	Lecanu *et al*., 2002
Heptanone	Fruity, milk	Wang & Xu, 2009
Hexanal	Fatty, green, fruity	Wang & Xu, 2009
Hexane	Fatty	Gorraiz *et al*., 2006
Hexanoic acid	Sweaty, sour, cheesy, blue cheese	Lecanu *et al*., 2002; Wang & Xu, 2009
Isovaleric acid	Sweaty, smelly feet	Amoore, 1977; Jooste, 1986a
Isoveraldehyde	Malty, fruity, cocoa-like	Tian *et al*., 2007
2-Nonanone	Floral, grassy	Wang & Xu, 2009
1-Pentanol	Fruity	Arora, 1995
2-Pentanone	Fruity, acetone, sweat, orange peel	Arora, 1995; Lecanu *et al*., 2002
Trichloroethane	Sweetish chloroform-like	Tay *et al*., 1995

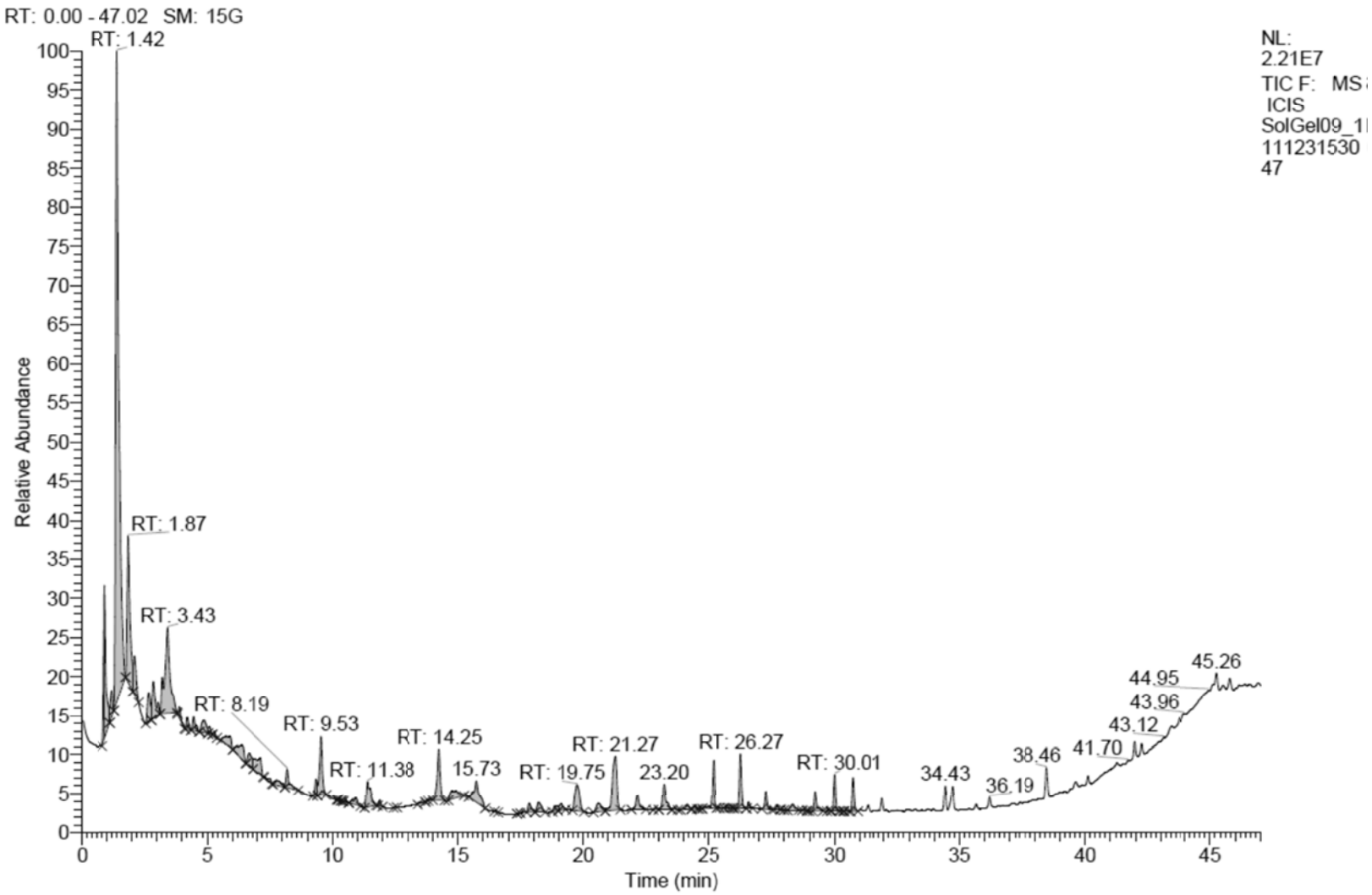

Figure 4.5 Chromatogram of fat-free milk control sample

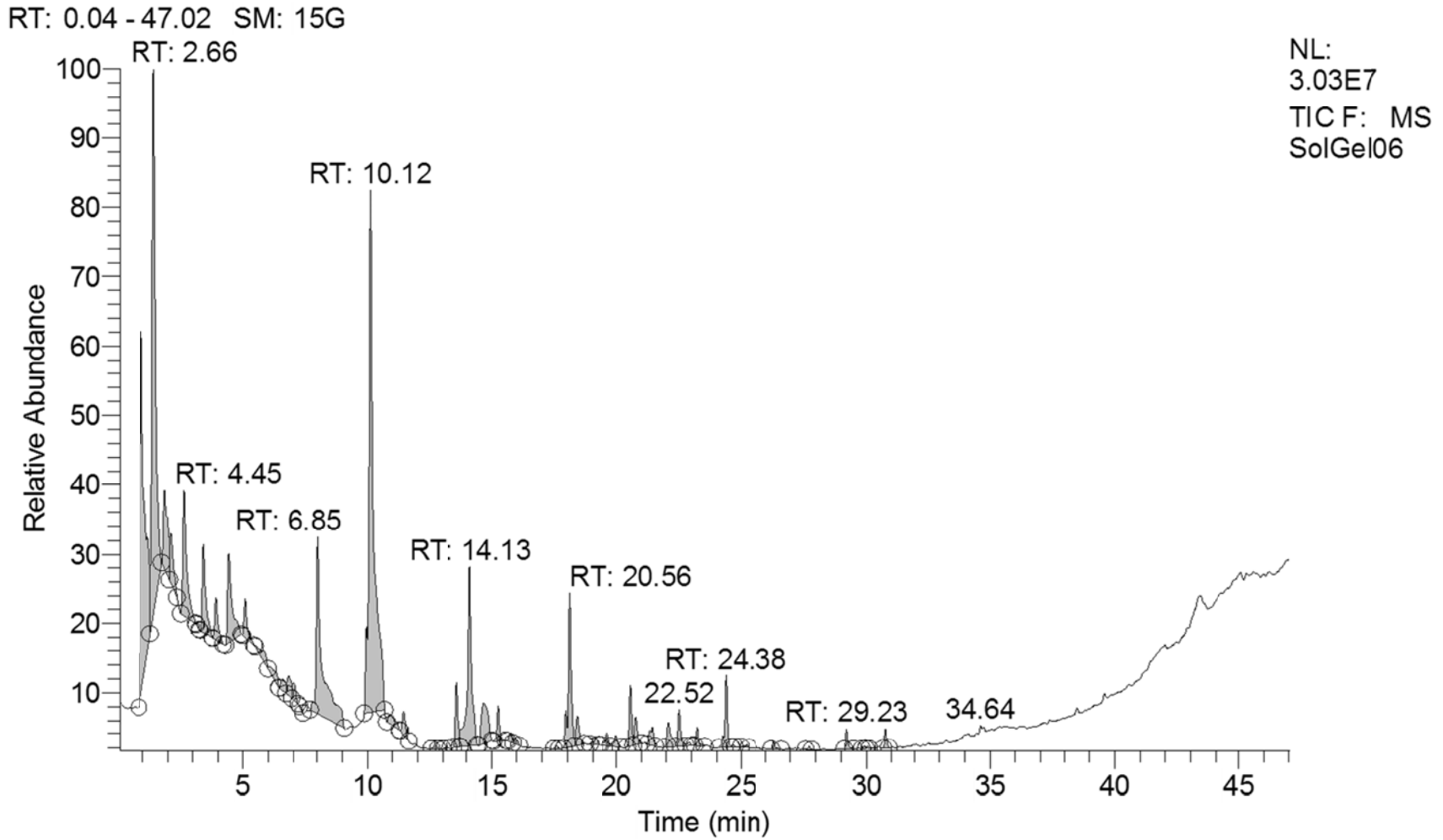

Figure 4.5 Chromatogram of *C. joostei* in fat-free milk incubated at 25 °C

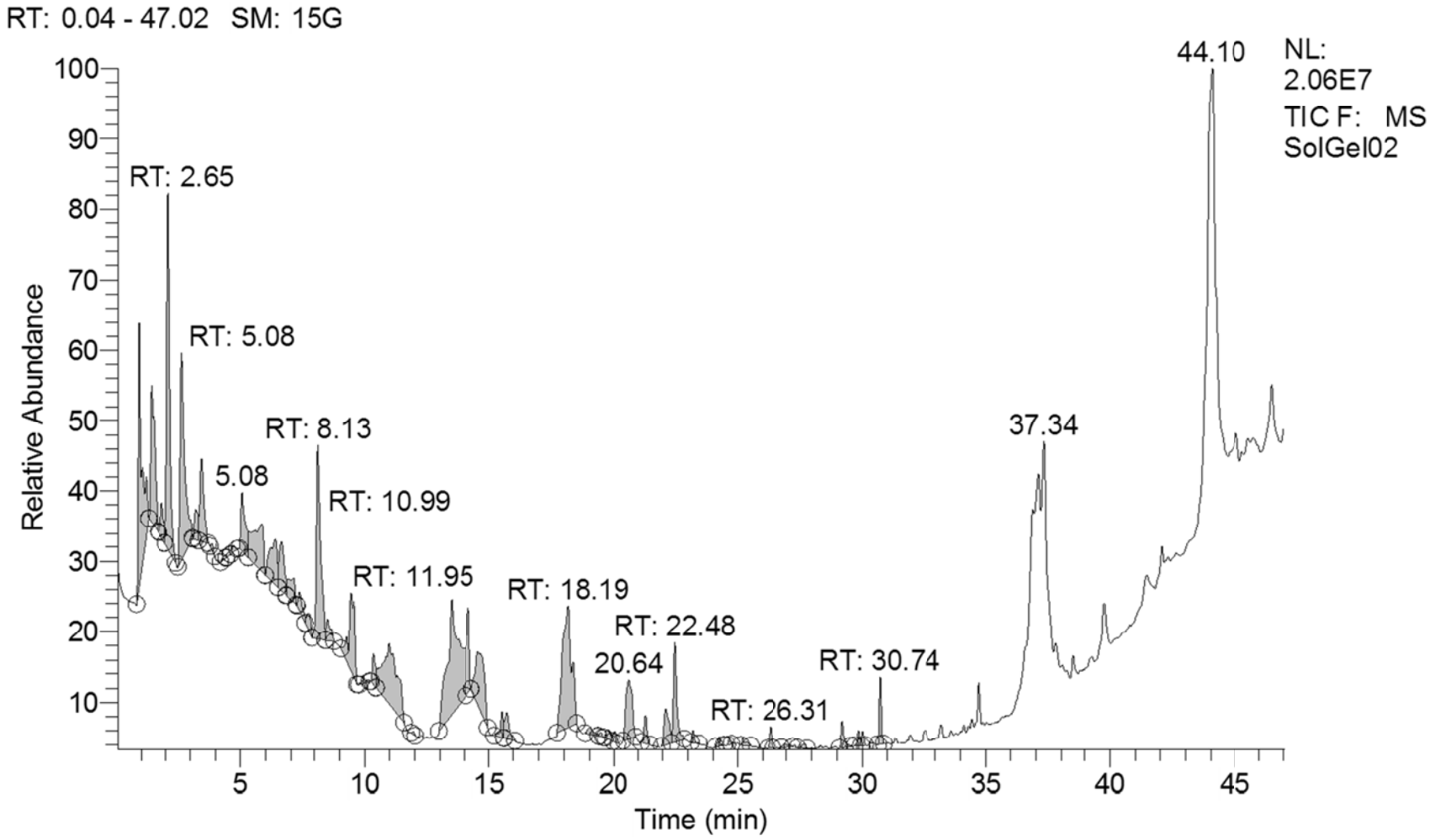

Figure 4.6 Chromatogram of *Ps. fluorescens* in fat-free milk incubated at 25 °C

Hexanoic acid and 2-heptanone could be responsible for the “blue cheese” odour that was observed in the samples during the sensory analysis (Lecanu *et al*., 2002). Ketones, that were present in many of the samples, are secondary products of lipolysis formed from the dehydration of keto acid – a product of lipolysis (Wang & Xu, 2009). They not only contribute to the fruity and floral odours, but could also diminish the sharp flavour of fatty acids due to their high concentrations and extremely low sensory thresholds (Wang & Xu, 2009). Acetone has been reported to be a component of the normal fresh raw milk odour (Nursten, 1997). Primary alcohols are probably formed by microbial reduction of the respective aldehydes and it is unlikely that they contribute to the normal odour of fresh milk, considering their relatively low concentrations (Moio *et al.*, 1993).

4.4 Conclusions

High levels of lipolysis were observed for all the inoculated samples. Although differences between the different sample treatments were observed, these differences were not significant enough to differentiate between the spoilage potential of *C. joostei* and *Ps. fluorescens* in the different milk samples. The low levels of lipid oxidation observed for all the samples and the fact that there were no significant differences between the samples of fat-free and full cream milk also proves that this method cannot be used to distinguish between the two organisms. Sensory evaluation on the other hand showed clear differences between the two organisms. Sensory scores attributed to *C. joostei* were significantly higher than those of the control samples and the samples inoculated with *Ps. fluorescens*. The odours described for the two organisms also showed distinct differences which could be helpful when indentifying the organism responsible for spoilage in milk.

Volatile compounds produced by the two organisms were identified. Acetic acid, butanoic acid, dichloromethane, 2-heptanone, hexanoic acid and 2-pentanone were present in all the inoculated samples. Isovaleric acid could be responsible for the production of the smelly feet odour observed during sensory analysis, while the ketones could have produced the fruity and flowery odours. Fatty acids are responsible for the sour odours. The results obtained from the analysis of the volatile

compounds did not show clear differences that can be used to distinguish between the spoilage potential of the two organisms in the different milk samples.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Spoilage of a food product is characterized by changes that render it unacceptable to the consumer from a sensory point of view (Gram *et al*., 2002). Microbial spoilage may be evident as visible growth (slime, colonies), as textural changes or as off-flavours and –odours. In refrigerated raw milk, spoilage is generally caused by the growth and activity of psychrotolerant bacteria (Champagne *et al*., 1994). The production of enzymes, particularly proteases and lipases, by these bacteria is the main mechanism of spoilage in refrigerated milk (Sørhaug & Stepaniak, 1997).

Various *Chryseobacterium* species, previously known as *Flavobacterium* species, have been reported to cause spoilage of food products. Food products that have been reported to contain *Chryseobacterium* include fish, meat and meat products, poultry and dairy products (Bernardet *et al.*, 2006). Other products include butter (Wolochow *et al*., 1942; Jooste *et al*., 1986a), creamed rice (Everton *et al*., 1968) and canned vegetables (Bean & Everton, 1969). *Flavobacterium* was found to cause bitter and fruity flavours in pasteurized, refrigerated milk and also discolouration and slime production in cottage cheese (Banwart, 1989).

To determine the spoilage potential of *Chryseobacterium* species in milk, the growth kinetics, protease and lipase activity, lipid oxidation, sensory effects and volatile compounds produced were investigated during this study and the results were compared to those of *Pseudomonas fluorescens* that is known as a major milk spoilage organism.

The specific growth rates and cardinal temperatures for *C. joostei*, *C. bovis* and *Ps. fluorescens* were determined by optical density measurements of the organisms grown in nutrient broth at temperatures ranging between 4 and 50 °C. It was found that *C. joostei* had the highest maximum specific growth rate followed by *Ps. fluorescens* and *C. bovis* in that order. *Chryseobacterium joostei* had a maximum growth rate of 0.98 h^{-1} between the temperatures of 28.9 and 31.3 °C. Growth was

still observed at 4 °C with a maximum growth temperature of 38.7 °C. *Chryseobacterium joostei* was previously found to grow at 5 °C but not at 37 or 42 °C (Hugo *et al*., 2003). For *C. bovis,* a maximum growth rate of 0.62 h^{-1} was observed at an optimum temperature of 30.7 °C. Growth was observed at 4 °C and the maximum growth temperature was found to be 40.2 °C. Growth between 7 and 37 °C was noted in the description of *C. bovis* by Hantsis-Zacharov *et al*. (2008a). A maximum specific growth rate of 0.85 h^{-1} was observed at 27.7 °C for *Ps. fluorescens*. Growth still occurred at 4 °C and this species had a maximum growth temperature of 36.6 °C. *Pseudomonas fluorescens* has an optimum growth temperature of between 25 and 30 °C (Pelleroni, 2005). At temperatures below 7 °C, *Ps. fluorescens* showed the highest growth rate indicating that it might be more psychrotolerant than the other species. It can, however, be said that all three organisms are psychrotolerant mesophiles due to the fact that growth was observed at 4 °C for all three organisms with optimum temperatures ranging between 25 and 30 °C.

Arrhenius plots for the three species showed that *C. joostei* had the lowest activation energy followed by *Ps. fluorescens* and *C. bovis*. This means that *C. joostei* was the least sensitive to temperature changes that may take place, while *C. bovis* was the most sensitive of the three tested organisms. From these results it can be concluded that *C. joostei* has the ability to grow over a wider temperature range without being affected by changes in the temperature and that this organism can therefore potentially cause spoilage over a wider range of temperatures than *Ps. fluorescens*.

The protease activity per mg protein was determined for *C. joostei*, *C. bovis* and *Ps. fluorescens*. The three organisms were incubated in UHT milk at temperatures ranging between 4 and 50 °C and activity was determined by means of the azocasein method. *Chryseobacterium joostei* showed the highest activity followed by *C. bovis* while *Ps. fluorescens* showed significantly lower activity than the *Chryseobacterium* species. All three species showed protease activity at 4 °C, with *C. joostei* having the highest optimum temperature for protease activity at 41.9 °C, followed by that of *Ps. fluorescens* at 31.2 °C and *C. bovis* at 28.0 °C. These results give a strong indication that *C. joostei* may have the ability to cause even more proteolytic spoilage than *Ps. fluorescens* over a wider temperature range when present in milk.

Determination of the lipolytic activity of *C. joostei* and *Ps. fluorescens* were done by measuring the amount of free fatty acids (FFAs) produced in fat-free and full cream milk incubated at either 4 or 25 °C. Both organisms showed high levels of lipolysis. Statistical analysis of the results showed that *Ps. fluorescens* incubated at 4 °C and *C. joostei* at 25 °C produced significantly higher amounts of FFA's than the uninoculated control sample in the fat-free milk. In the full cream milk, *C. joostei* grown at 25 °C showed levels of lipolysis that were significantly higher than the control and 4 °C samples. *Pseudomonas fluorescens* incubated at 25 °C also showed significantly higher FFA levels than the control sample. No significant differences were observed when comparing the fat-free and full cream milk samples. Lipopolytic activity is not often observed in flavobacteria, although activity has been reported for some species (Roussis *et al*., 1999).

Secondary lipid oxidation of the two organisms was determined using the TBA method. Very low levels of oxidation were observed for the samples. In the fat-free milk, the *C. joostei* samples incubated at 4 and 25 °C as well as the *Ps. fluorescens* sample incubated at 4 °C showed significantly higher values than the control samples. The *Ps. fluorescens* sample incubated at 25 ° C did not differ significantly from the control sample. There were also no significant differences between the different treatments in the fat-free and full cream milk or between milk types themselves. The reason for the very low TBARS values that were observed, may be that oxidation was still in progress at the primary level as indicated by the high levels of FFAs that were observed during the lipolysis study.

Sensory analysis on the fat-free and full cream milk inoculated with *C. joostei* and *Ps. fluorescens* showed that *C. joostei* produced stronger odours than *Ps. fluorescens*. All the samples that were inoculated with *C. joostei* differed significantly from the controls as well as from the *Ps. fluorescens* samples. It was found that sensory scores of the fat-free milk samples were slightly higher than those of the full cream samples. This may be as a result of higher protease activity in the fat-free milk (Deeth *et al*., 2002). Odours present in the different samples were described by the sensory panel. In the fat-free milk samples inoculated with *C. joostei* and *Ps. fluorescens*, odours described in each case included "smelly feet", "blue cheese" and "agar". At 25 °C, *C. joostei* also produced a "putrid" odour while *Ps. fluorescens*

produced “flowery” and “sour” odours. At 4 °C *Ps. fluorescens* produced “fruity” and “nutty” odours. Odours present in all the full cream samples were described as “smelly feet”, “sour” and “agar”. All *C. joostei* samples also produced “putrid” odours, while an odour resembling “blue cheese” was observed at 4 °C. The *Ps. fluorescens* sample at 4 °C produced “fruity” and “bitter” odours.

It has been reported that bacterial lipases are responsible for the production of “rancid” and “fruity” odours present in the milk (Forss, 1971; Hayes *et al*., 2002) while protease enzymes are responsible for the “unclean” and “putrid” odours (Mayerhofer *et al*., 1973; Shipe *et al.*, 1978). Isovaleric acid may be responsible for the “smelly feet” odour observed in the samples (Jooste *et al*., 1986a).

Volatile compounds produced by *C. joostei* and *Ps. fluorescens* were determined using headspace SPME-GC/MS analysis. The identified compounds included ketones – responsible for fruity and floral odours (Wang & Xu, 2009); fatty acids, including isovaleric acid that is responsible for the “smelly feet” odour (Amoore, 1977; Jooste *et al*., 1986a) and alcohols, that are unlikely to contribute to the odour of milk (Moio *et al.*, 1993). Acetic acid, butanoic acid, dichloromethane, 2-heptanone, hexanoic acid and 2-pentanone were present in all the inoculated samples. Isovaleric acid was present in all the inoculated samples, except in the fat-free *Ps. fluorescens* samples incubated at both 4 and 25 °C. The only sample that did not contain acetone, was the full cream milk sample incubated with *C. joostei* at 25 °C. Acetone concentrations in fat-free and full cream milk were higher in the control samples than in the inoculated samples. Results however, did not show clear differences between the two organisms in the different milk samples.

When considering all the results obtained during this study, it can be concluded that *Chryseobacerium joostei* should also be considered as a major spoilage ~~causing~~ organism in the dairy industry. This organism may be able to cause even more damage than *Pseudomonas fluorescens*, which is generally regarded as the major spoilage organism in refrigerated milk, due to the higher overall growth rate, protease activity and sensory scores that were observed in the *C. joostei* inoculated milk samples. It can also be stated that lipid oxidation and lipolytic activity cannot be used to distinguish between the spoilage potential of the two organisms, while sensory

analysis of the odours produced may be useful as a tool to identify the organisms when they are present in milk.

Future research may include:

- A study of the growth kinetics and spoilage characteristics determined during this study for other *Chryseobacterium* species that may be involved in food spoilage.
- A study of the effect of *Chryseobacterium* species in other food products.
- Determining the effect of other parameters including pH, water activity, oxygen concentration and nutrient content of the food on the growth of *Chryseobacterium* species in order to predict spoilage potential.
- Determining the effect of heat treatment on the stability of the protease enzymes produced by *Chryseobacterium* species.

CHAPTER 6

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SUMMARY

Chryseobacterium species have been reported to cause spoilage in food products such as fish, meat, poultry and dairy products. Some of these *Chryseobacterium* species can grow at temperatures below 7 °C which gives them the ability to grow and cause spoilage in milk kept at refrigerated temperatures. Protease and lipase enzymes produced by psychrotolerant species in milk are responsible for spoilage by the production of off-odours and –flavours. The aim of this study was to determine the growth and spoilage characteristics of *Chryseobacterium* species in milk and compare the results to those of *Pseudomonas fluorescens* which is regarded as the major organisms causing spoilage in milk.

The specific growth rates and cardinal temperatures for *C. joostei*, *C. bovis* and *Ps. fluorescens* were determined by optical density measurements. *Chryseobacterium joostei* had the highest maximum specific growth rate followed by *Ps. fluorescens* and *C. bovis*. All three organisms were able to grow at 4 °C, but *Ps. fluorescens* showed the highest growth rate temperatures below 7 °C. All three organisms can thus be classified as psychrotolerant mesophiles due to the fact that growth was observed at 4 °C with optimum temperatures ranging between 25 and 30 °C. Arrhenius plots for the three species showed that *C. joostei* had the lowest activation energy followed by *Ps. fluorescens* and *C. bovis*. This means that *C. joostei* was the least sensitive to temperature changes that may take place while *C. bovis* was the most sensitive of the three tested organisms. The protease activity per mg protein for the three organisms was determined with the azocasein method. *Chryseobacterium joostei* showed the highest activity followed by *C. bovis* while *Ps. fluorescens* showed significantly lower activity than the *Chryseobacterium* species. All three species showed protease activity at 4 °C.

Determination of the lipolytic activity of *C. joostei* and *Ps. fluorescens* were done by measuring the amount of FFAs present in fat-free and full cream milk incubated either at 4 or 25 °C. High levels of lipolysis were observed for all the inoculated samples. Although differences between the different sample treatments were observed, these differences were not significant enough to differentiate between the

spoilage potential of *C. joostei* and *Ps. fluorescens* in the different milk samples. Secondary lipid oxidation of the two organisms was determined with the TBA method. Although inoculated milk samples demonstrated higher levels of oxidation compared to non-inoculated samples, there were no signigicant differences between any of the inoculated milk samples.

Sensory analysis was done on the samples and *C. joostei* produced stronger odours than *Ps. fluorescens*. All the samples that were inoculated with *C. joostei* scored significantly higher spoilage scores than the control samples as well as the *Ps. fluorescens* samples. The odours described for the two organisms also showed distinct differences. In addition to the “smelly feet”, “blue cheese” and “agar” odours described for all the inoculated samples, *C. joostei* also produced “putrid” and “sour” odours while *Ps. fluorescens* produced odours described as “fruity”, “nutty” and “bitter”.

The volatile compounds produced by *C. joostei* and *Ps. fluorescens* were identified with headspace SPME-GC/MS analysis. Ketones, alcohols and fatty acids were the main compounds produced. Ketones are responsible for the production of fruity and floral odours. Fatty acids produce sour odours, with isovaleric acid being responsible for the smelly feat odour. Alcohols do not usually contribute to the odour production in milk. The results could not be used to differentiate between the two organisms in the different milk samples.

Keywords: *Chryseobacterium*, milk, *Pseudomonas fluorescens*, spoilage, growth, temperature, protease, lipase.

OPSOMMING

Daar is al gerapporteer dat *Chryseobacterium* spesies bederf veroorsaak in verskeie voedselprodukte soos vis, vleis, hoender en suiwel produkte. Sommige van hierdie spesies kan by temperature laer as 7 °C groei, wat hulle in staat stel om bederf te veroorsaak in melk wat by yskas temperature gestoor word. Proteases en lipases wat deur psigrotolerante bakterieë geproduseer word is verantwoordelik vir bederf deur die vorming van afgeure en –reuke. Die doel van hierdie studie was om die groei en bederf eienskappe van *Chryseobacterium* spesies te bepaal en die resultate te vergelyk met dié van *Pseudomonas fluorescens*. Laasgenoemde is bekend as die hoof organisme wat bederf in melk veroorsaak.

Die spesifieke groeitempo en kardinale temperature van *C. joostei*, *C. bovis* en *Ps. fluorescens* is deur optiese digtheid metings bepaal. *Chryseobacterium joostei* het die hoogste maksimum spesifieke groeitempo gehad, gevolg deur *Ps. fluorescens* en *C. bovis*. Al drie die organismes was in staat om by 4 °C te groei, maar *Ps. fluorescens* het die hoogste groeitempo gehad by temperature laer as 7 °C. Die organismes kan dus al drie geklassifiseer word as psigrotolerante mesofiele as gevolg van die feit dat groei by 4 °C plaasgevind het en optimum groei temperature tussen 25 en 30 °C was. Arrhenius grafieke vir die drie spesies het getoon dat *C. joostei* die laagste aktiveringsenergie het, gevolg deur *Ps. fluorescens* en *C. bovis*. Hierdie bevindinge dui daarop dat *C. joostei* minder sensitief is vir verandering in temperatuur, terwyl *C. bovis* die mees sensitiefste spesie was. Die protease aktiwiteit per mg proteïen vir die drie organismes is deur middel van die azocasein metode bepaal. *Chryseobacterium joostei* het die hoogste aktiwiteit gehad, gevolg deur *C. bovis*. *Pseudomonas fluorescens* het aansienlike laer aktiwiteit gehad. Al drie die species het by 4 °C steeds aktiwiteit getoon.

Bepaling van die lipolitiese aktiwiteit van *C. joostei* en *Ps. fluorescens* is gedoen deur bepaling van die aantal vrye vetsure. Hoë vlakke van lipoliese is waargeneem vir al die geïnokuleerde monsters. Alhoewel verskille tussen die monsters waargeneem is, was hierdie verskille nie betekenisvol genoeg om te onderskei tussen die bederf potensiaal van *C. joostei* en *Ps. fluorescens* in die verskillende melk monsters nie. Sekondêre lipied oksidasie van die twee organismes is bepaal deur die TBA metode.

Alhoewel die geïnokuleerde melk monsters hoër oksidasie vlakke getoon het in vergelyking die monsters wat nie geïnokuleer was nie, was daar geen beduidende verskille tussen enige van die geïnokuleerde monsters nie.

Sensoriese analise is op die monsters gedoen en *C. joostei* het sterker reuke geproduseer as *Ps. fluorescens*. Al die monsters wat met *C. joostei* geïnokuleer was, het beduidend hoër bederf tellings getoon as die kontrole en *Ps. fluorescens* monsters. Die reuke wat beskryf is vir die twee organismes het ook beduidende verskille getoon. Bo en behalwe die “stink voete”, “bloukaas” en “agar” reuke wat vir al die monsters beskryf is, het *C. joostei* ook “vrot” en “suur” reuke geproduseer, terwyl *Ps. fluorescens* “vrugte”, “neut” en “bitter” reuke geproduseer het.

Die vlugtige verbindings wat deur *C. joostei* en *Ps. fluorescens* geproduseer is, is geïdentifiseer deur middel van ‘headspace’ SPME-GC/MS analise. Ketone, alkohole en vetsure was die hoof verbindings wat geproduseer is. Ketone is gewoonlik verantwoordelik vir die vorming van vrugte en blomme geure, terwyl vetsure verantwoordelik is vir die suur reuke. Alkohole dra normaalweg nie by tot die reuk produksie in melk nie. Die resultate kan nie gebruik word om te onderskei tussen die twee organismes in die verskillende melk monsters nie.

Kernwoorde: *Chryseobacterium*, melk, *Pseudomonas fluorescens*, bederf, groei, temperatuur, protease, lipase.