Mechanisms of Defence Against Tannins by

Streptococcus caprinus

Lisa Anne O'Donovan

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For my parents

Pat and Terry O'Donovan.

Without their love, support and their unwavering belief in me,

the completion of this thesis would not have been possible.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Lisa Anne O'Donovan

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Foreword

Tannins are large polyphenolic compounds found in a wide variety of plants. They contain a large number of phenolic residues that allow them to form effective cross-links with protein and other macromolecules. The role of tannins is diverse and their presence in a number of important forage plants may have a large influence on the nutritive value of these plants. In grazing ruminants, high concentrations of tannins adversely affect ruminant nutrition by forming insoluble tannin-protein/mineral complexes which are poorly digested in the rumen and lower digestive tract. Tannins have also been shown to inhibit microbial growth and metabolism. The inhibition of ruminant digestion by dietary tannins is a significant factor limiting livestock production in many areas of the world.

Not all animals however, appear to be adversely affected by tannins. It has been observed that some, for example wild goats and other feral ruminants appear to thrive on plants containing high concentrations of tannins and it has been proposed that these animals harbour novel microorganisms that aid in their ability to tolerate tannins. One such organism, *Streptococcus caprinus* was isolated from the rumen of a feral goat grazing tannin-rich *Acacia* species. Characterisation of this bacterium may provide essential insight into the ability of some ruminants to tolerate tannins.

Abstract

This thesis aimed to identify and characterise the mechanism(s) by which *Streptococcus caprinus* tolerated the presence of tannins in its environment. *S. caprinus*, isolated from the rumen contents of feral goats known to be grazing tannin rich material was found to tolerate high (>7% w/v) levels of tannins in its growth medium compared with the more common ruminal streptococcal species, *S. bovis* (<0.75% w/v). *S. caprinus* was also found to produce zones of clearing when grown on nutrient agar containing the hydrolysable tannin, tannic acid (0.5% w/v). Growth studies revealed the lag period of *S. caprinus* increased and the rate of growth decreased with increasing tannin concentration. Fractionation of tannic acid determined that large molecular weight components were responsible for growth inhibition. *S. caprinus* was found to be more tolerant to the presence of simple phenolic acid monomers than *S. bovis*, although the lag periods were similarly increased. The effect of tannic acid on lactate production, enzyme activities (lactate dehydrogenase and proteinase) and bacterial protein production has been described. Tannic acid-substrate binding studies were conducted to determine the conditions required for association and dissociation of these complexes. Further characterisation determined that *S. caprinus* was able to degrade gallic acid, the major phenolic monomeric component of tannic acid while *S. bovis* could not. Characterisation of one enzyme present in the degradative pathway of gallic acid, gallate decarboxylase was achieved. This enzyme was found to be specific for gallic acid and tannic acid. Gallate decarboxylase activity was induced by the presence of tannic acid and gallic acid but not by other phenolic acid compounds. Electron microscopic analysis

revealed that tannins affected the size and shape of both *S. caprinus* and *S. bovis*. Extracellular material was observed surrounding *S. caprinus* and interconnecting bacterial cells. An increase in the amount of tannins resulted in a proportional increase in extracellular material produced by *S. caprinus*. Characterisation of this material determined it to be exopolysaccharide. Removal of the exopolysaccharide increased *S. caprinus* susceptibility to tannins.

Thus it appears *S. caprinus* employed at least two mechanism to overcome the potential detrimental effects of tannins: degradation of tannic acid and production of a protective exopolysaccharide.

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Abbreviations

aka	also known as
BSA	bovine serum albumin
BSTFA	n-o-bis (trimethylsilyl)-trifluroacetamide
CSLM	confocal scanning laser microscopy
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
e.g.	example
EPS	exopolysaccharide
et al.	*et alia* (and others)
FESEM	field emission scanning electron microscopy
GAME	gallic acid methyl ester
GLC	gas-liquid chromatography
ie	that is
IPTG	isopropylthiogalactoside
LDH	lactate dehydrogenase
mBHI	modified brain heart infusion
MS	mass spectroscopy
NAD	b-nicotinamide adenine dinucleotide
NADH	b- nicotinamide adenine dinucleotide (reduced form)
nd	not determined
ng	no growth
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PVP	polyvinylpyrolidone
PEG	polyethylene glycol
SDS	sodium diacyl sulphate
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TMS	trimethylsilyl

Symbols and units

%	percentage
°C	degree Celsius
≅	approximately equal to

±	plus or minus
µF	micro Farad (capacitance)
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
nm	nanometre
pM	picomolar
pmoles	picomoles
/	per
A	absorbance
α	alpha
β	beta
cm	centimetre
Da	Dalton
G	gauge
g	grams
h	hour
bp	base pairs
Kb	kilobase
kDa	kilodaltons
KeV	kilo electron volts
kg	kilogram
KV	kilovolts
l	litre
log	logarithmic
M	molar
Mb	megabase
mg	milligram
min	minute
ml	millilitre
mM	millimolar
N	normal (molar)
nm	nanometre
OD	optical density
PSI	pounds/square inch
rpm	revolutions per minute
sec	second
std	standard
U	units
V	volts
v/v	volume/volume
W	watt
w/v	weight/volume
x g	times gravitational force

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Introduction and review of literature

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Introduction

Ruminant animals gain their energy from the efficient digestion of plants. The presence of a large population of microorganisms existing in symbiotic association with the ruminant animal are responsible for this breakdown and enables the animal to exist on fibrous, low protein feeds which are not readily digestible by most non-ruminant animals. These forage plants sometimes contain toxic or antinutritive substances, which inhibit digestion and may lead to a decrease in animal productivity.

This project focuses on one class of plant secondary compounds, tannins, which are known to be potentially toxic to ruminants and may adversely affect ruminal nutrition. In the following literature review, a general overview of ruminant digestion is provided, followed by a discussion of tannins and their impact on ruminant digestion.

Review of literature

1.1 Ruminant digestion and metabolism

The ruminant digestive system is considered the most advanced among herbivore populations. The stomach of the ruminant is divided into four compartments: the rumen, the reticulum, omasum and the abomasum. The rumen constitutes 10-15% of the total mass of the animal (Krause and Russell 1996) and harbours a large population of predominantly anaerobic and facultative anaerobic microorganisms (bacteria, protozoa, fungi, mycoplasmas, bacteriophage and other viruses) which exist in a symbiotic relationship with the animal. The microorganisms are primarily

responsible for the breakdown of foodstuffs. Large, fibrous plant material is retained in the reticulo-rumen for prolonged periods thereby ensuring that the resident microflora have sufficient opportunity to degrade the cellulosic cell wall constituents (Hacker and Ternouth 1987) such as cellulose, hemicellulose and pectins. During rumen fermentation, foodstuffs are converted into short chain, volatile fatty acids (VFA's), ammonia, methane, carbon dioxide, cell material, and heat. Animal performance is dependent on the balance of these products, and the types and activities of microorganisms in the rumen ultimately control this balance. The volatile fatty acids are used by the animal as an energy source, while the microbes serve as an important source of amino acids for protein synthesis. Ammonia, methane and heat by contrast represent a loss of either nitrogen or energy to the animal (Russell and Cook 1995). About 60% of digestible dry matter is broken down in the rumen and this results in a loss to the animal of about 70% of the potentially available plant protein and most of the plant fibre (Waghorn *et al* 1990). Carbon dioxide and methane are lost by eructation (belching) and the volatile fatty acids are mainly absorbed through the rumen wall. The microbial cells, together with undegraded food components, pass to the abomasum and intestines where they are digested by enzymes secreted by the host animal. The peptides and amino acids from this digestion are then absorbed into the blood stream of the animal.

The rumen environment is highly buffered to a pH of around 6 to 7 and the steady supply of food along with the continuous removal of fermentation products and food residues helps to maintain relatively constant conditions in which extremely dense populations of microbes develop. The composition of rumen microflora however,

changes with time through turnover of the digesta and the nature of the diet. Microbes with specific advantages over others dominate under favourable conditions. The most predominant microbial group within the rumen environment are bacteria, which can achieve populations of 10^{10}- 10^{11} /ml (Krause and Russell 1996). Bacterial diversity within the rumen ecosystem is large: Bryant and Burkey (1963) described 22 genera and 63 species. Recent evidence indicates another level of diversity; genetic diversity within a given phenotype may be considerably greater than species diversity (Krause and Russell 1996).

1.2 Tannins and other phenolic compounds

1.2.1 Biosynthesis and occurrence

The term tannin was introduced in 1796 and referred to substances with the ability to tan leather. It is now generally used to include any naturally occurring compound of high enough molecular weight (>500) and containing a large number of phenolic groups to enable it to form effective cross-links with protein and other molecules (Kumar and Singh 1984, Spencer *et al*, 1988; Haslam 1989; Reed 1995). Tannins are widespread in the plant kingdom (pteridophytes, gymnosperms and angiosperms), and have been shown to accumulate in the cell vacuoles of a number of plant tissues. However, they are often found in higher concentrations in woody, lignified tissues (Bernays *et al* 1989, Haslam 1989; Jean-Blain 1998; Bhat *et al* 1998). Tannins are considered to be plant secondary substances as they are not involved in metabolic pathways. The precursors for all phenolic compounds are synthesised in the endoplasmic reticulum of the plant cell via either the shikimic acid or the acetate-malonate metabolic pathways. With the shikimate-derived aromatic compounds,

monohydric phenols are characteristically p-hydroxy compounds, whereas acetate-derived phenols generally possess a hydroxy group in the meta position. The phenolic compounds are then primarily deposited in cell vacuoles or in the cell wall (lignin). Synthesis of the aromatic amino acids tyrosine and tryptophan occurs via the shikimate pathway and this suggests a close relationship of phenolic biosynthesis with normal plant metabolism (Jung and Fahey 1983).

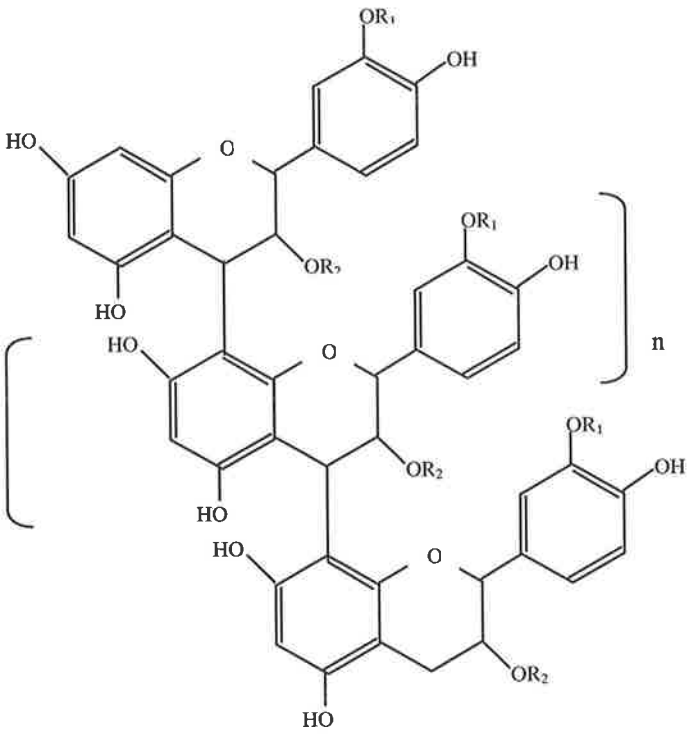
The structure of tannins can vary with their location in the plant structure and these variations may result from abiotic oxidations, epimerisation, depolymerisation or polymerisation or condensations in plant tissue (Reed 1995). The concentrations of tannins and lignin are reported to be regulated by an environmental component as well as a genetic one; increasing nutrient stress in the soil produces increases of similar magnitude in the concentration of both polyphenolics (Barry and Manley 1986). In arid and semi arid regions, the concentration of tannins and lignin in plants is much higher than in temperate zones (Barry and McNabb 1999). Also, higher levels of all phenolics, including tannins and lignins have been found in plants growing in the sun relative to shade. This may indicate that the polymers play a role in drought tolerance (Barry and McNabb 1999). Tannins may also act as defensive compounds by protecting the plants possessing them from damage by herbivorous organisms either by direct action against grazing or by protecting damaged areas from insect attack by increasing the concentrations of tannins in these regions (Augner 1995; Fassler and Lascano 1995). Phenol synthesis increases in plants in response to infection and the eventual accumulation of toxic levels of these compounds is considered an important

mechanism through which plant tissue limits the spread of pathogens (Nicholson *et al* 1986; Woodward and Coppock 1995).

Tannins have been divided into two broad groups, the hydrolysable tannins and the condensed tannins based on structural differences and their reactivity towards hydrolytic reagents. Both gross chemical differences, such as those distinguishing condensed tannins from gallotannins, and subtle differences, such as molecular weight or steriochemical configuration (Clausen *et al* 1990; Jean-Blain 1998) can influence the biological activity of tannins.

Condensed tannins or the proanthocyanidins are the most widely distributed tannins in vascular plants and are the principal forage tannins. They are synthesised via the acetate-malonate pathway, by condensation of flavonoid precursors such as catechin, epicatechin or gallocatechin (Figure 1.1, McLeod 1974). While the proanthocyanidins are not readily degraded, upon heating in acid solution they may be converted to anthocyanidins, cyanidin and delphinidin (Kumar and Vaithiyanathan 1990, Bernays *et al* 1989). Condensed tannins have been demonstrated in legume pasture species such as *Lotus corniculatus* (Birdsfoot trefoil) and *Lotus pedunculatus* (Lotus major), in *Onobrychis viciifloia* (Sainfoin), in several species of acacia, such as *Acacia aneura* (Mulga), *Acacia saligna* (Degen *et al* 1995) and in sorghum grain (Kumar and Singh 1984). Condensed tannins exhibit a wide range of molecular weights, ranging from about 2000 in the sorghum grains (Kumar and Singh 1984) and 6000 in lotus to 28 000 in sainfoin (Waghorn 1990).

Figure 1.1 Condensed tannin structure



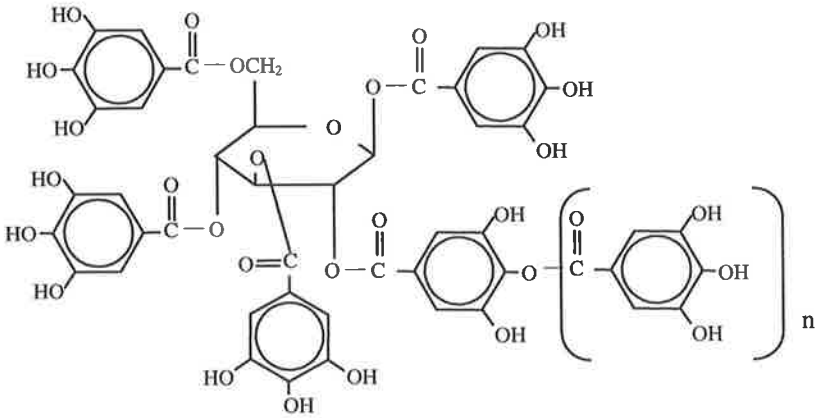
n = 4 – 5

$R_1 = R_2 = H$	Catechin
$R_1 = R_2 = C\text{-}OH$	Gallocatechin

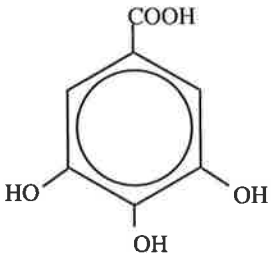
Hydrolysable tannins have a central carbohydrate core, usually glucose, which serves as a polyalcohol to which a number of phenolic carboxylic acids such as gallic acid or hexahydroxy-diphenic acid, are bound by ester linkages (Figure 1.2, McLeod 1974, Bernays *et al* 1989; Bhat *et al* 1998). Hydrolysable tannins can be readily broken down by certain enzymes and hydrolysed by acids and bases (Kumar and Vaithiyanathan 1990). Hydrolysis by hot mineral acid yields the sugar and the constituent phenolic acid and serves as the basis for further subdivision of these tannins, eg. hydrolysis of gallotannins yield gallic acid (Figure 1.2) and ellagitannins produce hexahydroxy-diphenic acid that is isolated as its stable dilactone, ellagic acid (Figure 1.2, Bernays *et al* 1989). Tannic acid is a gallotannin consisting of esters of gallic acid and glucose, containing up to 5 galloyl groups esterified directly to the glucose molecule (mono-, di-,...pentagalloyl glucose), with additional galloyl groups esterified to the core galloyl groups (hexa-, heptagalloyl glucose,...) also known as B-penta-O-galloyl-D-glucose. Tannic acid is the model compound for this group of tannins and is reported to be the best commercially available tannin standard but is also been shown to contain variable amounts of low-molecular weight galloyl esters and of non-tannin material. This classification system is likely to become less useful as more is learned about tannin chemistry. Catechin gallates, which consist of gallic acid esterified to catechin, occupy an intermediate position in the tannin hierarchy as they have properties of both hydrolysable and condensed tannins. These tannins are quite common in tropical shrub legumes (Mueller-Harvey *et al* 1987; Reed 1995) and tea leaves (Graham 1992). Recently gallic acid esters of proanthocyanidins have also been described (Porter 1994).

Figure 1.2 Hydrolysable tannin structure

Gallotannin

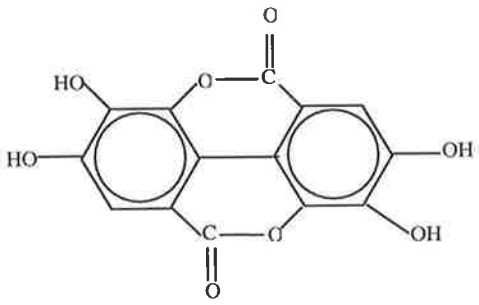


n = 1, 2, 3



Gallic acid

Ellagitannins



Ellagic acid

The low molecular weight phenolic acids are largely present as intermediary metabolites of tannins and lignin and are usually found in close association with these larger polyphenolics. These compounds include benzoic acid derivatives (C1-C1): p-hydroxybenzoic, gallic, vanillic, protocatechuic and syringic acids as well as the substituted cinnamic acids or phenylpropanoid compounds (C6-C3): p-coumaric, ferulic, sinapic and caffeic acid. Almost all occur in a bound form in plant cells (Krumholz and Bryant 1986, 1988). This combined form usually involves the linking of the hydroxyl group of the phenol to a glucose molecule, ie. phenolic glycosides, or as esters of aliphatic alcohols, phenols, phenolic acids and alcohols, alkaloids, flavonoids, lignins, tannins and sugars. They may also, however, be found alone and are reported to be a major component of many tropical and semi-tropical plant species (Harborne 1990; Murray *et al* 1996).

1.2.2 Mode of action

Natural tannins are powerful reducing agents and tend to absorb oxygen, especially in alkaline solutions, forming strongly coloured oxidation products (McLeod 1974). Both simple phenols and polyphenols form weak, reversible associations with a range of substrates including cellulose, proteins (Hagerman and Klucher 1986; Waghorn *et al* 1990, Jones and Mangan 1977; Reed 1995), enzymes (Kumar and Singh 1984; Ahmed *et al* 1991; Horigome *et al* 1988; Makker *et al* 1988; DeBruyne *et al* 1999), fats, nucleic acids and amino acids (Mole and Waterman 1987; Chung *et al* 1998). The bonding between the tannin and substrate molecules has been described as "dynamic" with individual linkages being continually broken and reformed in a random manner (McLeod 1974). Hydrogen bonding has been proposed as the most

likely mechanism for forming the linkages and this explains the reversibility and low specificity of the phenolic-substrate complex. Tannins have been found to have greater affinity for proteins, especially proline-rich proteins, than for cellulose and the other aforementioned substrates. The strength of these complexes depends on characteristics of both tannin and protein: molecular weight, tertiary structure, isoelectric point, and compatibility of binding sites (Hagerman and Butler 1981, Martin and Martin 1984, Martin *et al* 1985). The specificity of interaction is an inverse function of size, conformation and charge of the protein molecules (Hagerman and Butler 1981; Halsam 1989). The large number of phenolic groups in the tannin molecule provide many points of attachment for linkage by hydrogen bonding with peptides of adjacent protein chains to form protein-tannin complexes. Any ionised groups present are thought to introduce either attractive or repulsive forces, which help to reinforce or weaken the effect of hydrogen bonding. The net effect determines the stability of the tannin-protein complex. The protein-tannin association gives a compound that is less hydrophilic than the protein itself and aggregation eventually results in precipitation from solution. In an excess of either proteins or tannins, cross-linking between separate protein molecules via tannins is not completed and soluble tannin-protein complexes can occur without precipitation. The protein-precipitating capacity of the tannins depends on the molecular weight of the tannins and increases progressively from 500 up to 12, 000 Da (McLeod 1974). However when the molecular weight is very large (>20, 000 Da), tannins have reduced affinity for proteins and lose their protein precipitating capacity (Kumar and Singh 1984, Barry and Manley 1986, Zhu *et al* 1997). The tannin-protein complexes have been shown to be pH dependent in the range of pH 5 to 8. Thus both the protein and polyphenols

can, in principle, be recovered unchanged from the complex at pHs outside this range. However, this reversibility decreases with time (Burritt *et al* 1987; Perez-Maldonado *et al* 1995) and under some conditions, eg. alkaline pH which ionises the phenolic hydroxyl and destroys the hydrogen bonding ability of the tannin (Hagerman and Butler 1989), or oxygen. In these cases, the polyphenols may become oxidised to a quinone and may then form covalent linkages with the amino or sulphydryl groups of proteins, making the association irreversible. Detergents that disrupt hydrophobic interactions can also dissociate the complexes formed through hydrogen bonding.

1.2.3 Analytical methods

Isolation and quantification of soluble phenolics and tannins in plants are essential to studies on their nutritional and ecological effects (Martin and Martin 1982, Waterman and Mole 1994; Nelson *et al* 1997, 1998). However the commonly used methods of colorimetric analysis have many problems (Martin and Martin 1982). Equating phenolics with tannins is also a problem, because many phenolics that react with analytical reagents are not tannins by strict definition (they do not precipitate protein), but they can be converted to tannins by oxidative polymerisation. Therefore it is necessary to estimate total phenolics and tannins in addition to their biological effects. There are a growing number of analytical methods for the analysis of tannins in plants (Waterman and Mole 1994; Dawra *et al* 1988; Bae *et al* 1993). However, no single method will give satisfactory results for quantitative analysis in relation to nutritional effects because the chemical properties that are involved in the reactivity of polyphenols in colorimetric and/or precipitation assays may differ from the properties that underlie their nutritional or toxic effects. Sample preparation has a large

influence on the determination of tannins and related polyphenols in plants (Mulimani and Supriya 1994). Tannins and related polyphenols in fresh samples are likely to have low complex formation and oxidative polymerisation and high solubility in aqueous organic solvents, however, tannins from air or oven-dried samples are frequently found to be complexed with plant cell wall material (Mulimani and Supriya 1994).

The most commonly used colorimetric procedures for the analysis of tannins are the Folin-Dennis reaction (Folin and Ciocalteu 1927), the vanillin-HCl reaction (Broadhurst and Jones 1978), and the butanol-HCl reaction (Bate-Smith 1975). These colorimetric procedures all share the analytical problem of the lack of suitable standards, limiting their usefulness for quantitative analysis of tannins.

1.3 Effect of tannins on animal nutrition

1.3.1 Positive effects

Several studies have shown that the presence of condensed tannins in the herbivore diet may result in improved digestion (Barry *et al* 1986, Waghorn *et al* 1987; Lees 1992; Barry and McNabb 1999). Synthesis of microbial protein in the rumen does not always compensate for excessive degradation of plant protein to ammonia and protein can therefore become limiting. In sheep and cattle grazing fresh forages, the first limiting nutrients for wool, meat and milk production are usually amino acids required for synthesis of the protein components of these tissues, such as sulphur containing amino acids. The binding of tannins to dietary protein to form predominantly insoluble complexes that are stable at rumen pH, protects the dietary protein from

bacterial deamination in the rumen (Bernays *et al* 1989, McNabb *et al* 1993; Fassler and Lascano 1995; Barry and NcNabb 1999). Less plant protein is lost as ammonia during rumen digestion and therefore more plant protein and hence non-ammonia nitrogen passes to the abomasum and intestines. The more acidic pH in these areas allows the dissociation of the tannin-protein complex allowing enzymatic digestion of the protein to occur (Goodchild and McMeniman 1986, McSweeney *et al* 1988). These plant proteins have higher proportions of limiting amino acids and are more suited to the animals' requirements for milk, meat and wool protein synthesis, and are thus of higher biological value than microbial proteins (Waghorn 1990). Therefore, although the percentage of amino acid absorption from the intestine is reduced, the net effect of tannins in these forages is to increase the amount of essential amino acids absorbed by 20 - 50% compared to plants without tannins (Waghorn 1990; Murray *et al* 1996). Further studies have indicated other positive effects due to the presence of tannins in herbivores' diets, such as the inhibition of cyanogenesis in animals fed *Carica papaya* and the prevention of alkaloid absorption into the blood stream (Bernays *et al* 1989). In addition, the precipitation of soluble proteins effectively removes one of the primary factors involved in foam stabilisation and thus reduces the likelihood of bloat (Bernays *et al* 1989, Waghorn 1990, Jones and Mangan 1977; Lees 1992; Reed 1995; Barry and McNabb 1999).

1.3.2 Negative effects

Studies on animals have demonstrated a number of detrimental biological effects of tannins. These include a decrease in both growth rate and body weight gain, perturbation of mineral absorption, and inhibition of digestive enzymes (Butler 1992;

Yan and Bennick 1995; Wood and Plumb 1995; Murray *et al* 1996; Aerts *et al* 1999). Thus, the presence of tannins in a large number of nutritionally important plants hampers their utilisation as ruminant feed. This is largely the result of the ability of tannins to complex with proteins, carbohydrates and minerals. An inverse relationship has also been found between the protein-precipitating capacity of tannins in plants and the palatability, voluntary feed intake and digestibility of crude protein and dry matter (Robbins *et al*, 1990; Reed 1995).

Tannins are released during mastication of plant tissue and may render the tissue unpalatable by precipitating salivary proteins and binding to the mucous epithelium, causing reduced lubrication and an astringent taste in the mouth. Unpalatability due to astringent tannins may lead to a reduction in voluntary feed intake, even though the plants may contain high levels of carbohydrates and protein (Provenza *et al* 1990). Opposing this view, Waghorn (1990) argues that palatability is not a factor in mediating the effects of tannins on intake. In his experiments, sheep preferred high (>5%) tannin containing leaves of *Lotus peduncitatus* to stems with a lower amount of tannins. However, in his experiments, Waghorn (1990, Waghorn *et al* 1994b) does not take into account the fact that leaves and stems are different tissues or that the digestibility of leaves is higher than that of stems. These other factors may override palatability in this case. Provenza (1990) has proposed that it is not the binding of tannins to salivary proteins in the mouth which leads to the aversion of plants containing tannins, but the subtle differences in tannin structures which result in adverse postingestive consequences (see later) which causes the animal to reduce intake. Other arguments for reduced feed intake are that tannins may reduce the

permeability of the gut wall by reacting with the outer cellular layer of the gut so that the passage of nutrients through the gut wall is diminished (Bernays *et al* 1989). The degree of permeability change depends on the amount of tannin entering the gut and this will eventually give signals of physical distension, which is an important feedback in the ruminant for controlling feed intake.

Concentrations of tannins in fodder plants exceeding approximately 6% of the dry matter have a detrimental effect on the nutritive value for ruminants by reducing feed digestibility, leading to a reduced feed intake (Pritchard *et al* 1988). Waghorn (1990), showed that a concentration of 5.5% tannin (dry weight) added to the feed of sheep resulted in a 12% reduction in dry matter intake and reduced absorption of amino acids in the small intestine leading to increased faecal nitrogen levels. He concluded that the gains achieved through protection of plant protein from rumen degradation were considerably reduced at these concentrations of tannins. VanHoven and Furstenburg (1992), also illustrate the rapid decline in VFA energy yield by fermentation as the tannin values in the substrate increases to and above the 6% level. They conclude that in animals forced to consume a diet containing tannins higher than this percentage, tannins could interfere with fermentation processes to such an extent that available metabolisable energy from this source would become limiting. Chelation of some minerals by tannins may result in a mineral deficiency. Increased secretion of mucus and cell damage caused by tannins may result in sodium depletion (Bernays *et al* 1989).

Barry *et al* (1986) expanded on the proposed dynamic situation (1.2.2, McLeod 1974) and outlined an equilibrium situation between the tannin-protein complex and 'free' tannin (Figure 1.3). They concluded that the 'bound' and 'free' tannins were respective indices of the nutritionally beneficial and detrimental effects of tannins in forage diets consumed by ruminant animals, with 'bound' tannin increasing amino acid supply and 'free' tannin probably responsible for depressions in rumen carbohydrate digestion and voluntary intake. This proposal is supported by Perez-Maldonado and Norton (1996) who studied condensed tannin metabolism in the digestive tract of sheep and goats. In their experiments they assumed that condensed tannins in plants exist in three forms with an interchange taking place between the forms during metabolism. These were:

(i) free

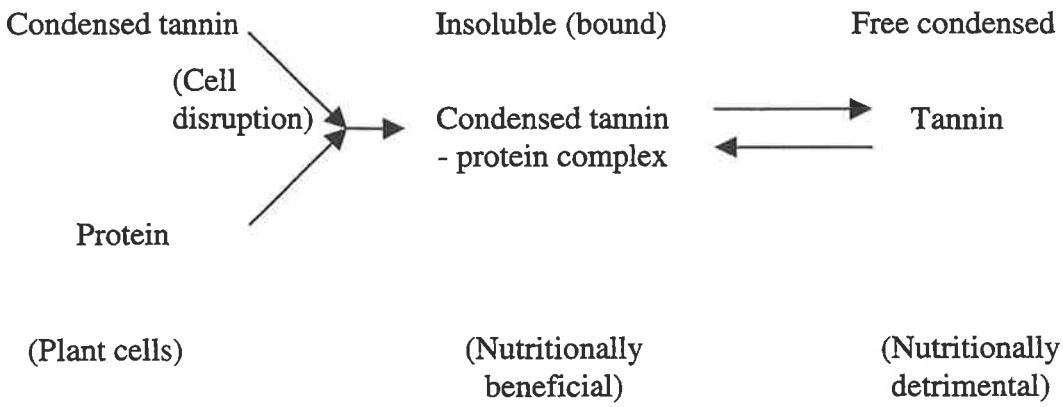
(ii) protein-bound

(iii) fibre-bound

In this model the authors propose that only free condensed tannin may undergo degradation or absorption, since the protein and fibre complexes are too large to be absorbed or metabolised (Figure 1.4).

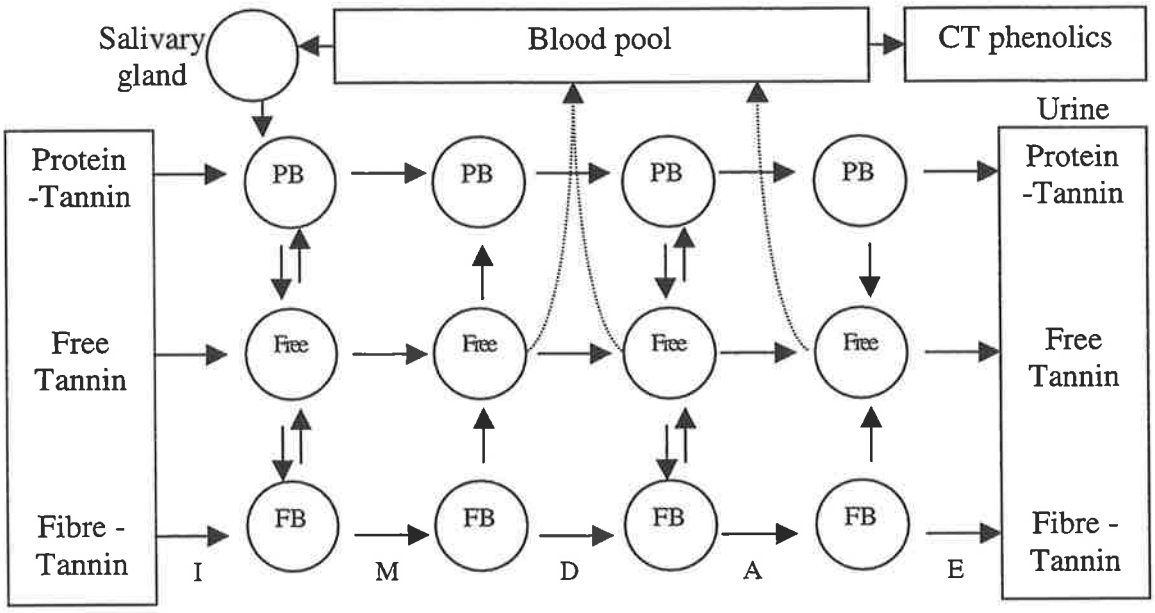
Tannins readily combine with dietary proteins, salivary proteins, digestive enzymes and rumen microbes and, when in a complexed form, are most unlikely to undergo normal metabolism (Perez-Maldonado and Norton 1996). The large size and reactivity of the tannin molecule prevents direct absorption of tannin from the digestive tract, however a continuous consumption of tannin can cause gastritis and irritation and also oedema of the intestine which allows the tannins to cross the intestinal membrane (Kumar and Vaithiyanathan 1990, McLeod 1974). This may

Figure 1.3 **Proposed equilibrium reaction between condensed tannin and protein**



Proposed mechanism of condensed tannin reaction with plant proteins and free tannin formation during disruption, and the suggested roles of bound and free condensed tannin in ruminant nutrition (Barry and Manley, 1985).

Figure 1.4 **Proposed pathway of condensed tannin passage through the digestive tract of ruminants.**



Model describing the exchanges of condensed tannin (CT) between free, protein bound (PB) and fibre bound (FB) tannin pools through the digestive tract of ruminants. I, intake; M, mastication; D, ruminal digestion; A, abomasal digestion; E, excretion. (Perez-Maldonado and Norton, 1996)

result in potentially harmful interactions leading to toxicity if prolonged consumption of tannin-rich plants occurs. Both hydrolysable and condensed tannins may exert toxic effects.

Hydrolysable tannins are toxic and cause poisoning in animals if sufficiently large amounts of tannin containing plant material, such as leaves of oak (*Quercus* spp.) and yellow-wood (*Terminalia oblongata*) are consumed (Garg *et al* 1992; Murdiati *et al* 1990, 1991). They can be broken down by the ruminal microflora to yield gallic acid, hexahydroxy diphenic acid and/or ellagic acid, which can be absorbed into the body. The major lesions associated with hydrolysable tannin poisoning are haemorrhagic gastroenteritis, necrosis of the liver, and kidney damage with proximal tubular necrosis (Hill *et al* 1998, Zhu and Filippich 1995). Acute intoxication from excessive consumption of oaks and other tree species that contain around 20% hydrolysable tannin results in high mortality and morbidity in cattle and sheep. The actual toxic compound is still uncertain and most likely will differ among tree species (Reed 1995). Pyrogallol, a minor urinary product in sheep fed *Terminalia oblongata* (Murdiati *et al* 1992), produces similar lesions to tannic acid when fed to rabbits.

Condensed tannins must be depolymerised before they can be absorbed into the body unless they are absorbed as part of an oedema reaction. Clausen *et al* (1990) has proposed that depolymerisation may occur under the acidic conditions found in the digestive tract of most mammalian herbivores. Both the rate of depolymerisation and the products formed are dependent upon tannin structure and they suggest that it may be product toxicity rather than the amount of tannin present in the plant material

ingested that is responsible for deterring herbivore feeding. The toxicity of condensed tannins to ruminants is difficult to separate from their profound effect on the digestion of protein and carbohydrates. The consumption of tropical tree legumes such as species of Acacia are associated with increased mortality of ruminants (Rittner and Reed 1992). These plants may contain high levels of proanthocyanidins and low levels of protein and while the condensed tannins are not absorbed, they may effect the mucosa of the digestive tract, leading to a decrease in the absorption of other nutrients and essential amino acids. The amino acids most susceptible are methionine and lysine (Reed 1995). As methionine is involved in the detoxification of cyanide via methylation to thiocyanate, decreased methionine availability could increase the toxicity of other plant compounds such as cyanogenic glycosides.

Absorbed phenols are usually detoxified by means of conjugation of hydroxyl groups with glucuronate or sulphate anions. Hydroxylation and methylation may also occur (McLeod 1974). Liver and kidney damage may result if the detoxification mechanisms are inadequate to handle the absorbed phenolic compounds (Robbins *et al* 1987, 1987b). The phenolics present in plants may therefore be part of a broad defence against large herbivore attack with both gastrointestinal lumen functions (decreasing protein digestion, microbial activity, gut wall permeability) and internal cellular and organ effects. Food selection by ruminants is likely to be influenced by an acceptable body phenolic burden as determined by the rate of detoxification and elimination.

1.4 Effect of tannins on microbial activity

High concentrations of tannins have been reported to affect microbial activity in the rumen, an important consideration since the productivity of ruminants is primarily dependent on the metabolic activity of the overall microbial population. Kumar and Vaithiyanathan (1990) have proposed that high levels of tannins (6%) may have bacteriostatic and bactericidal effects on rumen microbes either by directly complexing with their cell envelope, or indirectly by reducing the availability of protein nitrogen and sulphur for microbial use so that microbial growth is inhibited. This results in a lower rate of plant fibre degradation in the rumen. I have previously shown that growth of *Streptococcus bovis* was inhibited by tannic acid at a concentration less than 5 mg/ml (Brooker *et al* 1994).

Tannins may also complex with many animal and microbial enzyme systems, ie. both the enzyme and the substrate, which reduces the activity of the enzyme and also the availability of protein in the diet. For example, cellulase activity, proteolysis and urease action have been shown to be inhibited by tannins (Bernays *et al* 1989; Makker *et al* 1988; Jones *et al* 1994), as has gas and volatile fatty acid production in the rumen (Kumar and Singh 1984; Woodward and Reed 1997). Kumar and Singh (1984) propose that phenolic compounds can affect enzymes by either:

(i) reducing the solubility of the enzyme protein by forming insoluble protein-phenolic complexes or

(ii) inhibiting the enzyme activity by forming a soluble but inactive enzyme-inhibitor complex.

In vitro studies have shown that tannins inhibit digestive enzymes such as trypsin and amylase as well as the zymogen, enterokinase (Bernays *et al* 1989, Kumar and Viathiyanathan 1990). However it is difficult to demonstrate that this enzyme inhibition is significant *in vivo*. Mahansho *et al* (1983) suggested that the inhibition of digestive enzymes by the binding action of tannins might not be responsible for the antinutritional effects of tannins on ruminant digestion. He proposes that:

(i) enzymes are protected from tannin inhibition by membrane components, and

(ii) dietary tannins have the opportunity to complex with a wide variety of dietary proteins and other proteins of the digestive tract, before being exposed to the major digestive enzymes (Mehansho *et al* 1983).

This implies that most tannin will be bound and inactivated before being exposed to the digestive enzymes. Further arguments imply that even if digestive enzymes are subject to inhibition by dietary tannins, the effect is likely to be prevented or reversed by surfactants such as bile acids (Mole and Waterman 1985).

Although interest has focused primarily on tannins and lignin, evidence is accumulating that the simple phenolic monomers, which exist both in cell solubles and as constituents of lignin or plant cell walls, may have a significant influence on animal nutrition (Jung and Fahey 1983; Vadiveloo and Fadel 1992). Primarily, they have been reported to interact with proteins as do the tannins; decreasing microbial activity and digestion and resulting in the inefficient utilisation of foodstuffs (Kondo *et al* 1991, Jung 1988). Certain of the phenolic monomers have been reported to be toxic to microorganisms (Bourneman *et al* 1986; Jones *et al* 1994; McAllister *et al*

1994). Natural and related phenolic compounds have shown antimicrobial properties against bacteria and fungi (Jung and Fahey 1983; Nakahara *et al* 1993), and phenolics have long been used as food preservatives to inhibit microbial growth. Phenolic monomer constituents of lignin have also been implicated as inhibitors of growth and cellulose degradation by ruminal microorganisms (Chesson *et al* 1982). Other reports, however, underline the importance of removing the phenolic monomers from complex hemicellulosic and lignified plant fractions in order for microbial degradation of cell wall components to occur. Bourneman *et al* (1986) reports that the release of phenolic monomers such as coumaric and ferulic acids from plant fractions will increase microbial digestion of these plant cell components.

Several authors have reported changes in bacterial cell morphology induced by tannins and simple phenolic monomers (Jones *et al* 1994; McAllister *et al* 1994; Stack and Hungate 1984). The authors found that in the presence of phenolic compounds the cells showed different surface structures, the bacteria were more gram variable and some lysis and escape of cell contents were visible. The effects of tannins and the simple phenolic compounds on the morphology of these strains implicate the cell wall as a target for tannin toxicity. Tannins induce changes in morphology of several species of ruminal bacteria. Electron microscopy indicated that proanthocyanidins from sainfoin were bound to cell coat polymers in *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, *Prevotella ruminocola*, and *Ruminobacter amylophilis* but abnormal cell growth and division were observed only in *Streptococcus bovis* and *Butyrivibrio fibrosolvens* (Jones *et al* 1994).

1.5 Control of tannin toxicity

1.5.1 Dietary supplements

The concentration of tannins present in many important forage plants hinders their utilisation as ruminant feeds, especially in arid and dry tropical regions where these forages constitute an important feed source. In these circumstances, a reduction in the effective level of dietary tannins and thus their detrimental effects would be desirable.

The interaction of dietary proteins with tannins leading to the formation of indigestible tannin-protein complexes results in a loss of available protein to the animal. The addition of protein in the diet has been shown to alleviate some of the growth-depressing effects of tannins (Kumar and Singh 1984). However, a large quantity of protein would be required to annul this effect. In ruminants, urea supplementation of tannin-rich forages has been shown to result in an increase in palatability and digestibility of nutrients (Kumar and Vaithiyanathan 1990), however, the amount of urea required for this effect varies from plant to plant making quantification difficult. Urea improves feed quality by providing an extra nitrogen source and by destabilising the hydrogen bond interactions, which participate in the formation of protein-tannin complexes (Kumar and Singh 1984). This renders protein free from the complex for further utilisation by animals.

Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) have been shown to selectively bind tannins and prevent the tannins from complexing with dietary protein or microbial enzymes. This effectively removes the tannins from the diet and thus reduces their detrimental effects (Pritchard *et al* 1988, Pritchard *et al* 1992, Garrido *et*

al 1991; Silanikove *et al* 1997). PEG has also been shown to displace dietary protein from tannin-protein complexes and this exchange reaction was found to be dependent on both the amount of tannin in the complex and the age of the complex prior to addition of PEG (Makkar *et al* 1995). Increases in these factors decreased the exchange. When PEG was incorporated into the diet of sheep existing on *Acacia aneura* (mulga), improvements in feed intake and wool growth and a reduction in rate of liveweight loss were observed (Pritchard *et al* 1988, 1992). The dietary incorporation of PEG appears to improve nutrient availability by increasing protein availability and microbial activity in the rumen, which results in an increased amount of microbial protein available for digestion in the lower gut. Alternatively, PEG binds free tannins in the abomasum and small intestine thereby negating their detrimental effect on protein digestion and absorption.

1.5.2 Animal defence

Animals that regularly utilise tannin containing plant materials for food have developed mechanisms for overcoming their potentially detrimental effects (Bernays *et al* 1989). Several mechanisms have been proposed to account for tannin-tolerance. In many insect species the potential adverse effects of tannins on digestion are avoided by gut conditions, especially alkalinity and detergents that prevent the formation of complexes between tannins and digestive enzymes or food proteins (Martin *et al* 1985, Austin *et al* 1989). In some insect larvae, the periotrophic membrane acts as a barrier to the passage of tannins (Barbehhenn and Martin 1992). The proposed mechanisms for this are:

(i) adsorption on the interior surface;

(ii) ultrafiltration of unbound tannins and

(iii) ultrafiltration of tannins that are bound to high molecular weight solute molecules, such as the proline-rich proteins produced by some vertebrates (Austin *et al* 1989).

This mechanism for avoiding the potential toxicity of dietary tannins contrasts with the mechanisms found in some arachnids, in which the major anti-tannin adaptation is believed to be hydrolysis of hydrolysable tannins to gallic acid or ellagic acid. Gallic acid, which may freely penetrate the periotrophic membrane, has been shown to have beneficial effects on the growth of other tree-feeding insects. In tree locusts, absorbed phenolics are not toxic but are utilised in the synthesis of the cuticle (Bernays *et al* 1989).

Rats and mice have been found to adapt to dietary tannins by the induced synthesis of several salivary glycoproteins by the parotid gland. These salivary proteins are rich in proline (25-45%), glutamine/glutamate (25%) and glycine (25%) which apparently diminish the antinutritional effects of dietary tannins by binding to them (Mehansho *et al* 1983). Robbins *et al* (1987) have carried out studies on deer, sheep and cattle and demonstrated that these salivary glycoproteins could be involved in the improved utilisation of forages that contain tannins. East African bovidae that feed on woody dicots that contain high levels of tannins have larger salivary glands relative to body weight than grazing bovidae (Hofmann 1973). The salivary tannin-binding proteins have been shown to protect other proteins from tannin interaction at all stages of the digestive process (Robbins *et al* 1987a, Austin *et al* 1989). This indicates that the

tannin-proline-rich-protein (PRP) complexes are stable over the pH range of the entire digestive tract, ie. pH 2 to pH 9. Salivary PRPs contain large amounts of carbohydrate which gives the protein an open configuration and a relatively high molecular weight that enables the protein to freely interact with the tannin molecule through the formation of hydrogen bonds (Asquith *et al* 1987). Removal of the carbohydrate groups causes the proteins to assume a more compact conformation, which has a much lower affinity for tannin. Robbins *et al* (1987a) proposed that the predominantly grass diet and behavioural avoidance of tanniferous forages by domestic sheep and cows would not necessitate development of physiological defences against tannins. This was supported by Bernays *et al* (1989) who reported that the parotid salivary glands in ruminants (per unit body mass) were three times larger in browsers than in grazers, and of intermediate size in mixed feeders. Austin *et al* (1989) compared the saliva of deer, a browsing animal, to that of domestic sheep and cattle which are grazing animals, and found that the deer, which normally ingests dietary tannin, produces tannin-binding proteins, while the cattle and sheep did not. Deer were thus able to minimise nitrogen loss and tannin toxicity by the production of salivary proline-rich proteins. Yan and Bennick (1995) also report on a group of low molecular weight proteins rich in histidine, called histadins which are present in human saliva and which appear to bind tannins more effectively than the proline-rich salivary proteins. In animals consuming tannins, the binding of tannin to salivary proteins would protect the gut mucosa and prevent any other deleterious processes such as chelation of essential minerals in addition to decreasing anti-digestive effects. Also, tannin-binding proteins may impede the breakdown of tannins to smaller phenolic products that can be absorbed into the blood and exert toxic effects on the

liver and kidneys. Secretion of other endogenous glycoproteins along the digestive tract should also be investigated. However, it is likely that microbial adaptation may be the primary mechanism by which ruminants can tolerate high levels of tannins in their diet.

1.5.3 Microbial defence

1.5.3.1 Secretion of tannin binding polymers

Changes in production of the glycocalyx of ruminal bacteria in response to tannins from Birdsfoot trefoil were observed by electron microscopy (Chiquette *et al* 1988). These changes may indicate that bacteria produce glycoproteins that are analogous to the salivary glycoproteins that are secreted by rats in response to dietary tannins (Scalbert 1991), but the microscopic observations need to be verified by quantitative and qualitative measurements. The production of a fungal spore matrix containing glycoproteins with a relatively high proline content and high affinity for phenols has been reported by Nicholson *et al* (1986). Studies of the composition of the spore mucilage revealed that these glycoproteins have a carbohydrate and amino acid composition similar to those of animal mucins. The authors propose that passive binding may reduce the accessible concentration of toxic phenols to levels that are not inhibitory and hence provides a mechanism through which fungi inactivate toxic phenolic compounds in their environment.

1.5.3.2 Production of an extracellular matrix

Tannins have been reported to induce changes in the morphology of several species of ruminal bacteria (Jones *et al* 1994; McAllister *et al* 1994b). Electron microscopy

indicated that proanthocyanidins from sainfoin were bound to cell coat polymers in *Streptococcus bovis*, *Butyrivibrio fibrisolvens*, *Prevotella ruminocola*, *and Ruminobacter amylophilis* but abnormal cell growth and division was observed only in *Streptococcus bovis* and *Butyrivibrio fibrosolvens* (Jones *et al* 1994). *P. ruminocola* cells grown with condensed tannins were interconnected by condensed extracellular material which was absent from cells grown without condensed tannins. The authors suggest that this material may protect the organism from the effects of tannins. Bae *et al* (1993b) found that *F. succinogenes* S85 cells grown in the presence of condensed tannins were also seen to possess large amounts of electron dense surface-associated material that appeared to offer the bacteria protection from the effects of tannins. The authors suggest that this material is the result of the formation of complexes between the condensed tannins and the cell surface. Stack and Hungate (1984) demonstrated that *R. albus* 8 cells grown in the presence of hydroxy-cinnamic acid (HCA), a simple phenolic monomer, show a surface structure different to that of cells grown without the phenolic acid. The presence of HCA appears to increase the size of the capsular material surrounding the cells. The authors suggest that the ability of the microorganism to surround itself in a highly hydrated extracellular polysaccharide (EPS) layer may provide it with protection by either reacting with and neutralising the phenolic acid or by creating a diffusion barrier to the phenolic acid.

Production of an extracellular polysaccharide matrix (EPS) is a common characteristic of many procaryotes (Sutherland 1972, 1982, 1998; Costerton *et al* 1981, Costerton *et al* 1995). Microorganisms may excrete EPS's that form tight, cell-associated (integral) capsules or dispersed (peripheral) slime matrices, generally considered to be

a loose network of unordered gel or fibrils which extend from the cell surface and which can slough into the aqueous phase (DeVuyst and Degeest 1999). In some cases both capsular and unattached polysaccharides may be produced by the same microbe making it difficult to distinguish between the two forms. Capsule polymers are highly flexible and extend, often for great distances, radially from the cell wall (Beveridge and Graham 1991). They are used as cementing substances to bind bacteria into microcolony formats, or to bind the cells to substrates and surfaces, form flocs and eventually develop large biofilms, which shroud visible surfaces. The organic constituents are branched or unbranched homo- or heteropolymers, which are typically repeating oligosaccharide units, although proteinaceous types have also been identified (Troy 1979). Structural diversity arises from a broad range of monosaccharide components and is increased by potential non-carbohydrate substituents and linkage types, usually mixed α- and β-linkages in the main chain. Their most abundant constituent, however, is water, typically 99%, and this characteristic makes them difficult to preserve intact for ultrastructural study as the polymer strands readily collapse on to the surface of the cell during dehydration. Staining with specific electron scattering agents such as ruthenium red, alcian blue, or silver methenamine as well as lectins and specific antibodies, cross link the polymers of these exopolysaccharide matrices and thus prevent radical condensation during the dehydration steps of preparation for electron microscopy. In thin sections, the stains usually reveal a fibrillar amorphous aggregate compacted against the cell wall, while the antibody preparations facilitate the demonstration of the real dimensions of the glycocalyces keeping the polymer strands extended (Bayer and Thurow 1977; Lambe *et al* 1984; Kennedy and Sutherland 1987). The most effective linkages between

capsule polymers seem to be electrostatic and hydrophobic-hydrophilic forces which are essential for the maintenance of the structure, although highly acidic types (eg. rich in carbonyl groups) can be naturally cross-linked by metal divalent ions such as Mg^{2+} or Ca^{2+} (Lambe *et al* 1984, 1994).

Like capsules, bacterial slimes are abundant in nature and include a wide range of externalised homo- and heteropolymers. Slimes may sometimes be formed from an excess of capsular material produced by the bacteria or which the bacteria have failed to anchor to their surface. Such polymers are sloughed off into the surroundings and float freely until they become associated with other solid surfaces (Costerton *et al* 1995).

The nature of the linkage between EPS and cell wall has proved difficult to identify (Whitfield 1988, Jacques *et al* 1990). In several *E. coli* and some *N. meningitidis* capsular exopolysaccharide a diacyl glycerol moiety has been identified (Whitfield 1988). It has also been suggested that lipids link the capsular material of *Haemophilus influenzae* to the cell surface. Surface appendages, which mediate attachment of streptococci such *as S. pneumoniae*, *S. mutans*, *S. suis*, and Group B streptococci, to host surfaces are often associated with a fibrillar layer outside the cell wall, historically termed the fuzzy coat. More recent studies on the morphology of the fuzzy coat of *S. salivarius* and *S. sanguis*, showed that this layer consists of densely packed fibrils with a loose, amorphous appearance (Jacques *et al* 1990; Lambe *et al* 1994)

Extracellular polymers have been shown to vary considerably in their chemical structure, however, five distinct groups have been classified and are listed below (Whitfield 1988).

Group I A polysaccharide formed entirely of one monosaccharide type (homopolysaccharides) and produced by bacteria utilising sucrose as a specific substrate. In the absence of sucrose or a few closely related substrates, the bacteria can grow but are unable to form the polysaccharide, e.g. dextrans and related polysaccharides.

Group II Heteropolysaccharides (containing more than one component monosaccharide) metabolised by a specific carbon substrate.

Group III Homopolysaccharides metabolised from any of several carbon substrates. Formation appears to occur intracellularly and the polysaccharide is secreted into the extracellular environment.

Group IV Heteropolysaccharides formed from repeating unit structures. These make up the largest and most heterogenous group of exopolysaccharides.

Group V Heteropolymers that are made up of non-repeating units, e.g. bacterial alginate.

There is a pronounced tendency of bacteria to lose the protective glycocalyces on subculture (Costerton *et al* 1987, 1995), however capsules have been identified on a number of Gram-positive and Gram-negative bacteria (Beveridge and Graham 1991). Physiological studies have shown that the type and quantity of capsular material is dependent on carbon, nitrogen and phosphorous sources, on oxygen and pH levels,

and on growth temperature and growth phase (Cerning 1990; Kojic *et al* 1992; Roberts *et al* 1995; Manca *et al* 1996).

Bacterial EPS are synthesised in different growth phases and under a variety of growth conditions, depending on the organism studied (Bae *et al* 1993; Bonet *et al* 1993; Quessy *et al* 1994). The biosynthetic process involved can be divided into two principal categories, based largely on the site of synthesis and the nature of the precursors: synthesis outside the cell or synthesis at the cell membrane. Homopolysaccharides, such as dextrans, levens and mutans produced by *Leuconostoc mesenteroides* and *Streptococus mutans* are synthesised by relatively few enzymes, outside the cell. For example,

n sucrose → dextransucrase → (dextran)n + n fructose

n sucrose → levansucros → (levan)n + n glucose

Both polymers show a requirement for sucrose or an alternative oligosaccharide as an activated precursor.

The structure and synthesis of heteropolysaccharide capsules and slimes are more complex. Heteropoplysaccharide synthesis differs from homopolysaccharide synthesis in that they are produced at the cytoplasmic membrane using precursors found intracellularly. Sugar nucleotides play an essential role in heteropolysaccharide synthesis due to their role in sugar interconversions as well as sugar activation, which is necessary for monosaccharide polymerisation. The biosynthesis of these polysaccharides, obtained from studies using cell-free enzyme systems, involves four stages: synthesis of nucleotide sugar diphosphate intermediates; stepwise assembly of

the repeating oligosaccharide subunit of the polymer by transfer of monosaccharides from the corresponding nucleotide to the carrier lipid located in the cell membrane; addition of accessory compounds, such as pyruvate, acetate, and succinate; transfer of the growing polysaccharide chain from its carrier lipid to its new subunit. Very little is known about the mechanism of polymerisation, but it is believed to occur on the inner face of the cytoplasmic membrane, after which the EPS is transported to the cell surface through Bayer adhesion zones between the outer and inner membranes. Thus, EPS synthesis requires enzymes for production of each nucleotide sugar precursor, separate transferases for each monosaccharide in the subunit, one or more polymerases, and proteins involved in export of the polysaccharide.

The molecular basis of EPS synthesis and regulation, while vague, has been described in a few bacterial systems. In *E. coli* K1, the capsular polysaccharide genes are clustered at the kps locus of approximately 15 kb of DNA (Silver *et al* 1984). In other bacteria, such as *X. campestris*, the EPS genes may be organised into more than one cluster (Whitfield 1988) and in *R. meliloti*, the EPS genes are divided between the chromosome and a megaplasmid (Kojic *et al* 1992).

Bacteria that produce EPS have been identified in a variety of ecological niches and it is apparent that the precise role of EPS is dependent on the natural environment of the microorganism. It has been suggested that the ability to produce EPS was a direct and logical response to selective pressures in the natural environment (Whitfield 1988; Costerton *et al* 1995). The fact that the role of EPS has not been clearly established probably suggests that it is diverse and complex. Several functions, however have

been attributed to capsular material although rarely have they been elucidated at the molecular level.

In a variety of environments survival depends on the ability of a microorganism to adhere to a surface. The presence of EPS in adherent biofilms on inert and biological surfaces has been recognised for some time, although the widespread occurrence of these biofilms and their commercial implications in microbiological problems as diverse as the fouling of pipelines and the onset of dental carries is only now being recognised (Costerton *et al* 1987, 1995). The selective adherence of some bacteria to a singular substrate can be mediated by capsular material. For example, the growth phase specific capsule of Rhizobium spp. can differentiate between lectins of various legumes and is important for adherence, leading to subsequent nodulation (Beveridge and Murray 1976). Nonselective adherence by capsules can also benefit the cell. Growth environments, which are highly abrasive or fast moving, require strong adherence of bacteria to a substratum, eg. the bacterial inhabitants of dental plaque (Mackie *et al* 1979), and ungulate rumen's (Costerton *et al* 1981; McAllister *at al* 1994). As a consequence, microcolonies form and proliferate within a singular capsular mass, which is cemented to the substratum. EPS has also been reported to function in the movement of gliding bacteria. Capsular material of the gliding bacteria has been implicated both in their mechanism of movement and in the horizontal drag involved with the movement (Beveridge and Graham 1991). Most of the functions ascribed to EPS, however, are of a protective nature. The ability of a microorganism to surround itself in a highly hydrated EPS layer may provide it with protection against desiccation, may confer resistance to bacteriophage and phagocytic

amoebae and inhibit predation by protozoans (Beveridge and Graham 1991; Costerton *et al* 1995). This suggests that encapsulated pathogens possess enhanced virulence and are able to better resist the phagocytotic and immunological defences of the host. Alginate biosynthesis has been studied extensively in *Pseudomonas aeruginosa*, where it functions as a major virulence factor in strains affecting the lungs of cystic fibrosis patients (Whitfield 1988; Penaloza-Vazquez *et al* 1997). The protective capsule surrounding *P. aeruginosa* has been well characterised and shown to provide a protective barrier against antibiotics and host immune defences and may also function to trap nutrients and water. A number of environmental signals for the induction of alginate biosynthesis have been identified and include dehydration, high osmolarity, starvation, and a biofilm mode of growth (Whitfield 1988). Under these conditions some EPS genes are amplified. Costerton *et al* (1987, 1995) suggested that expression of an exopolysaccharide which has been described by X-ray diffraction as a thick, continuous, highly ordered, hydrated, polyanionic polysaccharide matrix, must be expected to influence the access of molecules and ions to the cell wall and cytoplasmic membrane and thus may afford protection to the underlying cells by retarding diffusion of antimicrobial compounds. The anionic electrostatic charge character of a number of bacterial EPS's enables them interact strongly with cations of the environment to collect and store soluble metal ions (Dudman 1977).

EPS's do not appear to function as energy sources, since slime-forming bacteria are not usually capable of catabolising the polymer, which they synthesise. Unrelated organisms in mixed cultures can however use EPS produced by various organisms. The relationship between encapsulation and role in virulence has been studied and it

has been proposed that the degree of hydrophobicity of the bacterial surface dictate the susceptibility to become engulfed by neutrophils. Encapsulated organisms having a more hydrophilic surface were shown less susceptible to phagocytosis by human neutrophiles (Whitfield 1988). This hypothesis has been used to explain protection of soil bacteria against predation by amoeba and flagellated soil protozoa (Dudman 1977).

Induction of EPS by particular substances has also been described (Mittelman and Geesey 1985). Kidambi *et al* (1995) has reported that plant associated pseudomonads, such as *P. syringae sp. syringae* F85 that are commonly exposed to copper bactericides have developed a resistance or tolerance to copper salts due to the increased production of EPS.

1.5.3.3 Synthesis of tannin-resistant enzymes

Bae *et al* (1993) indicated that the extracellular endoglucanase from the ruminal bacterium *Fibrobacter succinogenes* was more susceptible to inhibition by condensed tannins from Birdsfoot trefoil than the cell associated enzyme. These results suggest that bacterial enzymes may be more susceptible to tannin inhibition when isolated in cell free systems than when the integrity of the cell envelope is maintained. Proanthocyanidins from sainfoin inhibited the cell-associated protease activity of *Streptococcus bovis* and *Butyrivibrio fibrosolvens* but not in *Prevotella ruminocola* or *Ruminobacter amylophilis* (Jones *et al* 1994). The authors fail to give a reason for the difference in enzyme susceptibilities but suggest that the resistance of *P. ruminocola and R. amylophilis* proteases to high concentrations of condensed tannins is of interest

because of the functional importance of these species in ruminal proteolysis. Other enzyme systems known to be affected by tannins include salivary α-amylase (Yan and Bennick 1995), and pancreatic amylase (Ahmed *et al* 1991), glucosyltransferases of *S. mutans* (Nakahara *et al* 1993) and tyrosinase activity (Yu *et al* 1996).

1.5.3.4 Biodegradation of tannins

Degradation of both hydrolysable and condensed tannins has been best described in fungal systems (Wantanabe 1965; William *et al* 1986; Patel *et al* 1990; Saxena *et al* 1994; Bhat *et al* 1998). The pathways of aerobic degradation of these tannins and their phenolic monomers (gallic acid, catechin, quercetin and their intermediates like phloroglucinol) have been described and are similar to the pathways found in aerobic bacterial degradation of such compounds (Bhat *et al* 1998; Figure 1.5 and Figure 1.6). Degradation is initiated by oxygenases that hydroxylate and cleave the aromatic nucleus through addition of molecular oxygen (Haddock and Ferry 1993). Gallic acid monomers are readily utilised as substrates by oxidative breakdown to simple aliphatic acids, which then enter the citric acid cycle (Field and Lettinga 1992). The aerobic breakdown of flavonoid compounds derived from condensed tannins occurs through a number of pathways (William *et al* 1986).

The degradation of tannins in the absence of molecular oxygen has also been documented (Evans and Fuchs 1988; Fuchs *et al* 1994; Heider and Fuchs 1997; Harwood *et al* 1998; Figure 1.5 and Figure 1.6) and several ruminal microorganisms with the ability to degrade phenolic monomers have been isolated. *Eubacterium oxidoreducens* is a strictly anaerobic ruminal bacterium that degrades gallate,

Figure 1.5 **Proposed pathways for hydrolysable tannin degradation**

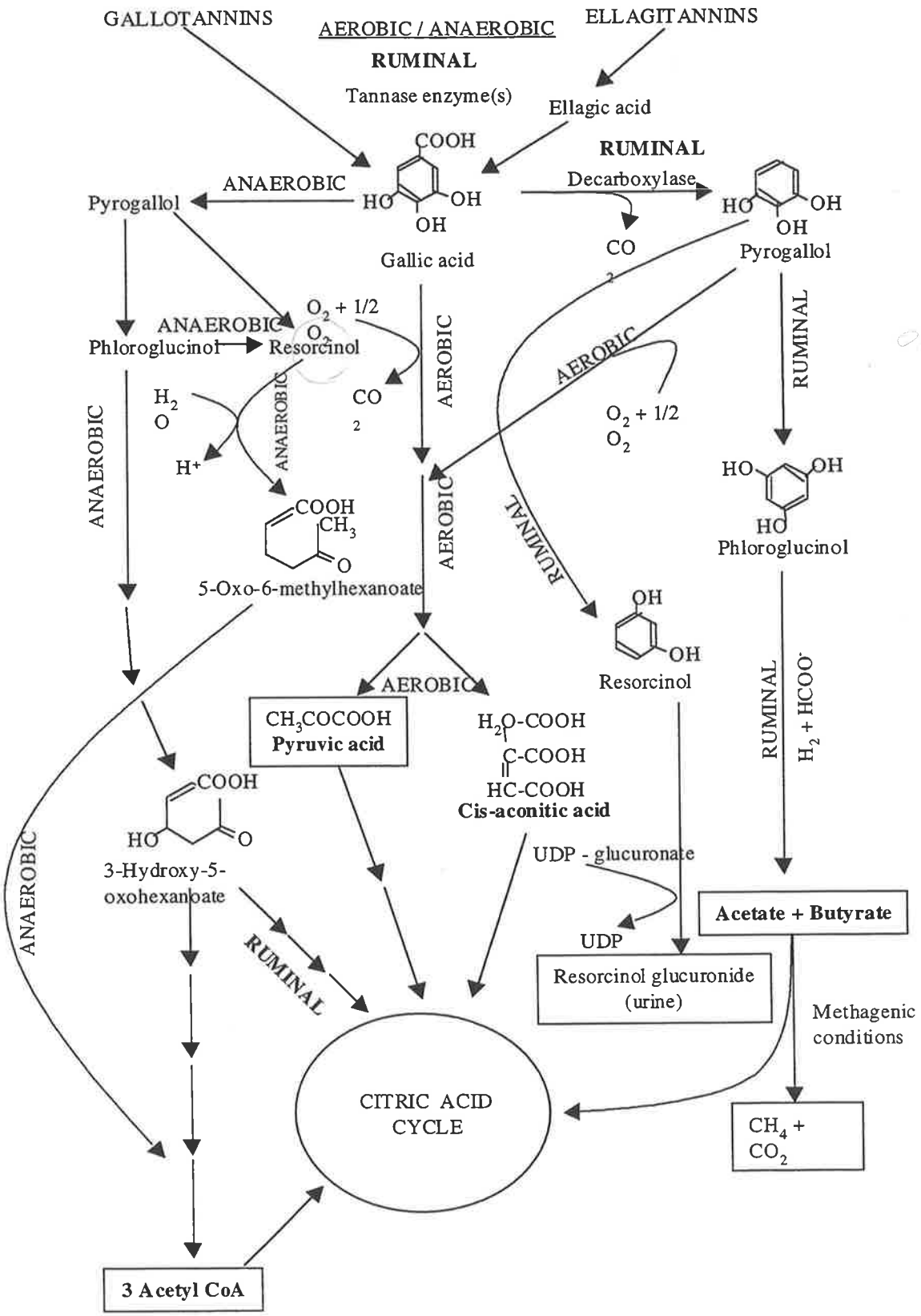
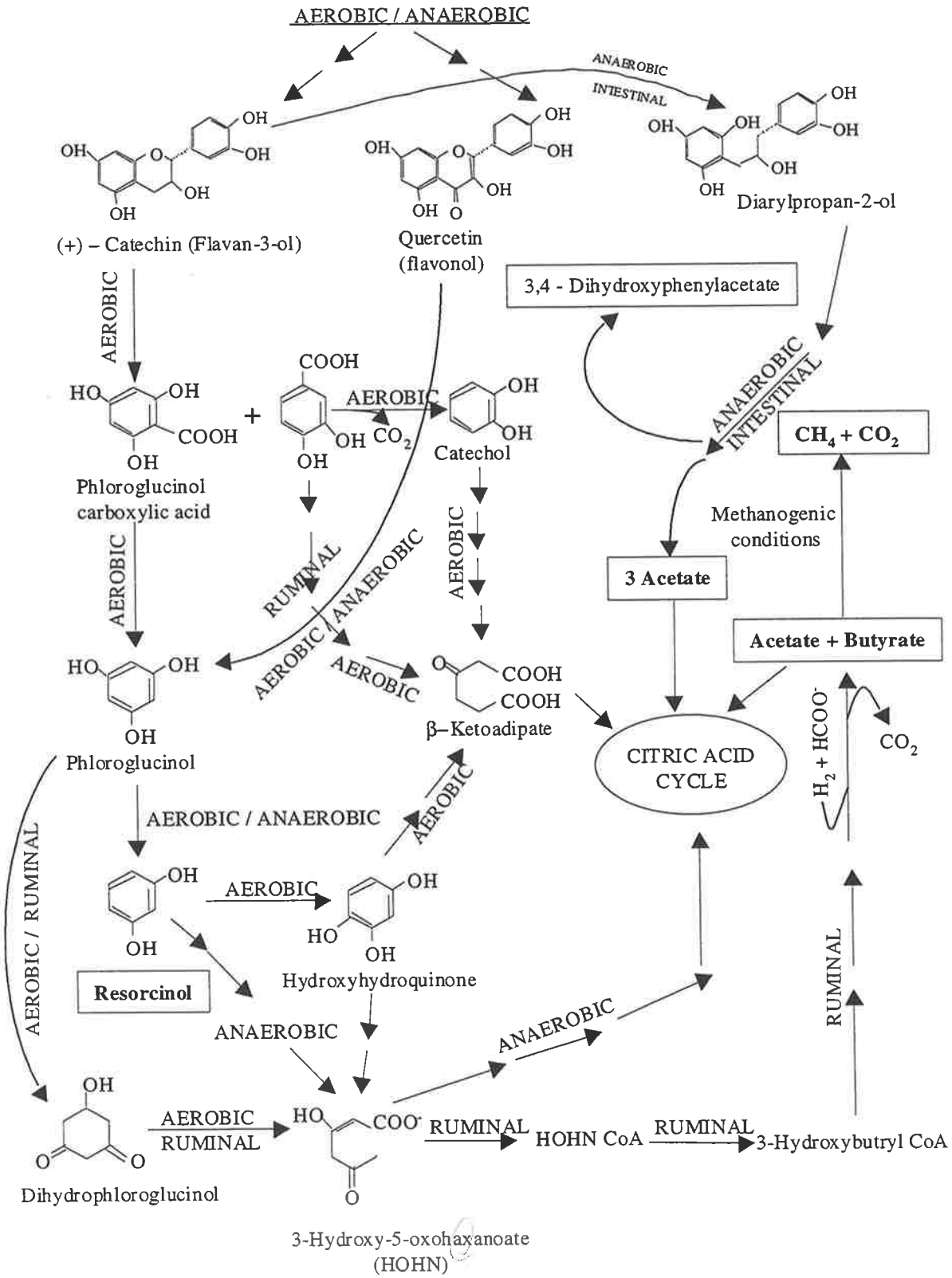


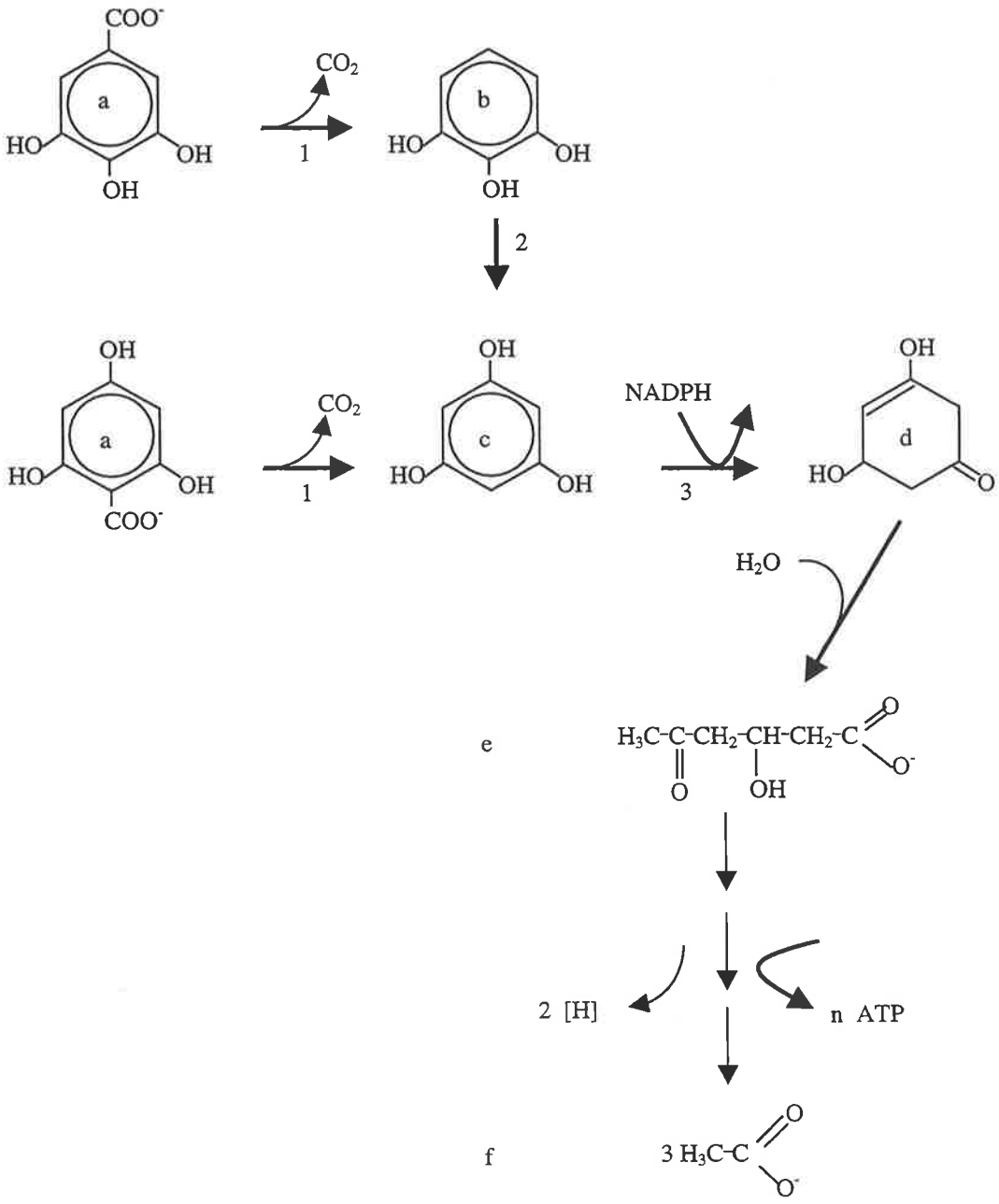
Figure 1.6 **Proposed pathways for condensed tannin degradation**



phloroglucinol, and pyrogallol to acetate and butyrate in the presence of hydrogen and formic acid (Krumholz and Bryant 1986). Tsai and Jones (1975) isolated Streptococcus strains and three Coprococcus strains from the bovine rumen that were capable of degrading up to 80% of phloroglucinol, present as the only added carbon source, within 2 days. *Syntrophococcus sucromutans* is another bacterium which demethoxylates various phenolic compounds (Krumholz and Bryant 1986b) and *Pelobacter acidigallici* (Schink and Pfennig 1982), isolated from sewage and anaerobic aquatic sediments, has been shown to degrade a number of trihydroxybenzoids with acetate as the major product. A number of Selenomonas species capable of degrading tannic acid and gallic acid have been isolated from the rumen of East African ruminants (Odenyo and Osuji 1998) and recently three phylogenetically distinct groups of ruminal bacteria were isolated from four species of ruminants in Europe, North America and South America (Nelson *et al* 1998). These reports suggest that climate, geography or host animal does not restrict the presence of tannin tolerant bacteria.

Breakdown of the aromatic compounds appears to proceed via several independent pathways (Krumholz *et al* 1987; Brune and Shink 1990; 1992; Nelson *et al* 1995). Brune and Schink (1992) have reported on the elucidation of the steps involved in the enzymatic breakdown of trihydroxybenzoids to their respective trihydroxybenzenes in *P. acidigallici*. The pathway is initiated by a decarboxylation step, which is followed by the degradation of phloroglucinol, the aromatic nucleus, via a novel pathway (Figure 1.7). Determination of the enzyme activities appeared to be dependent on the strict exclusion of oxygen in the assay systems.

Figure 1.7 **Proposed pathway for the degradation of trihydoxybenzenes in *P. acidigallici***



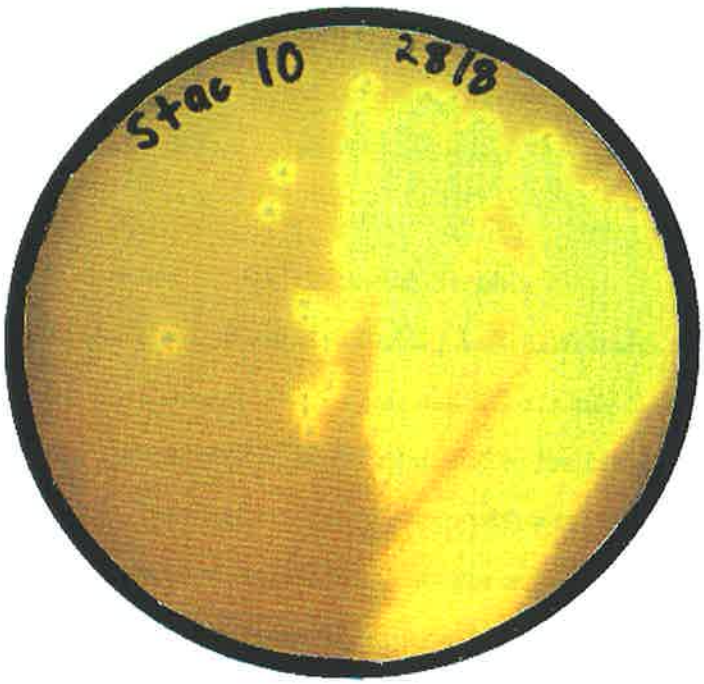
The steps involved in the degradation of trihydroxybenzes in *P. acidigallici* have been characterised. The compounds represented are a, trihydroxybenzenes; b, pyrogallol; c, phloroglucinol; d, dihydrophloroglucinol; e, 3-hydroxy-5-oxo-hexanoate; f, acetate. The enzymes involved are 1, a trihydroxy decarboxylase; 2, tetrahydroxybenzene-pyrogallol hydroxyltransferase; 3, phloroglucinol reductase.

Some fungal strains, belonging to the genus of *Aspergillus* (Barthomeuf *et al* 1994), the yeast *Candida* (Aoki *et al* 1976) and more recently, the fungi *Trichoderma viridae* and *Fusarium solanii* (Bajpai and Patil 1996) have been shown to produce an enzyme 'tannase' (tannin acyl hydrolase) which hydrolyses gallotannin to yield gallic acid. The enzyme was found to be induced by methyl gallate and tannic acid but not by other simple phenols such as gallic acid, salicylic acid or methyl salicylate (Otuk and Deschamps 1983). Tannase is now reported to be widespread in various fungi, bacteria and yeasts. It is produced both as membrane bound and extracellular forms (Deschamps 1989; Field and Lettinga 1992; Lekha and Lonsane 1997). Recently, tannin acylhydrolase activity has been described in detail from an anaerobic ruminal bacterium, *S. ruminantium* subsp. *ruminantium*, isolated from goats browsing tannin-rich forage (Skene and Brooker 1995). This is the first report of tannase activity in a ruminal microorganism. Nelson *et al* (1995) has reported on the characterisation of a Streptococcal species isolated from goat ruminal contents that can degrade tannic acid to gallic acid and pyrogallol within four hours. The authors suggest that tannin-acyl hydrolase is responsible and have proposed a pathway describing the degradation of tannic acid however, they fail to provide any enzymatic evidence in support of their claim. Osawa (1990, 1991) and Osawa and Mitsuoka (1990) have reported that mannitol fermenting strains of Streptococcus and Enterobacterium (Osawa 1992), both of which occur frequently in the alimentary tract of the koala, had the unique property of degrading protein complexed with tannic acid. The basis for this claim came from the formation of a cleared region when the bacteria were grown on media agar containing tannic acid. The formation of these cleared regions has also been ascribed to tannin acyl hydrolase although again no evidence for the enzymatic basis

has been described (Osawa and Walsh 1995). The Streptococcal species was originally assigned to *S. bovis* (biotype 1), however, following rRNA sequencing, the bacteria was found to be significantly different from *S. bovis* and has been reassigned as a novel species and named *S. gallolyticus* (Osawa *et al* 1995). *S. gallolyticus* has been found in the faeces of a number of other browsing animals, such as the ringtail possum, deer, pig and guinea pig (Osawa and Sly 1992). Matthew *et al* (1991) isolated a number of bacteria from the rumen fluid of feral goats, known to be browsing tannin-rich Acacia species that appeared capable of tolerating high concentrations of tannins. Studies in our laboratory (L. O'Donovan, Honours Thesis, Brooker *et al* 1994) have focussed on a Streptococcal species obtained from this consortium. The bacterium was selected on the basis of its increased tolerance to tannins and its ability to produce cleared regions when grown on media agar containing tannic acid (Figure 1.8). The bacteria were found to be significantly different from the more common ruminal species, *S. bovis* to warrant a new species classification and were named *S. caprinus*. Recently, *S. caprinus* and *S. gallolyticus* have been found to be subjective synonyms based on their levels of 16S rRNA sequence similarity (98.3%), DNA-DNA homology (>70%) and the phenotypes of their type strains (Sly *et al* 1997).

Reports describing bacteria capable of degrading condensed tannins under anaerobic conditions are sparse. However, recently, Perez-Maldonado and Norton (1996b) have reported substantial disappearance of labelled condensed tannins from *Desmodium intorum* in the gastrointestinal tract in sheep and goats.

Figure 1.8 **Growth of *S. caprinus* on mBHI media containing tannic acid**



S. caprinus was isolated from crude goat rumen fluid on the basis of its ability to form cleared regions following growth (39 °C, 24 h, anaerobic conditions) on mBHI media agar containing 0.5% w/v tannic acid.

1.6 Future implications

There is growing interest among researchers in using techniques of molecular genetics to increase the productivity of known forage plants or to enable the use of previously unutilised or under-utilised plants as feed resources (McSweeney *et al* 1999). The introduction of tannins into productive species of forage legumes such as alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*) has been proposed (Reed 1995) in an attempt to overcome the problems of bloat and inefficient utilisation of protein in these species. However, a better understanding of the relationship between tannin structure and function is required to determine the effects of tannins on the nutritive value and toxicology of forage legumes before manipulation of tannins through breeding and selection through genetic engineering can be done. There may be a fine line between the potentially positive effects of tannins and their negative effects on intake, digestion, and performance.

Conversely, there are plants such as *Calliandra calothrysus* (Calliandra), *Chamaecytisus palmensis* (Tagasaste) and Acacia sp., which contain high levels of protein but which also include antinutritional components such as high concentrations of tannins which make them unsuitable as a feed resource. Mulga (*Acacia aneura*) is of particular importance in Australia as it covers a large proportion of Central, South Central and Northern Australia and comprises an important feed source for sheep and cattle in times of drought. However, despite its high leaf protein content (10-15% DW), mulga is nutritionally inadequate for domestic animals due to the low digestibility of this protein (35-40%). Consequently, sheep consuming mulga are not able to meet their protein requirements, resulting in low wool growth rates, loss of

liveweight and an increased death rate after an extended period of mulga feeding. The low protein digestibility has been directly attributed to the high concentration of tannins present in mulga (7-17% DW).

Tannin tolerant organisms have not been found in the rumen contents of domestic sheep and cattle and it has been proposed that these bacteria, either individually or as a consortium may be responsible for the tannin tolerance of the animal (Matthew *et al* 1991; Brooker *et al* 1994, Osawa 1990, 1991). The identification of microbes from the gut of native and feral animals which are capable of tolerating or degrading plant tannins, has shifted attention to the possibility of introducing these microbes to domestic ruminants with the aim of conferring tannin-tolerance to these animals. This approach has previously been successful with the transfer of mimosine tolerance from Hawaiian goats to Australian cattle by Jones and Megarrity (1986). This was accomplished through the transfer of rumen contents from goats to cattle and a number of Gram negative bacteria that are involved in the mimosine breakdown pathway have been isolated and characterised (Allison *et al* 1990; Hammond 1995). Preliminary studies involving the transfer of rumen fluid from feral goats to domestic sheep resulted in improved digestibility (8-10% DW) of tannin-containing mulga. This led to an increase in feed intake, nitrogen balance and digestibility and a decrease in liveweight loss (Miller *et al* 1997).

Extensive characterisation of *S. caprinus,* and other bacteria involved in conferring tannin tolerance to domestic ruminants, as well as an understanding of the mechanism(s) that the bacteria employ in dealing with tannins, is necessary before

transfer of the bacteria to domestic livestock can occur on a larger commercial scale. The results obtained in this study may also enable the manipulation of more predominant rumen bacteria, such as *S. bovis* to achieve tannin tolerance.

1.7 Objectives of this study

This research project set out to

- fully characterise the effect of tannins on the goat ruminal bacterium, *Streptococcus caprinus*
- determine the mechanism(s) of tannin tolerance employed by *S. caprinus*
- isolate the genes responsible for tannin tolerance by *S. caprinus*

Preliminary characterisation of *S. caprinus* has been carried out (L. O'Donovan, Honours Thesis, Brooker *et al* 1994) and for continuity and enhanced understanding of this study, those results shall briefly be described here.

Description of *Streptococcus caprinus* sp.nov. (caprinus; L., of the goat)

Gram positive, facultatively anaerobic cocci mainly occurring in short chains and present in the goat rumen at a population of 10^5-10^6/ml of goat rumen contents. The organism is morphologically similar to *S. bovis* however, preliminary analysis using DNA-DNA hybridisation, %G-C content and ribosomal RNA sequencing indicate that there are significant differences between the goat rumen bacteria and *S. bovis*. The species is able to grow and form clear zones on a complex media containing tannic acid, and is able to grow in a complex media with at least 2.5% of tannic acid and condensed tannins derived from *Acacia aneura*. The species grows on glucose, starch, cellobiose, galactose, mannose, trehalose, sucrose, lactose, fructose, maltose,

raffinose and inulin as sole carbon sources, but is unable to grow on rhamnose, glycerol, xylose, sorbitol, inosotol, L-arabinose or tannic acid. L-Lactate is the predominant acid produced by *S. caprinus* from a medium containing glucose, although acetic acid and ethanol are also produced. In a basal medium with glucose, *S. caprinus* is able to use trypticase or casamino acids as well as NH_4Cl as a sole nitrogen source. The type strain (ACM 3969) is deposited in the Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, St. Lucia, Queensland.

Chapter 2

Materials and methods

Chapter 2 Materials and methods

General protocols

2.1 Bacterial strains

Streptococcus bovis type 2B (*S. bovis*) was obtained from K. Gregg, University of New England, NSW, Australia. *Streptococcus caprinus* type strain 2. 2 (*S. caprinus*) and *Escherichia coli* (*E. coli*) type strains DB11 and ED8299 were obtained from J. Brooker, University of Adelaide, SA, Australia. *Selenomonas ruminantium* subsp. *ruminantium* (*S. ruminantium*) was obtained from I. Skene, University of Adelaide, SA, Australia. *Butyrivibrio fibrisolvens* H17c was obtained from T. Whitehead, USDA, Peoria, IL, USA.

2.2 Preparation of anaerobic media

2.2.1 Broth cultures

In the majority of experiments in this study, either mBHI media or NB media were used (Appendix). Anaerobic broth media were prepared by mixing all components in a glass Schott bottle, adjusting to the correct pH and then boiling in a microwave oven for approximately 30 sec. The bottle was then capped and placed inside a Coy anaerobic chamber with an internal atmosphere of 95% v/v CO_2, 5% v/v H_2. Anaerobic conditions were maintained with a palladium catalyst and circulator unit. Once inside the hood, the lid of the bottle was loosened and the bottle was left inside the hood for a period of 2 hours for gas exchange to occur and the media to become anaerobic. Aliquots of media were then dispensed into Hungate tubes (15 ml), which

had been stored in the anaerobic atmosphere over night. The tubes were then capped, removed from the anaerobic hood and sterilised by autoclaving for 15 min at 120 °C.

2.2.2 Plate cultures

Nutrient plates for anaerobic bacteria were prepared by adding agar (1.5% w/v) to medium (Appendix) before autoclaving. The medium was cooled to approximately 45 °C before plates were poured in a laminar flow cabinet. Once the agar medium had set, the plates were transferred to the Coy anaerobic hood and left overnight to allow it to become anaerobic via gas exchange.

2.2.3 Media containing tannins and other phenolic acids

Crude extracts of condensed tannins were supplied by P. Martin, Queensland Department of Primary Industries, Brisbane, Queensland by extracting *Acacia aneura* leaves with 70% v/v acetone: water according to the method of Broadhurst and Jones (1978). *Acacia aneura* condensed tannin and tannic acid (Sigma) were stored under anaerobic conditions and stock solutions (w/v) of these and the simple phenolic acids were prepared immediately prior to use in anaerobic dilution solution (Appendix). The solution was neutralised and filter sterilised and added to liquid broth via syringe or to plate medium just before pouring, at the final concentration required.

2.3 Bacterial growth conditions

Frozen bacterial stocks were held in 20% (w/v) glycerol at -80°C. Stock cultures mBHI on nutrient agar plates were subcultured from frozen stocks.

Unless otherwise stated, 15 ml Hungate tubes containing 10 ml of bacterial growth medium (Appendix) were inoculated under anaerobic conditions with 0.1% (v/v) of a mid log bacterial culture grown in the same medium. Cultures were incubated at 39 °C for a specified time. Growth was monitored spectrophotometrically at A_{600} using a spectrophotometer (LKB Biochrom NOVASPEC, Cambridge England) that had been modified to accept 15 ml Hungate tubes. Inoculum absorbance readings (0 h) were subtracted from subsequent absorbance values following incubation with the bacteria. In the presence of phenolic acids, growth was quantified on mBHI nutrient agar plates by serial dilution and viable colony count. These values were subtracted from a viable colony count made immediately following inoculation to provide the net change in bacterial numbers. Each experiment was carried out in triplicate and done twice. The results represent the averages of these trials.

2.4 Preparation of whole cells and cell-free extracts

Mid log cultures (100 ml) of bacteria grown in mBHI were placed on ice to arrest growth before being centrifuged at 3,000 x g for 10 min at 4 °C. The culture supernatant was collected and concentrated by lyophilisation. All subsequent steps were carried out either at 4 °C or on ice. The cells were washed (0.1 M sodium phosphate buffer pH 6.8, Appendix) and suspended in 20 ml of the same buffer. When phenolic acids were present in the growth media, cells were initially washed in an equal volume of CE buffer (0.1 M, pH 10, Appendix) containing polyvinyl pyrrolidone (PVP, Calbiochem, 50 mg/ml) before washing in phosphate buffer. A portion of cells were removed, boiled for 10 min in a water bath and included as a negative control. For preparation of cell-free extracts, glass beads (0.1 g, 106 µm,

Sigma) and PVP (0.1 g) were added to the cell suspension and the mixture was sonicated at 200 W using a Branson Sonifier 450 (USA) for 6 x 30 second bursts with 2 min rest intervals between cycles to keep the suspension cool. Lysates were centrifuged at 3,000 x g for 10 min and the resultant supernatant (crude cell free extract) was centrifuged at 20,000 x g for 10 min to produce a cell membrane fraction and a cytosolic fraction. Protein concentrations in bacterial culture supernatant, cell membrane and cytosolic fractions were estimated using the Bradford assay (Bradford 1976) with bovine serum albumin (BSA) Fraction V (Sigma) as the standard.

2.5 Determination of bound tannin

Determination of the amount of tannic acid present in the tannic acid-protein precipitate was achieved using the method described by Hagerman and Butler (1981). A volume of 1 ml of 0.1% SDS in 5% (v/v) triethanolamine was added to dissolve the tannic acid-protein pellet. Subsequently, 100 µl of colour reagent (0.1 M $FeCl_3$ in 0.01 M HCl) was added and the A_{510} measured. The amount of tannic acid in bound form was determined from a tannic acid standard curve prepared at the same time.

2.6 Statistical analysis

Differences in the levels of tolerance to phenolic acids between *S. caprinus* or *S. bovis*, cell and capsule sizes and in the amount of extracellular polysaccharide produced by the bacteria were determined using student's t-test. Slopes of exponential growth, determined by linear regression analysis, are reported as the growth rates. The growth rates were analysed using analysis of variance for organisms by phenolic concentration interactions.

Experimental procedures used in chapter three

2.7 Growth in the presence of tannic acid

Tannic acid (Sigma) was added to crimp top bottles containing 100 ml sterile mBHI or NB medium containing NH_4Cl as the sole nitrogen source (Appendix). Tannic acid was added to the medium to a final concentration of:

S. caprinus: 0, 0.5, 1.0, 2.0, 3.0, 5.0 and 7% (w/v)

S. bovis: 0, 0.1, 0.2, 0.5, 0.75 and 1.0% (w/v)

The tubes were inoculated with *S. caprinus* or *S. bovis* as described. Cultures were incubated at 39 °C and aliquots (1.0 ml) were removed at hourly intervals. Growth was quantified on mBHI plates.

2.8 Growth in the presence of condensed tannins

Acacia aneura condensed tannin (crude extract) was added to mBHI medium to a final concentration of

S. caprinus: 0, 0.25, 0.5, 0.75, 1.0, 2.0 and 4.0% w/v.

S. bovis: 0, 0.25, 0.5, 0.75 and 1.0 w/v

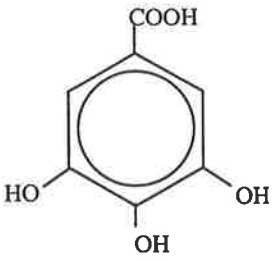
Triplicate tubes were inoculated with *S. caprinus* or *S. bovis* and after 0, 6, 12, 24 and 48 hours incubation, aliquots (0.5 ml) were removed and growth was quantified on mBHI plates as previously described.

2.9 Growth in the presence of other phenolic acids

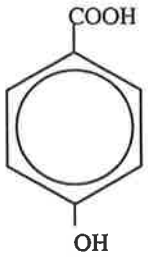
Various phenolic acids, as shown in Figure 2.1, were prepared immediately prior to use in anaerobic dilution solution (Appendix). The solution was filter sterilised and added to mBHI medium to a final concentration of 0, 0.25, 0.5, 1.0, 2.0, 4.0, and

Figure 2.1 Phenolic compounds used in this study

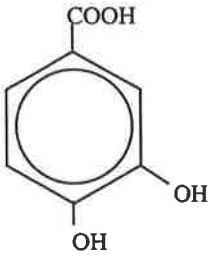
Gallic acid



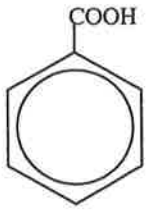
p-Hydroxybenzoic acid



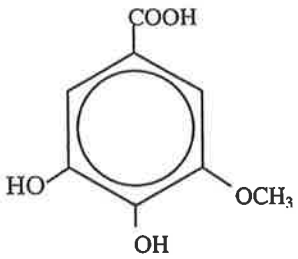
Protocatechuic acid



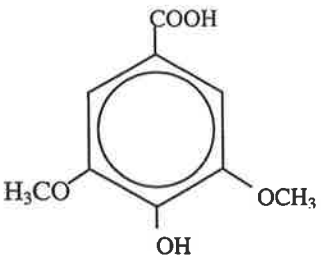
Benzoic acid



Methylgallate



Syringic acid



Vanillic acid

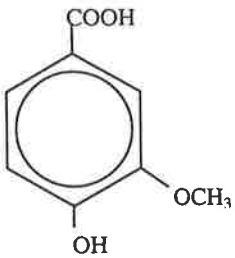
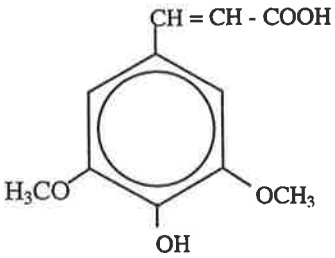
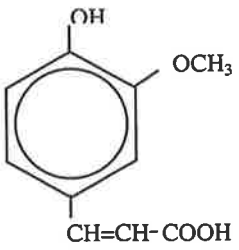


Figure 2.1 Phenolic compounds used in this study

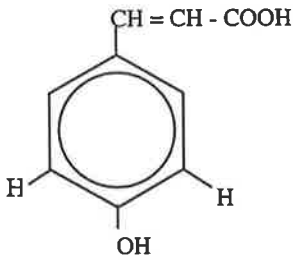
Sinapic acid



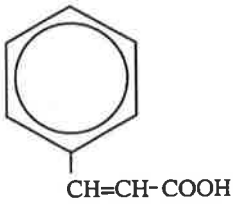
Ferulic acid



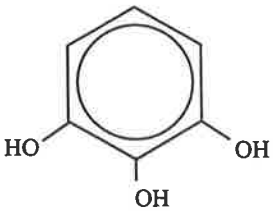
p-Coumaric acid



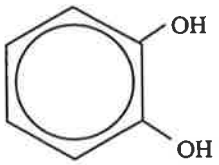
Cinnamic acid



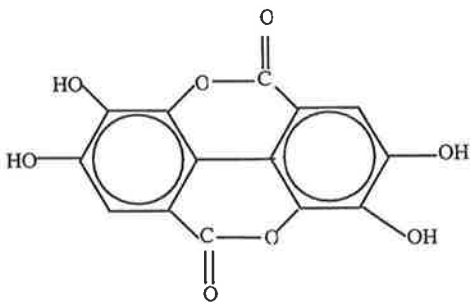
Pyrogallol



Catechol



Ellagic acid



5.0%. The tubes were inoculated with *S. caprinus* or *S. bovis*. Aliquots (0.1 ml) were removed and growth determined over a 48 h period by viable colony count as described above.

2.10 Ability to utilise tannins and other phenolic acids

Tannic acid, condensed tannin and the phenolic acids displayed in Figure 2.1 were incorporated as the sole carbon source to NB medium at a final concentration of 0.5% (w/v). The tubes were inoculated with *S. caprinus* or *S. bovis*. Growth was quantified on mBHI plates as previously described. When growth was detected, 0.1 ml was subcultured into a second tube to ensure no carry over of nutrients from the initial inoculum.

2.11 Adaptation to the presence of tannins and other phenolic compounds

Tannic acid, ferulic acid and p-coumaric acid were added to NB medium (0.75, 1.0% w/v) and inoculated with either *S. caprinus* or *S. bovis*. The cultures were incubated at 39 °C and growth was quantified at 12 h intervals by viable colony count as previously described. This constituted data for day 1. After 24 h growth, 0.1 ml from each of the first cultures was inoculated into a second set of tubes containing the test medium and cell growth was recorded for day 2. For day 3, a similar procedure was followed with 0.1 ml from cultures of day 2 used to inoculate a third set of test medium. Therefore the adaptation for three periods of 24 h each was evaluated.

2.12 Fractionation of tannic acid and effect on bacterial growth

2.12.1 Size fractionation

Dry Sephadex G-25 was allowed to swell in 95% ethanol for a minimum of 5 h before slowly poured into a glass column (60 x 4 cm) and washed with a 5 ml/min downward flow of this solvent. The void volume was determined using blue dextran (2 mg/ml) before the tannic acid sample (2 mg/ml in 95% ethanol) was applied to the top of the column in a volume of 3 ml. Fractions (5.0 ml) were collected, lyophilised, weighed and resuspended in dH_2O to a concentration of 1 mg/ml. The presence of 'tannin' was determined in each fraction by their dropwise addition to a 1% gelatin solution. Precipitation of the gelatin was determined visually and was taken to indicate a tannin component. Fractions were stored under anaerobic conditions until further use. The fractions were added to 3 ml mBHI media to a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 2.0, 4.0% w/v. The tubes were inoculated with *S. caprinus* or *S. bovis* and the effect on growth determined by viable colony count.

2.12.2 Charge fractionation

Fractionation of tannic acid was carried out using the procedure described by Graham (1992). Dry Sephadex LH-20 was prepared in 50% ethanol and poured into a glass column (60 x 4 cm) as described above. Tannic acid (2 mg/ml in 50% ethanol) was applied to the column, which was then extensively washed with 50% ethanol at a flow rate of 30 ml/h. Fractions (5 ml) were collected and the A_{280} was recorded. The column was then eluted at the same rate with 50% aqueous acetone (200 ml), fractions (5 ml) were collected and the A_{400} was determined. Results were graphed and the fractions making up individual peaks were combined. The fractions were lyophilised,

weighed and assayed for tannin as previously described. Fractions were stored under anaerobic conditions until use. The fractions were added to 3 ml mBHI media to a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 2.0, 4.0% w/v. The tubes were inoculated with *S. caprinus* or *S. bovis* and the effect on growth determined by viable colony count.

2.12.3 Protein binding ability

Tannic acid was added to mBHI medium to a final concentration of 1.0%. The precipitate that formed immediately upon addition of the tannic acid was allowed to consolidate by standing at room temperature for 1 h. The medium was centrifuged at 3,000 x g for 10 min and the supernatant removed under anaerobic conditions. In separate experiments, the supernatant was passed through various size filters (0.22 µm, 0.45 µm [Millex-GS, Millipore], and Whatman No. 3 filter paper). The amount of tannin in the pellet was determined as described (2.5). Fractions were stored under anaerobic conditions until use. mBHI medium (10 ml) was added to the tannic acid-protein pellet, and these tubes, along with those containing filtered supernatant fractions were inoculated with *S. caprinus* or *S. bovis* and the effect on bacterial growth determined.

2.12.4 Specific tannic acid-binding substances

Tannic acid-binding substances: polyvinylpyrollidone (PVP), iron, magnesium, lead acetate, were added as filter sterilised solutions to mBHI to a final concentration of 0.1 or 0.5% (w/v). Tannic acid was then added to the tubes in increasing concentrations

(0.5, 1.0 and 1.5% w/v), inoculated with either *S. caprinus* or *S. bovis* and growth determined by viable cell counts.

2.13 Preparation of trimethylsilyl (TMS) derivatives of phenolic compounds

Tannic acid, condensed tannins and isolated fractions obtained in 2.12. were extracted with an equal volume of ethyl acetate and freeze dried. Methyl gallate (0.1 mg/ml) was added as an internal standard. Pyridine (0.25 ml, Sigma) and n-o-bis(trimethylsilyl)-trifluroacetamide (BSTFA, 0.25 ml, Sigma) were added to the tubes and the mixture was allowed to stand for 30 min at room temperature. The samples were diluted in chloroform prior to analysis by GLC-MS.

2.14 Gas liquid chromatography, mass spectrometry (GLC-MS) of phenolic compounds

Trimethylsilyl derivatised samples were analysed on a Hewlett Packard GLC equipped with flame ionisation detectors and fitted with a BP-1 (SGE, 0.25) capillary column. Column conditions were as follows: carrier gas, helium; temperature 60 °C for 1 min then an increase to 200 °C at 5 °C/min followed by an increase to 300 °C at 20 °C/min. Injector temperature, 260 °C; detector temperature, 280 °C;

Mass spectra were obtained in the electron impact mode and the chemical ionisation mode; masses were scanned from 50 to 650 in 0.5 sec; chromatograms were recorded and peak areas integrated with a Hewlett-Packard reporting integrator and identified by comparison with the internal library and authentic standards

Experimental procedures used in chapter four

2.15 Binding properties of tannic acid used in this study

2.15.1 Effect of substrate

Neutralised and filter sterilised tannic acid (4 mg/ml) was added in increasing amounts (0 to 400 µl) to 100 µl of BSA, gelatin or PVP (1 mg/ml each). The total volume of each tube was brought up to 500 µl and the mixture incubated under anaerobic conditions for 1 h at 37°C. The mixture was then centrifuged at 15,000 x g for 15 min and the supernatant decanted off. The pelleted material was washed in 0.5 ml 0.1 M phosphate buffer and the amount of tannin in the pellet was determined as previously described (2.5).

2.15.2 Effect of pH

Tannic acid (4 mg/ml) was dissolved in buffers (0.1 M, Appendix) of various pH. pH 3.5 sodium citrate buffer, pH 6.8 sodium phosphate buffer, pH 9.0 Tris buffer. The tannic acid solution was added in increasing amounts (0 to 400 µl) to 100 µl of PVP (1 mg/ml). The total volume of each tube was brought up to 500 µl with buffer and the mixture was incubated under anaerobic conditions for 1 h at 37°C. The amount of tannin in the precipitate was determined.

2.15.3 Effect of oxygen

Equal volumes (0.25 ml) of tannic acid (4 mg/ml w/v) and PVP (1 mg/ml w/v) were combined and incubated anaerobically or aerobically at 39 °C for 1 h. The amount of tannin in the precipitate was determined as described.

2.15.4 Effect of time

In a number of tubes, equal volumes (0.25 ml) of tannic acid (4 mg/ml w/v) and PVP (1 mg/ml w/v) were combined and incubated anaerobically at 39 °C as described above. At 30 min intervals the amount of tannin in the precipitate was determined.

2.15.5 Effect of temperature

In a number of different experiments, equal volumes (0.25 ml) of tannic acid (4 mg/ml w/v) and PVP (1 mg/ml w/v) were combined and incubated anaerobically at temperatures of 25, 39, and 60 °C. The amount of tannin in the pellet was determined at a number of time intervals.

2.16 The effect of tannic acid on lactate production

Tannic acid was added to mBHI medium to a final concentration of 0, 0.5, 1.0, 2.0 and 5.0% w/v. The tubes were inoculated with *S. caprinus* or *S. bovis* and incubated at 39 °C and growth was quantified at hourly intervals by viable cell count. At these times polyvinylpyrolidone (PVP, 50 mg) was added to duplicate aliquots (0.1 ml) to remove residual tannic acid. Caproic acid (50 µl, 0.1 M) was included as an internal standard and the mixture was centrifuged at 3,000 x g for 10 min. The amount of lactate present in the supernatant was determined on a Shimadzu 14A Gas Chromatograph combined with Delta Data System (SGE Analytical Products) and equipped with a bonded phase capillary column (BP21: 0.5 µm film, 0.25 m x 0.53 mm ID, SGE). 0.2 µl was injected onto the column. A calibration standard containing lactic, succinic and caproic acids were used to identify chromatographic peaks in the sample fractions. The concentration of component acids were calculated by comparing the

ratio of acid peak area to internal peak area with the corresponding ratios measured on the standard mixture.

Conditions: injector temperature 240 °C; detector temperature 280 °C; column temperature 85 to 140 °C at a rate of 6 °C/min with split ratio 1/60 and purge ratio 1/10. The carrier gas was nitrogen at a flow rate of 0.5 kg/cm^2.

2.17 The effect of tannic acid on protein production

SDS polyacrylamide gel electrophoresis (PAGE) was performed as described by Ausubel *et al.* (1989) with 4 % stacking and 10% resolving gels. Cell-free extracts (2.4) from both *S. caprinus* and *S. bovis* grown in mBHI containing tannic acid concentrations of 0, 0.25 and 0.5% w/v were applied to the gel (15-20 µg protein in a load volume of up to 15 µl). The gel was electrophoresed at 200 V for 45 min and stained for protein using Coomassie Blue R 250 (Sigma) for at least 1 h before being placed in destain (Appendix) to resolve the protein bands. The gel was dried using a Biorad gel drier and/or photographed for a more permanent record.

2.18 The effect of tannic acid on enzyme activity

2.18.1 Proteinase activity

2.18.1.1 Casein degradation

NB nutrient agar plates were overlaid with a casein-agar preparation (Appendix). Bacteria from mid log cultures of *S. caprinus* or *S. bovis* were spot-inoculated onto the casein overlay with sterile Pasteur pipettes. The plates were incubated at 39 °C overnight. Casein degradation was determined by flooding the plate with 1 N HCl, which caused precipitation of the undegraded casein. Colonies that showed zones of

clearing were scored as positive for casein degradation. *Butyrivibrio fibrisolvens* was used as a positive control.

2.18.1.2 Azocasein degradation

The assay mixture contained 0.5 ml of substrate [azocasein (0.8%) dithiothreitol (DTT, 2 mM) in 0.1 M sodium phosphate buffer pH 6.8, (Appendix)] and 0.5 ml of the enzyme source (0.5 to 4 mg protein/ml) in 1.8 ml Eppindorf tubes. Tannic acid was added to the assay mix in increasing concentrations (0 to 300 µg/ml). Assay mixtures were incubated for 3 h at 39 °C. The reaction was stopped by the addition of 0.5 ml of cold 1.5 M $HClO_4$, and the tubes were held on ice for 30 min. The precipitated protein was removed by centrifugation at 12,000 x g for 5 min, and 1 ml of supernatant fluid was withdrawn and combined with an equal volume of 1 N NaOH. The concentration of acid-soluble azopeptides in the resultant solution was determined by optical density measurement at 450 nm using a Shimadzu UV-160A spectrophotometer. Control assays were performed by incubating enzyme samples and the azocasein substrate separately and by combining these solutions at the time of acid addition. Protease K at a final concentration of 25 µg/ml was also included as a control. The assay was performed in triplicate and the specific proteinase activity was expressed as µg azocasein hydrolysed per hour per mg of protein, except when whole cells were used and then activity was expressed per viable cell count. The assay was tested for linearity with respect to time, pH and bacterial enzyme concentration. All assays were conducted within the linear region of the curve.

2.18.2 Lactate dehydrogenase (LDH) activity

The method of Bergmeyer (1972) was employed to determine lactate dehydrogenase activity in cell-free extracts of *S. bovis* and *S. caprinus*. The assay mixture (1 ml) contained 0.1 M phosphate buffer (pH 6.8), 0.2 mM NADH, 0.1 mM fructose-1,6-biphosphate and 1 to 50 µl crude cell-free extract (2.5). Sodium pyruvate (1 mM) was added to start the reaction. The A_{340} was recorded at 1 min intervals and the mean change in A_{340}/min/mg microbial protein was calculated to determine the specific activity. Control reactions, in which substrate was incubated alone and enzyme preparation added after boiling for 5 min were included to determine if any spontaneous conversion of the substrate occurred. LDH (Sigma, 35 µl of 1 mg/ml) was also used as a control. Validity of the activity assay was tested for linearity with respect to time, enzyme concentration and pH as previously described. Samples were measured in triplicate and the experiment was repeated to confirm results.

2.19 Genetic characterisation of tannin tolerance

2.19.1 Construction of plasmid libraries

Chromosomal DNA was isolated from a 100 ml broth culture of *S. caprinus* as described by Ausubel *et al* (1991). The DNA was partially digested with Sau3A to produce DNA fragments of various sizes as determined by agarose gel electrophoresis. Fragments of between 1 and 10 kb were isolated from the gel according to standard procedures (Ausubel *et al* 1991). The plasmid vectors, pUC19 and pMU1328 were totally digested with BamH1 and the resultant 5' ends were treated with calf intestinal phosphatase to prevent religation (Ausubel *et al* 1991). The *S. caprinus* DNA fragments were then ligated into this site and the ensuing plasmids

were respectively electroporated into electrocompetent *E. coli* ED8299 or *E. coli* DB11 cells. Electroporation conditions were: 12.5 kV/cm voltage, 25 μF capacitance and 200 ohms resistance. Transformed cells were placed into SOC media (1.0 ml, Appendix) and allowed to express for 1 h at 37 °C with gentle shaking. Cells electrotransformed with pUC19 were plated onto LB media agar (Appendix) containing ampicillin (Sigma, 50 μg/ml), X-gal (Promega, 40 μg/ml) and isopropylthiogalactoside (IPTG, Boehringer Mannheim, 40 μg/ml) and incubated at 37 °C overnight. Positive clones were identified through α-complimentation as white colonies. Cells transformed with pMU1328 were plated onto LB agar containing erythromycin (erm, Sigma, 10 μg/ml). Positive clones were amplified by growing for 1 h in liquid LB media containing erm. The plasmids were then re-isolated and electroporated into *S. bovis* electrocompetent cells. Transformed cells were placed into mBHI medium (1.0 ml) and incubated anaerobically at 39 °C until an increase in cell numbers were detected by monitoring the A_{600}. Cells were then plated onto mBHI medium containing erm (10 μg/ ml).

2.19.2 Screening of libraries

Colonies were screened for increased tannin tolerance (tannin concentrations > 0.75%) and/or clearing activity by replica plating onto either

1. modified M9 medium (*E. coli* transformants, Appendix) containing ampicillin, X-gal, IPTG and 0.5% tannic acid or
2. mBHI medium (*S. bovis* transformants) containing erm and 0.5 and 1.0% tannic acid and incubating overnight at 39 °C under anaerobic conditions.

Experimental procedures used in chapter five

2.20 Detection of gallic acid in the media

S. caprinus or *S. bovis* were grown in mBHI (50 ml) containing 0.5 and 1.0% tannic acid for 36 h. Uninoculated media containing tannic acid were included as controls. At regular intervals, aliquots (0.1 ml) were removed and the presence of gallic acid determined in the sample using the method outlined by Inoue and Hagerman (1988). Each aliquot was added to a glass tube containing 0.15 ml methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol). After exactly 5 min, 0.1 ml of 0.5 N KOH was added and after a further 2.5 min, the mixture was diluted to 2.5 ml with dH_2O. The A_{520} was measured after 10 min using a Shimadzu UV-160A spectrophotometer and quartz glass cuvettes (1.0 cm light path). A standard curve was prepared using increasing concentrations of gallic acid in mBHI media.

2.21 Tannin acylhydrolase assay

Tannin acyl hydrolase activity was determined in *S. caprinus* by the method described by Skene and Brooker (1995). Whole cells of *S. caprinus* following growth in the presence of 0 or 0.5% w/v tannic acid were prepared as described (2.4). The cell suspension (50 µl) was added to a 1.5 ml microcentrifuge tube containing 0.1 M phosphate buffer (pH 6.8, 900 µl). The reaction mixes were preincubated at 37 °C for 5 min and were initiated by adding 600 µl of either gallic acid methyl ester (GAME, 0.5% w/v), gallic acid lauryl ester (0.5% w/v) or tannic acid (0.5% w/v), made up the same buffer. The tubes were incubated for up to 2 h. Aliquots (0.1 ml) were removed from the tube at various intervals and the concentration of gallic acid was determined

using the rhodanine assay (Inoue and Hagerman, 1988) described in 2.20. *S. ruminantium* K2 was included as a control.

2.22 Gallate decarboxylase activity

2.22.1 Spectrophotometric analysis

2.22.1.1 Whole cells

Whole cell preparation of *S. caprinus* grown in 0, 0.5 or 1.0% gallic acid or tannic acid were prepared as described (2.4). The absorbance maxima for tannic acid and gallic acid in 0.1 M phosphate buffer (pH 6.8) was determined by conducting a spectrophotometric scan of wavelengths between 250 and 300 nm. The gallate decarboxylase assay mixture contained the phenolic acid (0.01% w/v), dithiothreitol (DTT, 2.5 mM), $MgCl_2$ (10 mM) and phosphate buffer in a 1 ml quartz glass cuvette which had been preincubated at 39 °C for 5 min. Cell suspensions (0 to 20 µl) were also preincubated and added to the cuvette to initiate the reaction. The reaction was monitored at the predetermined wavelength over a 15 min period using a spectrophotometer (LKB Biochrom NOVASPEC) positioned in a Coy anaerobic hood. *S. bovis* was included as a control. Specific activity was expressed as ρmoles phenolic acid decarboxylated/min/cell.

2.22.1.2 Cell free extracts

Whole cell preparations of *S. caprinus* grown in the presence of 0 and 0.5 w/v tannic acid or gallic acid were prepared (2.4). The cells were transferred to an anaerobic hood and lysed by grinding with 100 mg of acid washed sand using a mortar and pestle. Liquid nitrogen was included to keep the cell suspension cold. Lysates were

centrifuged at 3,000 x g for 10 min to remove the sand and unlysed cells and obtain the cell free extracts. Protein concentrations in bacterial cell free extracts were estimated using the Bradford assay (Bradford 1976) with bovine serum albumin (BSA) Fraction V (Sigma) as the standard. Extracts were diluted in 0.1 M phosphate buffer (pH 6.8) to a protein concentration of 0.1 to 1 mg/ml and determination of gallate decarboxylase activity was carried out as described above.

2.22.2 GLC analysis

2.22.2.1 Whole cells

Whole cell preparations of bacteria grown in the presence of 0 and 0.5% w/v tannic acid or gallic acid were prepared as described (2.4). Aliquots (50 µl) of cell suspension were added to 0.1 M phosphate buffer (pH 6.8, 1.0 ml) and preincubated at 37 °C for 5 min. Gallate decarboxylase activity was initiated by adding 0.5 ml of 0.5% w/v gallic acid or tannic acid dissolved in 0.1 M phosphate buffer (pH 6.8). Immediately after addition of the substrate, an aliquot (0.1 ml) was removed and extracted with 0.5 ml of ethyl acetate and freeze dried. Subsequent aliquots (0.1 ml) were removed, extracted with ethyl acetate and dried. TMS derivatives of the supernatant were prepared and the samples analysed by GLC as described (2.13 and 2.14). Breakdown products were identified by comparison with authentic standards.

2.22.2.2 Cell free extracts

Cell free extracts were prepared as described above (2.4). Extracts were diluted in 0.1 M phosphate buffer (pH 6.8) to a protein concentration of 0.1 to 1 mg/ml and pre-incubated for 5 min at 37 °C prior to initiating the assay. Gallic acid and tannic acid

(0.5% w/v dissolved in 0.1 M sodium phosphate buffer pH 6.8) was pre-incubated under the same conditions. The enzyme reaction was initiated by addition of the substrate. At zero time an aliquot (0.1 ml) was immediately removed to a glass tube and the reaction stopped by immersion in liquid nitrogen. Aliquots (0.1 ml) were removed from the reaction at various intervals over a 60 min period and placed in liquid nitrogen. The tubes were removed from the anaerobic hood and TMS derivatives of the supernatant were prepared and the samples analysed by GLC as described.

2.23 Specificity of gallate decarboxylase activity

Gallate decarboxylase activity was assayed in whole cell suspensions prepared from *S. caprinus* using a number of different compounds as substrates. Gallate decarboxylase activity was assayed primarily as described (2.22.1) except that the gallic acid substrate was replaced with: protocatechuic acid, hydroxybenzoic acid or syringic acid. The absorbance maximum for each acid was obtained by conducting a scan of wavelengths between 250 and 300 nm.

2.24 Upregulation of gallate decarboxylase activity

Specific activity of gallate decarboxylase activity was determined in whole cell suspensions prepared from *S. caprinus* grown in mBHI supplemented with 0 and 0.5% w/v of the following phenolic acids: tannic acid, gallic acid, condensed tannin, and ferulic acid. Cells were then washed and incubated in 0.5% gallic acid or tannic acid and the amount of pyrogallol produced was measured by GLC analysis as previously described (2.22.2). Cell numbers were determined by viable cell count on mBHI

medium. Results were expressed as pmoles pyrogallol produced/min/cell. Induction of gallate decarboxylase activity in whole cells prepared from *S. bovis* after growth in 1.0% gallic acid was also examined.

2.25 Production of CO_2

S. caprinus was grown in mBHI medium supplemented with increasing concentrations (0 to 4% w/v) of tannic acid or gallic acid. An 18 gauge needle attached to a 2 ml glass syringe was inserted into the free space of the tube through the rubber septa and the tubes were incubated at 39 °C. The amount (ml) of gas produced by the culture, as determined by displacement of the syringe plunger, was monitored over a 24 hour period. The nature of the gas was then analysed by GLC and identified by comparison with standards. Uninoculated media and tubes inoculated with *S. bovis* were included as controls.

2.26 Breakdown of other phenolic compounds

Bacterial cultures were inoculated into mBHI medium supplemented with the phenolic acids shown in Figure 2.1. Aliquots (0.5 ml) were removed throughout the growth period and immediately extracted with ethyl acetate. TMS derivatives of the supernatant were prepared and the samples analysed by GLC as described (2.13, 2.14).

Experimental procedures used in chapter six

2.27 Morphological examination of bacterial cells using light microscopy

S. caprinus or *S. bovis* were incubated into mBHI medium containing tannic acid, condensed tannin, gallic acid, ferulic acid, p-coumaric acid and pyrogallol. At various time intervals, bacterial cells (wet mount) were visualised using phase contrast optics by light microscopy. Changes in morphology were recorded.

2.28 Gram stain

Gram stains of *S. caprinus* and *S. bovis* in the presence and absence of 0.5% (w/v) tannic acid were performed using the procedure described by Tortora *et al* (1992)

2.29 Capsule stain

2.29.1 India Ink Method

S. caprinus or *S. bovis* were spotted (≅ 5 µl) onto a glass slide and combined with an equal volume of India ink. Cells were viewed at x 400, under oil immersion.

2.29.2 Anthony Method

Bacterial cells were spotted onto a microscope slide as before and allowed to air dry. The cells were stained using crystal violet (1%, 2 min) and then rinsed with a 20% aqueous solution of copper sulphate. Bacteria were visualised with the light microscope as described above.

2.30 Field emission scanning electron microscopy (FESEM)

S. caprinus or *S. bovis* were grown in mBHI medium in the presence of increasing concentrations of tannic acid, condensed tannin, gallic acid, ferulic acid, p-coumaric acid and pyrogallol. Throughout bacterial growth, aliquots were removed and the cells centrifuged at 3,000 x g for 10 min. Cells were resuspended in fixative solution (Karnovsky 1965, Appendix) containing ruthenium red (0.15% (w/v), Frehel *et al* 1988) and incubated at room temperature for 1 h at 4 °C. The cell suspension was centrifuged as before and the cells were washed twice (30 min each) in phosphate buffered saline (PBS) containing 4% sucrose and 0.05% ruthenium red. The pellets were postfixed for 2 h with 2% aqueous osmium tetroxide (ProSciTech) containing 0.05% ruthenium red. The cells were dehydrated through a graded series of ethanol solutions (50, 70, 90, 95 and 100%) with a final change of 1 hour in 100 % ethanol at room temperature. The samples were infiltrated with Peldri II (ProSciTech) and ethanol in the ratios of 1:1 and 2:1 and with 100% Peldri II for 45 min each, before being spotted onto aluminium SEM stubs (ProSciTech) and dried under vacuum. The samples were coated with gold-palladium-carbon and examined with a Phillips XL30 field emission scanning electron microscope operated at 15 kV. Images were stored directly onto compact disc.

2.31 Transmission electron microscopy (TEM)

Bacterial cells were prepared as above, except that they were infiltrated with Spurr low-viscosity embedding resin (Spurr 1969, Appendix) and ethanol in the ratios of 1:1 and 2:1 and with 100% resin for 45 min each. The cells were placed in 1 ml fresh 100% Spurr resin and polymerised at 60 °C for 24 h. Embedded preparations were

sectioned with a diamond knife (70 ηm) using a Leica Reichert Ultracut E microtome and placed on collodion-coated 400-mesh copper grids. The sections were stained with 2% aqueous uranyl acetate (ProSciTech) for 20 min followed by lead citrate for 5 min (Reynolds 1963). The sections were examined using a Phillips CM100 transmission electron microscope at an accelerating voltage of 60 kV and images were recorded on Kodak no. 4489 electron microscope film.

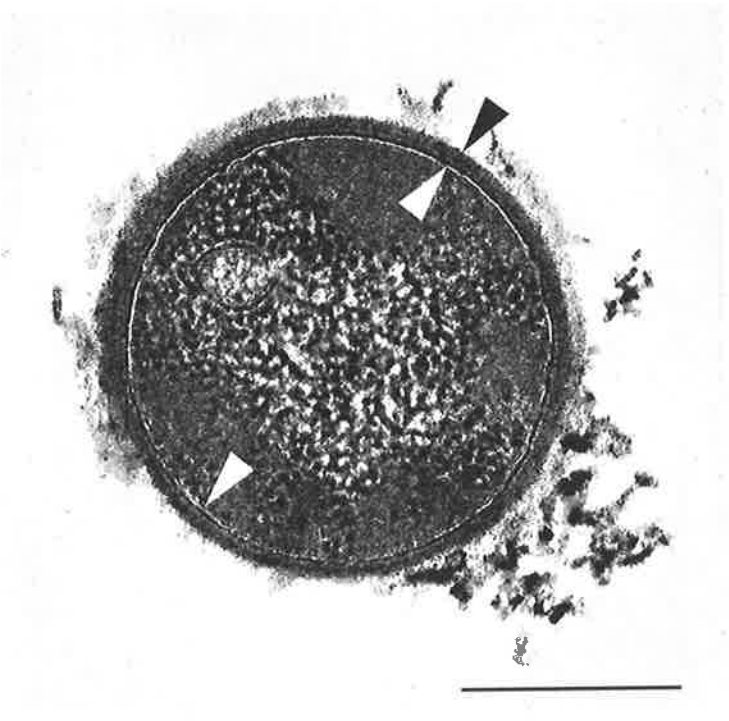
2.32 Image analysis

Images were transferred to the image analysis program Adobe Photoshop and the digitised images were optimised with respect to brightness and contrast. Following growth in phenolic acids, cell size and cell wall diameter were determined for 50 bacteria by measuring digitised micrographs of cell cross sections using Photoshop. Only cells that showed a distinct cross section of the cell wall were measured (Figure 2.2). The results were compared to that of the control (no phenolic acid) using the student's t-test.

2.33 Isolation and partial purification of exopolysaccharide (EPS)

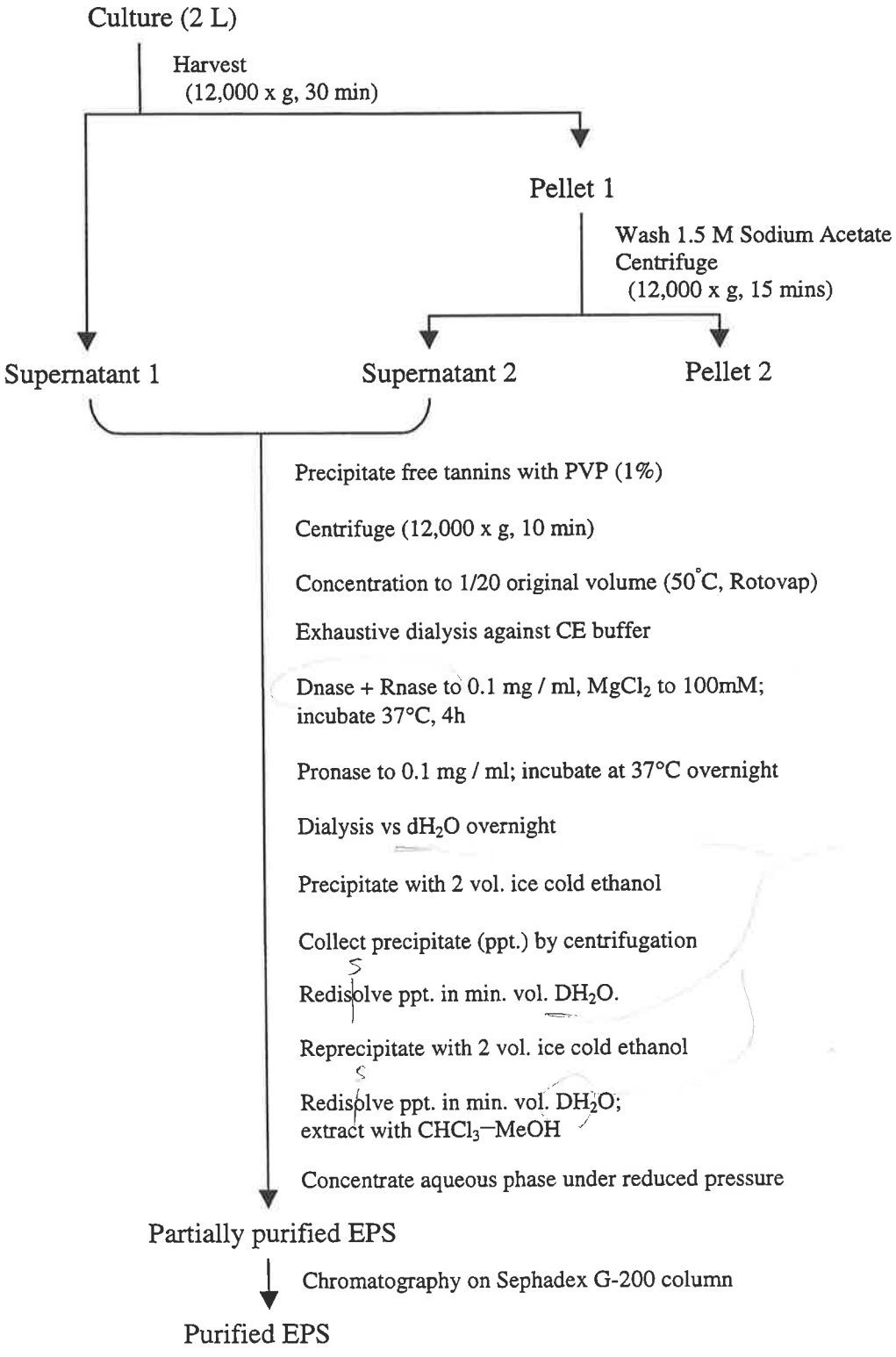
The procedure used for the isolation and purification of EPS from bacterial cells is described in Figure 2.3. Cultures (2 L) were grown in mBHI media in the presence and absence of tannic acid (0.25 to 5.0% w/v) for 24 hours and harvested by centrifugation (12,000 x g for 30 min). The supernatant was immediately removed and the bacterial cell pellet was resuspended in 1.5 mM sodium acetate (pH 4.2) to remove the cell associated EPS and re-centrifuged. The spent culture supernatant (supernatant 1) was combined with the sodium acetate wash fraction (supernatant 2)

Figure 2.2 **Transmission electron micrograph showing areas of measurement**



Transmission electron micrograph of a thin section of *S. caprinus* grown in mBHI media containing tannic acid and stained with ruthenium red. Note the distinct cross section. Cell size was measured from inner cell wall to inner cell wall (internal arrows). Cell wall thickness was measured from the outside of the cell wall to the inner cell wall. Bar = 0.5 μm

Figure 2.3 **Schematic diagram for the isolation and purification of bacterial extracellular polysaccharide**



and PVP was added to a final concentration of 1% before centrifuging (12,000 x g for 10 min) to precipitate any free tannin and to remove any remaining cells. The supernatant was then concentrated by rotary evaporation and dialysed extensively against CE buffer (pH 10, Appendix) to remove any EPS associated tannins. DNA, RNA and protein were removed from the supernatant before dialysis against dH_2O. The EPS was precipitated twice before being extracted with methanolic chloroform and concentrated under reduced pressure to give partially purified EPS. The cell pellet was taken to complete dryness under vacuum at 40 °C and subsequently weighed. The amount of EPS was then correlated to the dry weight of the cells.

2.34 Purification of EPS

EPS was further purified by chromatography on a Sephadex G-200 column (Pharmacia). EPS (2 mg/ml in 0.1 M NaCl) was applied to the equilibrated column (0.1 M NaCl) and eluted with 100 ml of NaCl at a flow rate of 20 ml/hr. Fractions (5.0 ml) were collected and analysed for total carbohydrate content by colorimetric analysis as described (2.5.9). Peak fractions were combined, dialysed overnight against dH_2O and lyophilised. The lyophilised samples were weighed and the neutral sugar content was determined by colorimetric assay.

2.35 Compositional analysis of EPS

2.35.1 Total carbohydrate analysis

Total carbohydrate analysis was determined by the phenol-sulphuric acid method of Dubois *et al,* (1956). A standard solution was made with increasing concentrations of glucose (10 to 100 µg/ ml). Samples (2 mg/ml) and standard were pipetted into Pyrex

tubes and the volumes adjusted to 0.5 ml with dH_2O. Phenol (0.5 ml, 5%) and concentrated H_2SO_4 (2.5 ml) were added to the tubes which were then kept at room temperature for 20 min. The A_{488} was recorded relative to a blank tube containing no glucose and the concentrations of sugars in the sample were determined from the standard glucose curve.

2.35.2 Monosaccharide determination

2.35.2.1 Acid hydrolysis

Sugar standards (ribose, arabinose, xylose, mannose, deoxy-galactose, glucose, and galactose, 1 mg/ml each, inositol (internal standard), 0.5 mg/ml) and EPS samples (2 mg/ml, inositol (internal standard), 0.5 mg/ml) were hydrolysed in 2 N HCl for 2 hours at 100 °C. HCl was removed by lyophilisation.

2.35.2.2 Aldononitrile acetate derivatives

Aldononitrile acetate derivatives were prepared by the procedure of Chaplin (1982). In a sealable glass vial, methanolic HCl (800 µl of 1 M) and methyl acetate (400 µl, Sigma) was added to the standards and sample. The tubes were vortexed and incubated at 80 °C for 16 h. After cooling, t-butyl alcohol was added (100 µl), and the mixture vortexed and left to evaporate in a fume hood at room temperature. Methanol (500 µl), pyridine (50 µl, Sigma) and acetic anhydride (50 µl, BDH) were added and mixed thoroughly. The vials were then incubated at room temperature for 15 min and then left to evaporate to dryness at room temperature in a fume hood.

2.35.2.3 Trimethylsilyl derivatives for carbohydrate analysis

Tri-Sil (100 µl, Pierce Cat. No 48999) were added and the samples were incubated at 70 °C for 10 min. The tubes were stored in a desiccator over silica gel until ready for analysis. Samples were analysed by GLC-MS after resuspension in n-Hexane (100 µl, BDH).

2.35.2.4 GLC-MS for carbohydrate analysis

Derivatised samples were analysed on a Hewlett Packard GLC equipped with flame ionisation detectors and fitted with a BP-21 (SGE, 0.25) capillary column. Column conditions were as follows: temperature 130 °C for 2 min then an increase to 320 °C at 6 °C/min for 10 min. Carrier gas, helium: injector temperature, 260 °C; detector temperature, 280 °C.

Mass spectra were obtained in the electron impact mode and the chemical ionisation mode; masses were scanned from 70 to 700 in 0.5 sec; chromatograms were recorded and peak areas were integrated with a Hewlett-Packard reporting integrator and identified by comparison with authentic standards.

2.35.3 Uronic acid content

Uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen (1974). A uronic acid standard curve using D-mannuronic acid lactone, was prepared (0 to 100 µg/ ml) in 0.4 ml dH_2O. The sample (2 mg/ml) and standards were hydrolysed in concentrated H_2SO_4 (2.4 ml) in a boiling water bath for 20 min and then chilled on ice. o-hydroxydiphenyl (OHDP, Sigma) solution (150 µl, 0.15% OHDP in 0.5% NaOH) was added to the tubes and the mixture vortexed. The A_{525} was recorded

against a blank containing no OHDP and the amount of uronic acid in the sample was determined by comparison to the standard curve.

2.35.4 Hexosamine content

Hexosamine content was determined by the method of Somme (1974). A hexosamine standard curve was prepared (0 to 100 µg/ ml) in dH_2O (0.1 ml) using D-glucosamine. The sample and standards were hydrolysed as previously described. Equal volumes (10 µl) of a saturated solution of $NaHCO_3$ and of freshly prepared aqueous acetic anhydride (5%) were added. After 10 min at room temperature, the tubes were immersed in boiling water for 3 min and then cooled on ice. Potassium tetraborate (50 µl, 5%) was added and the tubes incubated in a boiling water bath for 7 min and cooled as before. Morgan-Elson reagent (Appendix, 0.7 ml) was added and the tubes incubated at 37 °C for 20 min. The A_{585} was recorded against a blank and the amount of hexosamine in the sample was determined from the standard curve.

2.35.5 Pyruvate content

Pyruvate content was determined by the method of Slonecker and Orentas (1962) and Gutman and Wahlfield (1974).

2.36 The effect of phenolic compounds on EPS production

Bacteria were grown in mBHI medium containing increasing concentrations of gallic acid, ferulic acid and p-coumaric acid before isolation and partial purification of EPS was employed (2.33). The supernatant fluid was treated as described and the total carbohydrate composition was determined by colorimetric analysis (2.35.1). The cell

pellet was taken to complete dryness under vacuum at 40 °C and subsequently weighed. The amount of EPS was then correlated to the dry weight of the cells.

Chapter 3

Growth in tannins

Chapter 3 Growth in tannins

3.1 Introduction

Phenolic compounds, such as tannins, lignin and their intermediate degradation products have been found to occur in free or combined form within the plant kingdom (Akin *et al* 1993; Woodward and Reed 1997). After lignins, tannins are the second most abundant group of plant phenolics (Bhat *et al* 1998). They are found in leaves, fruits, bark and wood and as a result, are common constituents of the ruminant diet (Jones *et al* 1994; Woodward and Reed 1995, 1997; Sotohy *et al* 1997). Many of these phenolic compounds, such as condensed tannins and lignin, have been reported to be toxic to microorganisms, particularly rumen bacteria and extensive work has been done detailing their toxicity (Borneman *et al* 1986; Jung and Fahey, 1983; Kholdebarin and Oertli 1994; Field and Lettinga 1992; Bae *et al* 1993; Jones *et al* 1994; Nelson *et al* 1997). Further evidence suggests that the simple phenolic monomers, which exist both independently and as constituents of lignin or plant cell walls, have a significant influence on animal nutrition (Jung and Fahey, 1983, Akin *et al* 1993; Lowry *et al* 1996). The relative abilities of various microorganisms to tolerate tannins and other phenolic compounds differ (Bae *et al* 1993; Jones *et al* 1994; Scalbert, 1991). Certain bacteria can survive in the presence of various phenolic compounds and some can even utilise them as carbon sources (Deschamps 1989; Patel *et al* 1992; Nelson *et al* 1995, 1998; Osawa *et al* 1995; Osawa and Sly 1992; Brooker *et al* 1994) and these bacteria have an obvious advantage over those that are inhibited by their presence.

As mentioned in Chapter 1, feral goats in Australia are able to thrive on tannin-rich forages such as Acacia sp. which are poorly digested by domestic sheep and cattle. One possible explanation for this difference is that the goat possesses a unique rumen microbiota, which enables the animal to tolerate or degrade the phenolic compounds present in its diet. *S. caprinus* was isolated from the rumen contents of these feral goats and has been shown to tolerate high levels of tannins in its growth media. Preliminary characterisation of these bacteria has been published previously (Brooker *et al*, 1994), however, in this chapter, the ability of *S. caprinus* to tolerate tannins and other, more simple phenolic acids is presented in detail. Comparison with the more common, tannin sensitive ruminal bacteria, *S. bovis* is provided as a reference organism.

Both condensed tannin extracted from *Acacia aneura* and the hydrolysable tannin, tannic acid, were used in this study. The extraction procedure used in obtaining condensed tannin was difficult, time consuming and gave variable results. The composition of tannic acid has also been shown to vary depending on the extraction procedure used and the company supplying it. Gas liquid chromatography (GLC) was utilised in attempt to identify the composition of these tannins. Fractionation of tannic acid using gel filtration chromatography was undertaken in an effort to isolate the growth inhibitory compound present.

3.2 Results

3.2.1 Growth in the presence of tannins

Previously, it has been reported that *S. caprinus* can tolerate levels of tannic acid and condensed tannin in its growth medium of at least 2.5% w/v (L. O'Donovan, Honours Thesis, Brooker *et al*, 1994). The results presented here show that *S. caprinus* can in fact tolerate much higher levels of both hydrolysable and condensed tannins in its growth medium, particularly compared with the more common ruminal Streptococcus species, *S. bovis*. *S. caprinus* isolates grew in the medium containing at least 7.0% w/v tannic acid or 4.0% condensed tannin (the highest concentrations tested, Figure 3.1) whereas *S. bovis* cell numbers were significantly reduced by a tannic acid or condensed tannin concentration of 0.75% w/v ($P<0.05$, Figure 3.2) and even after 5 days incubation, no further increase in cell numbers were observed. At concentrations greater than this amount, growth of *S. bovis* was completely inhibited. These results were observed when either mBHI media was used or when the bacteria were grown in completely defined NB medium containing NH_4Cl as the sole source of nitrogen. A reduced precipitate was observed in this medium due to the lack of a protein source, however complexing of the tannic acid with other media components still occurred.

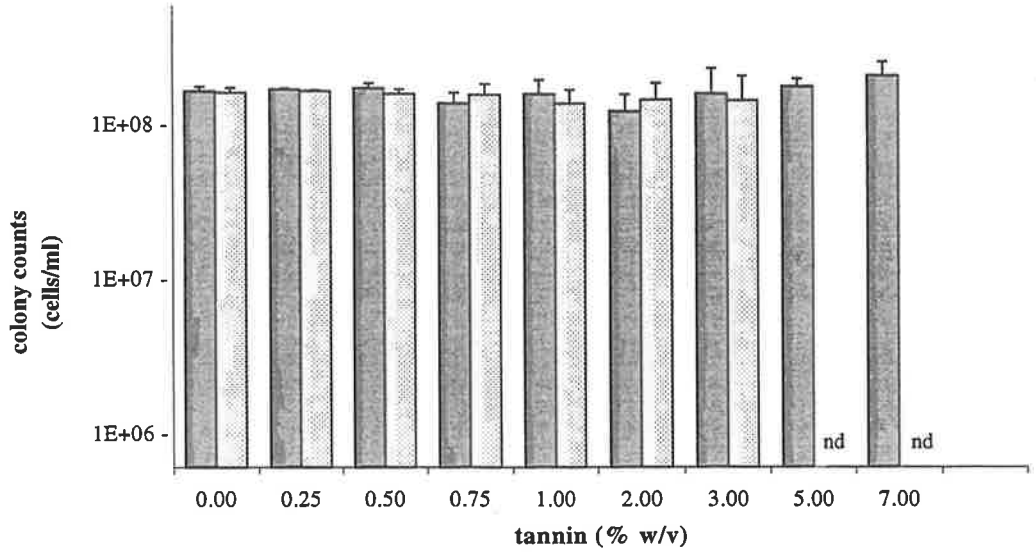
Significant differences ($P<0.05$) were observed in the growth lag period as the concentration of tannic acid or condensed tannin in the medium was increased. For *S. bovis*, growth in 0.1, 0.2, 0.5 and 0.75% tannic acid was preceded by a lag period of 2, 3, 8 and 16 hours respectively (significant $P<0.01$, Figure 3.3). *S. caprinus* also showed an increase in lag time at concentrations of tannic acid (Figure 3.4) greater

than 1.0% w/v or condensed tannin concentrations of 0.5% w/v (Figure 3.5). Average lag times were: 4, 7, 16 and 23 h for concentrations of tannic acid in the medium of 1.0, 2.0, 3.0, and 5.0% w/v. The presence of condensed tannin caused an even greater increase in the lag time for *S. caprinus*. At a concentration of 2.0% condensed tannin, the lag time was extended to 10 h.

Repeated subculturing of both *S. caprinus* and *S. bovis* into fresh media containing similar concentrations of either tannic acid or condensed tannin decreased the lag time required by both bacteria to achieve active growth. However, no increase in tannin tolerance was observed by *S. bovis*.

Bacterial growth rate as determined from the slope of the linear portion of the graph, indicate that the rate of growth of *S. bovis* was affected by the presence of as little as 0.1% w/v tannic acid in the growth medium. With an increase in the concentration of tannic acid, a proportional and significant decrease was observed in the rate of growth ($P<0.05$, Figure 3.6). Contrary to these results, the growth rate of *S. caprinus* remained unaffected by the addition of tannic acid to the growth medium until a concentration of 3% w/v was reached. This caused an approximate 20% decrease in the rate of growth compared to that of the control (no tannic acid addition). Condensed tannin appeared to have a more significant effect on the rate of growth of *S. caprinus*, with the addition of 2% w/v causing a decrease in growth rate of ~25% (significant $P<0.05$, Figure 3.6).

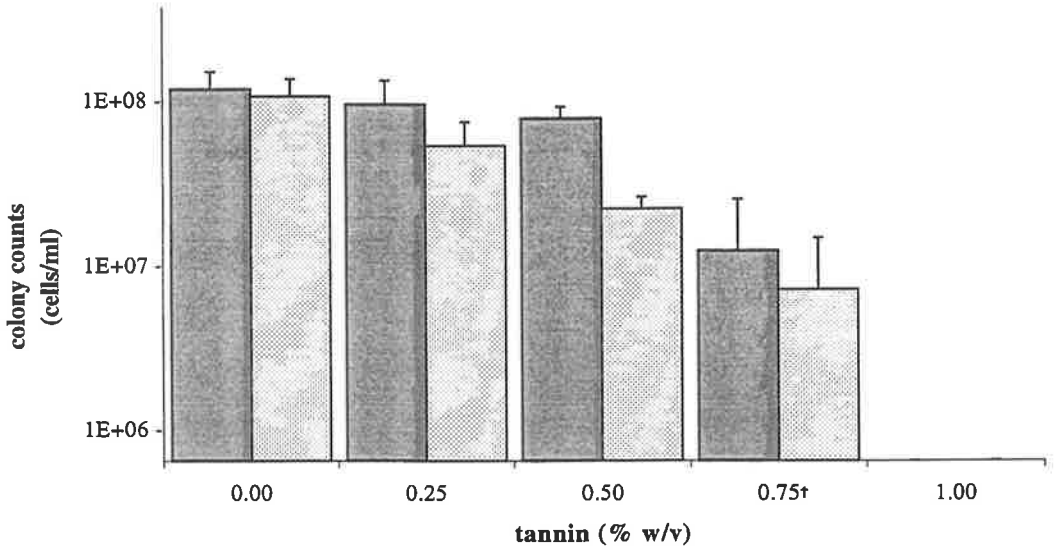
Figure 3.1 **Growth of *S. caprinus* in the presence of tannic acid and condensed tannin**



Tannic acid ▨ condensed tannin ⊡

S. caprinus was incubated in mBHI medium supplemented with increasing concentrations of tannic acid or condensed tannin. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error. nd: not determined.

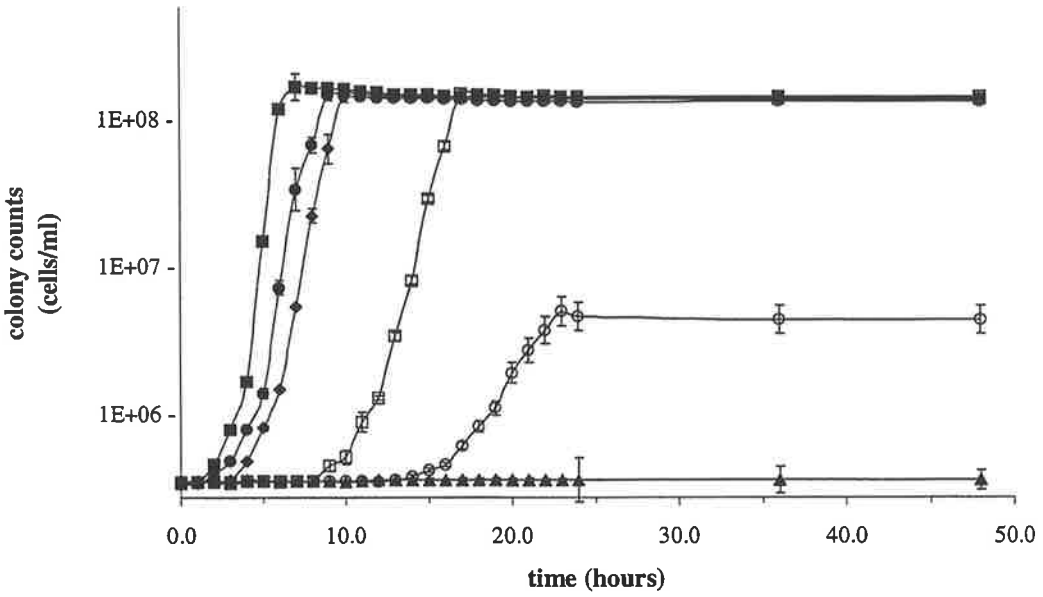
Figure 3.2 **Growth of *S. bovis* in the presence of tannic acid and condensed tannin**



Tannic acid ▨ condensed tannin ⊡

S. bovis was incubated in mBHI medium supplemented with increasing concentrations of tannic acid or condensed tannin. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.
† Significantly different to control (no tannin), $P<0.05$

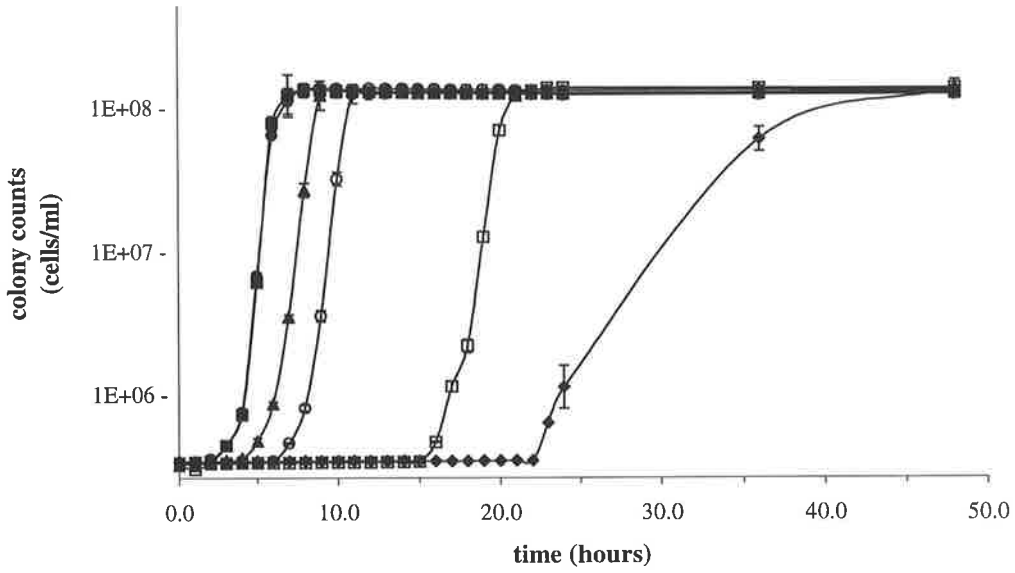
Figure 3.3 Effect of tannic acid on the growth of *S. bovis*



Tannic acid concentration (%, w/v) 0 ■ 0.1† ● 0.2† ◆ 0.5† □ 0.75† ○ 1.0† ▲

S. bovis was incubated in mBHI medium supplemented with increasing concentrations of tannic acid. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.
† Significantly different to control (no tannic acid), $P<0.05$

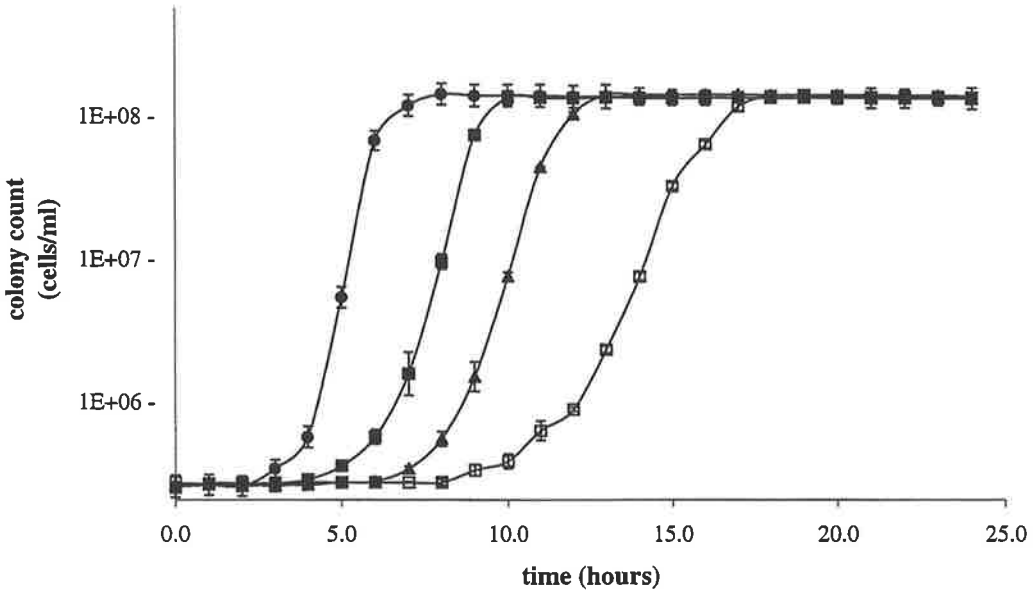
Figure 3.4 **Effect of tannic acid on the growth of *S. caprinus***



Tannic acid concentration (%, w/v) 0 ■ 0.5 ● 1.0† ▲ 2.0† ○ 3.0†□ 5.0† ◆

S. caprinus was incubated in mBHI medium supplemented with increasing concentrations of tannic acid. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.
† Significantly different to control (no tannic acid), $P<0.05$

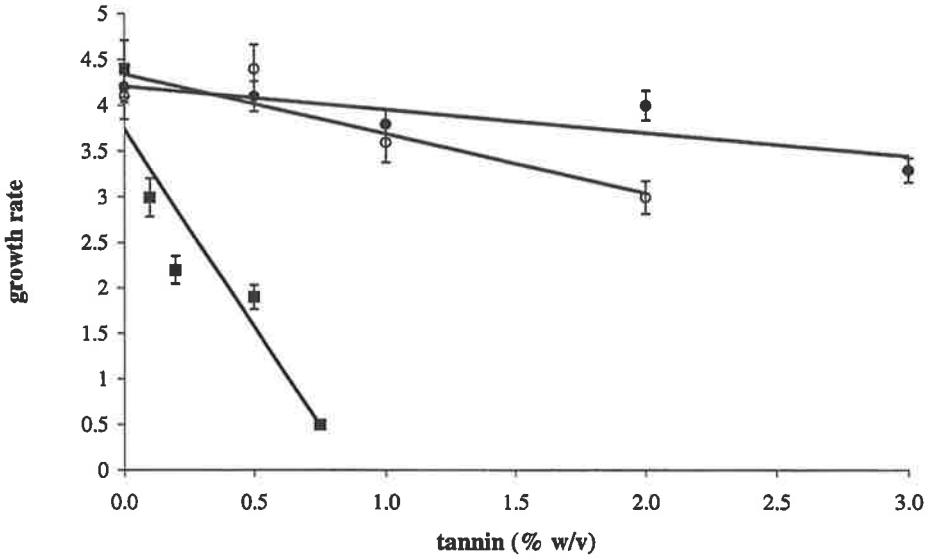
Figure 3.5 Effect of condensed tannin on the growth of *S. caprinus*



Condensed tannin concentration (%, w/v): 0 ● 0.5†■ 1.0†▲ 2.0†□

S. caprinus was incubated in mBHI medium supplemented with increasing concentrations of condensed tannin. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.
† Significantly different to control (no condensed tannin), $P<0.05$

Figure 3.6 **Effect of tannin on the growth rates of *S. caprinus* and *S. bovis***



S. caprinus tannic acid ● condensed tannin ○

S. bovis tannic acid ■

Bacterial growth rates were determined from the slope of the linear portion of the graphs depicted in Figures 3.3, 3.4 and 3.5. The values obtained were correlated with the concentration of tannin used.

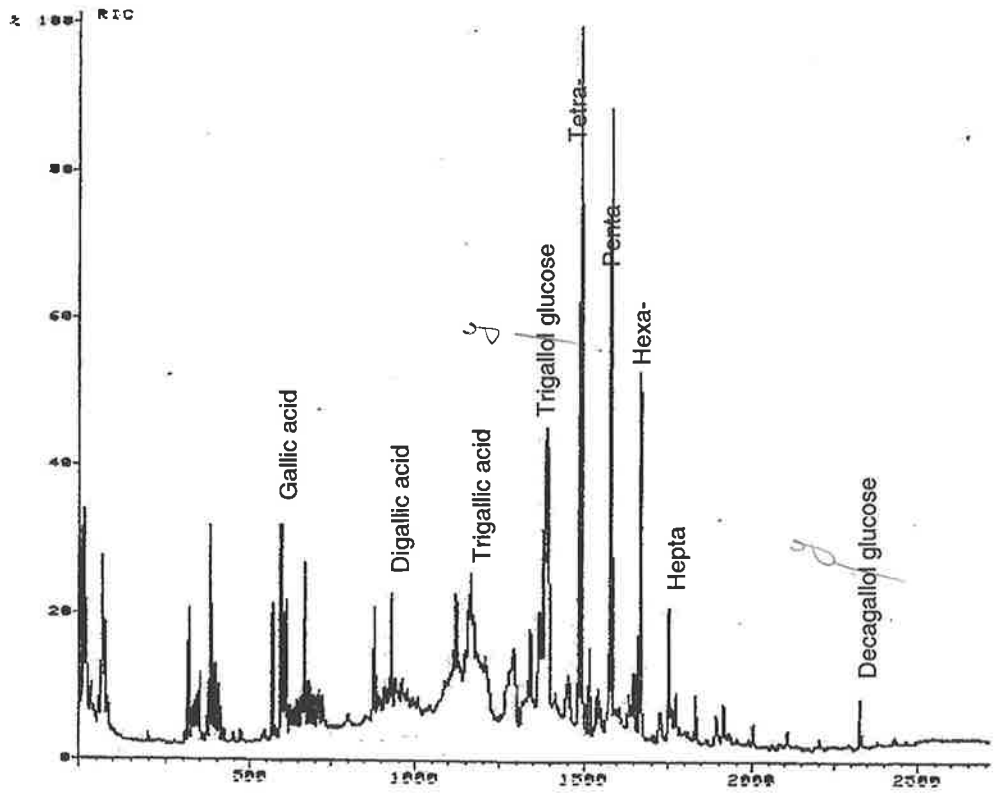
3.2.2 Fractionation of tannic acid

3.2.2.1 Column chromatography

Tannic acid was fractionated on the basis of its size using Sephadex G-25 and using the hydrophobic nature of Sephadex LH-20. In the first experiment, the tannic acid was separated into two fractions: high molecular weight components of tannic acid were eluted in the void volume and the low molecular weight components were collected after repeated washing of the column in 95% ethanol. The gelatin precipitation test indicated that the larger molecular weight components were responsible for protein precipitation. GLC-MS analysis revealed that the low molecular group was composed primarily of gallic acid and simple gallic acid esters whereas the high molecular weight group was made up of larger complexes of gallic acid. These include pentagalloyl glucose, hexagalloyl glucose and heptagalloyl glucose with smaller amounts of tetra- and octa-galloyl glucose present (Figure 3.7).

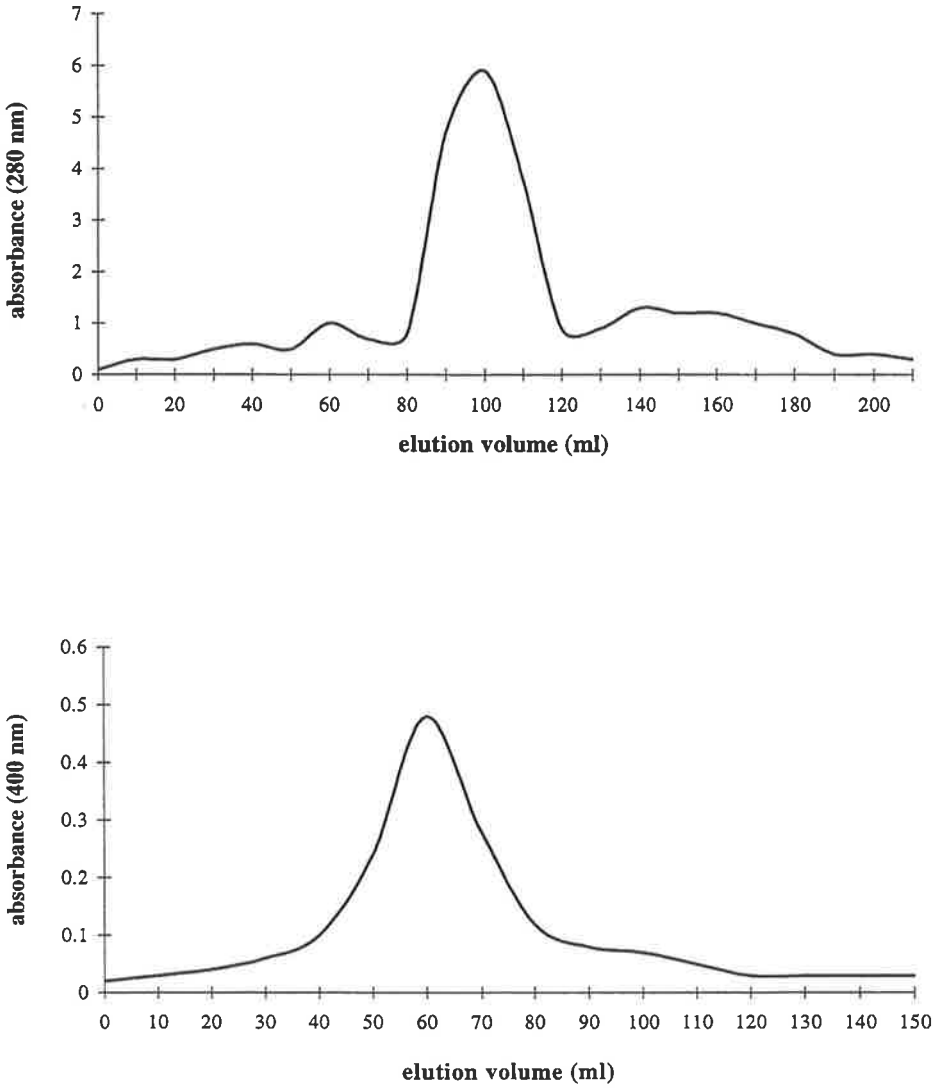
Two fractions were also obtained when tannic acid was separated on the basis of its hydrophobicity to Sephadex LH-20. The first fraction was eluted from the column with 95% ethanol and exhibited a unique absorbance peak at 280 nm. The second fraction remained tightly adsorbed as an immobile orange-brown band at the top of the column and was eluted from the column with 50% aqueous acetone. This fraction demonstrated an absorbance peak at 400 nm (Figure 3.8). 1.81 g of fraction one and 3.58 g of fraction two were obtained after lyophilisation, indicating that 90% of the initial tannic acid placed on the column was retrieved. Only the second fraction formed a precipitate when added dropwise to a 1.0% w/v solution of gelatin suggesting

Figure 3.7 Gas liquid chromatography of TMS derivatised tannic acid



The composition of tannic acid was determined by gas liquid chromatography/mass spectrometry as described in the text (2.13, 2.14)

Figure 3.8 **Elution profiles of tannic acid fractionation on a column of Sephadex LH-20**



Tannic acid (2 mg/ml in 50% ethanol) was applied to a column of LH-20 and extensively washed with 50% ethanol at a flow rate of 30 ml/h. 5 ml fractions were collected and the A_{280} was recorded. The column was then eluted at the same rate with 50% aqueous acetone (200 ml) and the A_{400} was determined in 5 ml fractions.

the presence of 'tannin' components. GLC-MS analysis of these fractions mirrored the previous results with the 'tannin', or protein binding components (Fraction 2) reflecting the higher molecular weight components of tannic acid and the 'non-tannin' components (Fraction 1) determined to be gallic acid.

In order to procure enough material, identical fractions from both chromatographic separations were combined, lyophilised and their effect on the growth of *S. caprinus* and *S. bovis* was determined by adding them in increasing concentrations to the bacterial growth media. The addition of the low molecular weight, 'non tannin' fraction appeared to have little effect on either *S. caprinus* or *S. bovis*, although the lag period of both bacteria increased from 1.3 and 1.2 h to 2.0 and 4.7 h respectively, when a concentration of 1.0% w/v was added to the growth media (Table 3.1). Conversely, the addition of the higher molecular weight or 'tannin' fraction to the growth media produced an inhibitory effect on the growth of *S. bovis* analogous to that obtained with tannic acid (Table 3.1, shown for 1.0% w/v). The addition of increasing concentrations of this fraction also resulted in a similar increase in the lag period of *S. caprinus*, however total bacterial cell numbers remained unaffected.

3.2.2.2 Comparison of precipitable and soluble components

Fractionation of tannic acid into its protein precipitating component and its soluble component was done by adding tannic acid (1.0% w/v) to growth medium, allowing the tannic acid-protein precipitate to form and then separating the two components by centrifugation. The amount of tannin present in the pellet was difficult to determine

Table 3.1 **Effect of tannic acid fractions on the growth of *S. caprinus* and *S. bovis***

Fraction tested[a]	Colony counts[b] (cells/ml)		Lag period (hours)	
	S. caprinus	*S. bovis*	*S. caprinus*	*S. bovis*
Nil	8.1E+08	8.3+08	1.3	1.2
Tannic acid	7.7E+08	ng[e†]	2.2[†]	–
Low molecular weight components[c]	8.5E+08	7.2E+08	2[†]	4.7[†]*
High molecular weight components[c]	7.8E+08	ng[†]*	2.1[†]	–
Precipitable[d]	8.2E+08	ng[†]*	1.8	–
Soluble (filtered)[d]				
Filter paper	8.3E+08	5.6E+06[†]*	1.9	10.6[†]*
0.45 µm	8.1E+08	3.9E+08	1.7	7.2[†]*
0.22 µm	7.8E+08	5.2E+08	1.6	3.3[†]*

[a] Fractions were added to mBHI media at 1.0% w/v

[b] Growth of *S. caprinus* and *S. bovis* was determined by serial dilution and viable cell count.

[c] Fractionation of tannic acid using column chromatography was carried out as described in the text (3.2.2.1).

[d] Fractionation of tannic acid into its precipitable and soluble components was carried out as described (3.2.2.2)

[e] ng: no growth detected

[†] Significantly different to control (no fraction added), $P<0.05$
* Significantly different between bacteria, $P<0.05$

accurately as results varied. However, approximately 70.2 ± 10.3% of the tannic acid appeared to be in bound form. In growth experiments, this tannic acid-medium complex inhibited the growth of *S. bovis* (Table 3.1), although it is also possible that a proportion of the complex dissociated upon addition to the liquid medium. This amount however, could not be quantified. The effect of the soluble fraction on the growth of the bacteria depended on the filter size used. Filtering the supernatant through Whatman filter paper inhibited the growth of *S. bovis* and increased the lag time (significant, $P<0.05$). Filtering the supernatant at 0.45 μm and 0.22 μm increased the lag time also (from 1.2 h to 7.2 and 3.3 h respectively, significant, $P<0.05$). However, after 24 h incubation, cell numbers were as normal. In *S. caprinus*, bacterial cell numbers were unaffected by the addition of either precipitable or soluble fractions, however, the lag times were slightly increased (not significant, $P=0.35$).

3.2.2.3 Addition of tannin-binding substances

The addition of various tannin-binding substances, such as PVP, iron, zinc or lead to the bacterial growth media enabled *S. bovis* to increase its tolerance to tannic acid slightly (not significant, $P=0.21$, results not shown) but had no visual effect on the growth of *S. caprinus*.

3.2.3 Growth in the presence of other phenolic acids

The tolerance of *S. caprinus* to tannins raised the possibility of a general ability to tolerate phenolic compounds and this hypothesis was explored in growth experiments with *S. caprinus* and *S. bovis* using a range of phenolic monomers reported to exert

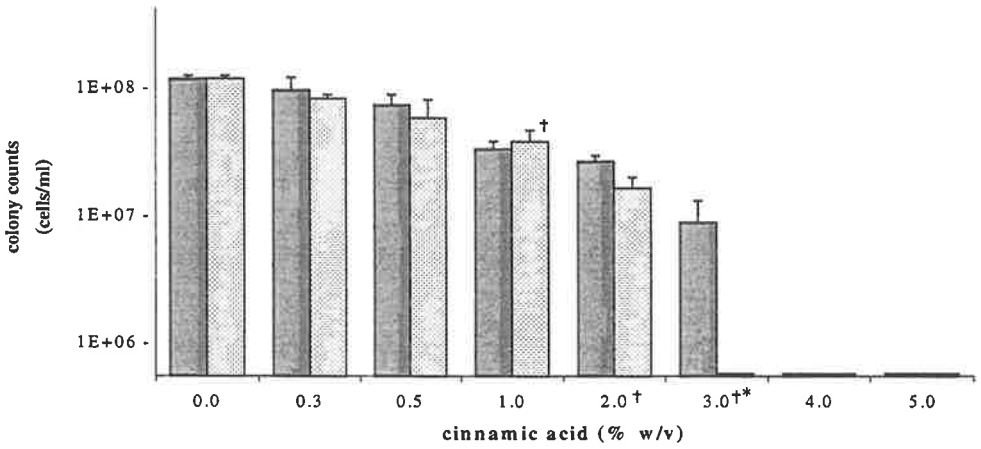
toxic effects on rumen bacteria (Borneman *et al* 1986). Phenolic monomers tested in these experiments are listed in Chapter 2, Table 2.1. Neither *S. caprinus* nor *S. bovis* were able to utilise any of the phenolic monomers as their sole energy source, determined by viable cell count on mBHI medium, nor did they adapt to the presence of these compounds in the growth media over a period of time. However, the addition of different phenolic compounds to the growth medium had different effects on the growth of *S. caprinus* and *S. bovis*. The results show that certain phenolic monomers were particularly influential and resulted in a significant alteration in the growth of *S. caprinus* and *S. bovis* (Figures 3.9a – j). Methyl gallate (Figure 3.9c) and p-coumaric acid (Figure 3.9b) had the greatest inhibitory effect on the growth of both bacteria compared with the control (no phenolic monomer). *S. caprinus,* however, showed an increase in tolerance to the presence of a number of the phenolic compounds compared with *S. bovis* (significant, $P<0.05$). These included: ferulic acid (3.0%, Figure 3.9d), cinnamic acid (3.0%, Figure 3.9a), sinapic acid (3.0%, Figure 3.9e), syringic acid (3.0%, Figure 3.9h) and gallic acid (5.0%, Figure 3.10) in its growth media compared with *S. bovis* (1.0%, 2.0%, 2.0%, 2.0% and 3% respectively).

Contrary to the inhibitory effect of the majority of phenolic monomers tested, the addition of gallic acid to the growth media appeared to stimulate the growth of *S. caprinus*, ie. an increase in growth rate and cell numbers was observed at a gallic acid concentration of 5.0% compared with the control (significant, $P<0.05$, Figure 3.10, Figure 3.11a).

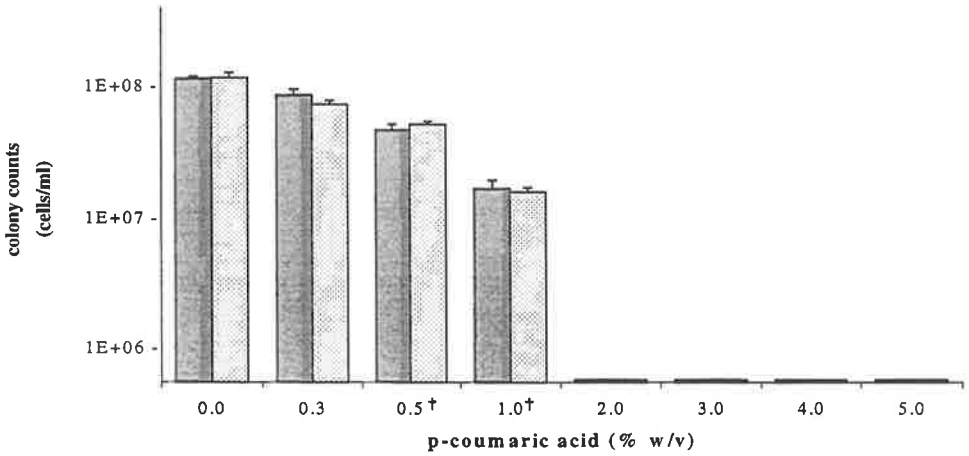
Growth curves were determined in *S. caprinus* and *S. bovis* in the presence of gallic acid, p-coumaric acid and ferulic acid. In all cases the presence of the phenolic acid increased the lag time of both bacteria, with *S. bovis* being, in general, more susceptible to the phenolic compounds than *S. caprinus* (significant, $P<0.05$, Figure 3.11a, b and c). Subculturing of the bacteria into media with similar phenolic acid concentration resulted in a decrease in the lag times.

Figure 3.9 Growth of *S. caprinus* and *S. bovis* in the presence of phenolic acids

(a) cinnamic acid



(b) p-coumaric acid



S. caprinus ▨ *S. bovis* ⊡

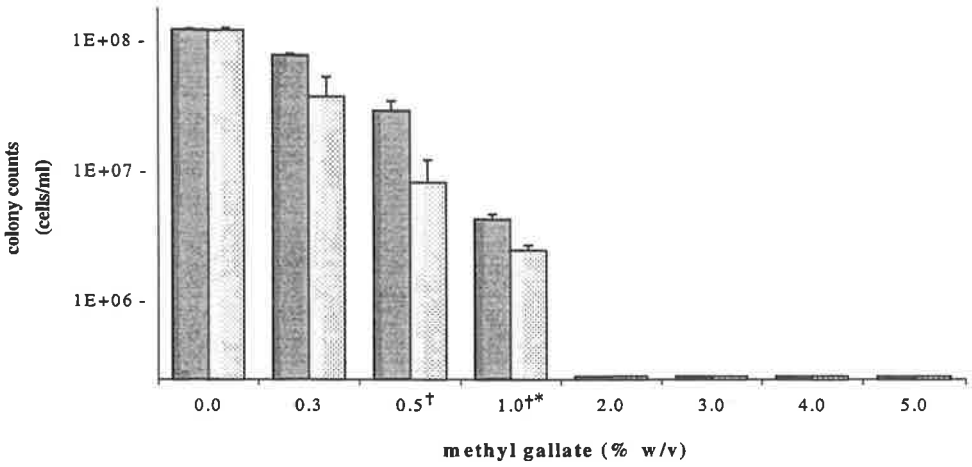
S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with increasing concentrations of phenolic acid. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no phenolic), $P<0.05$

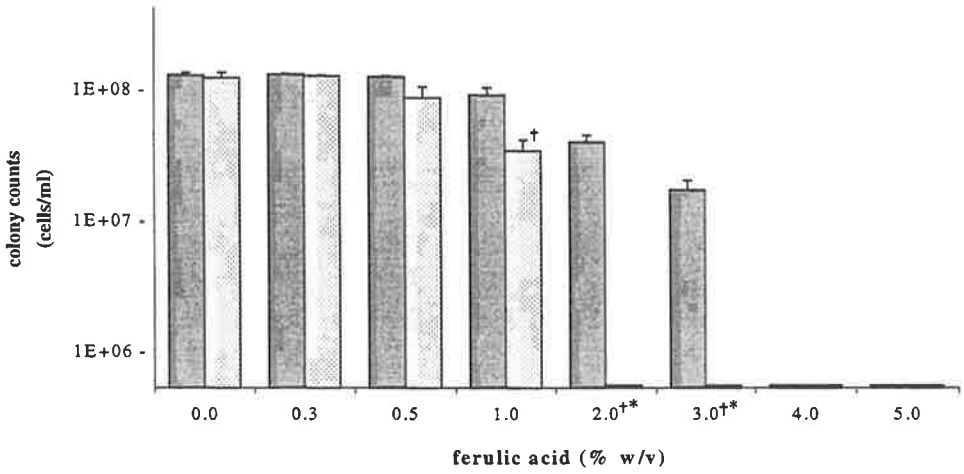
* Significantly different between bacteria, $P<0.05$

Figure 3.9 Growth of *S. caprinus* and *S. bovis* in the presence of phenolic acids

(c) methyl-gallate



(d) ferulic acid



S. caprinus ▨ *S. bovis* ⊡

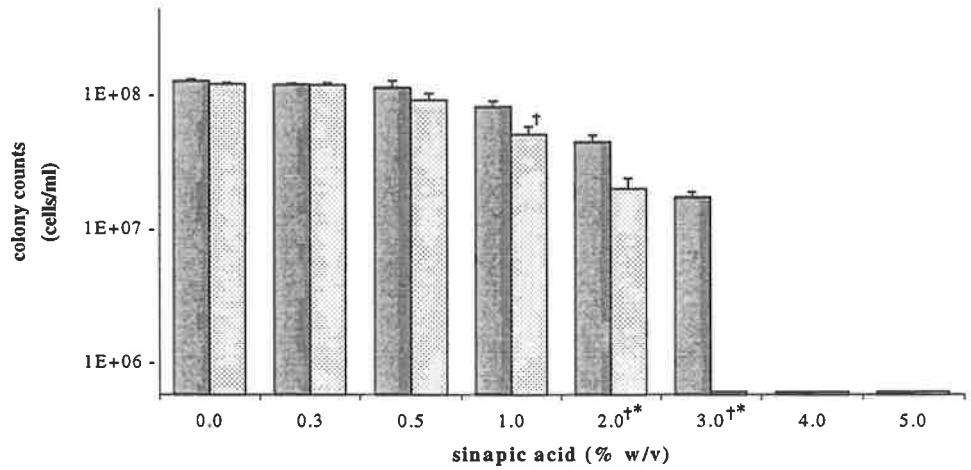
S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with increasing concentrations of phenolic acid. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no phenolic), $P<0.05$

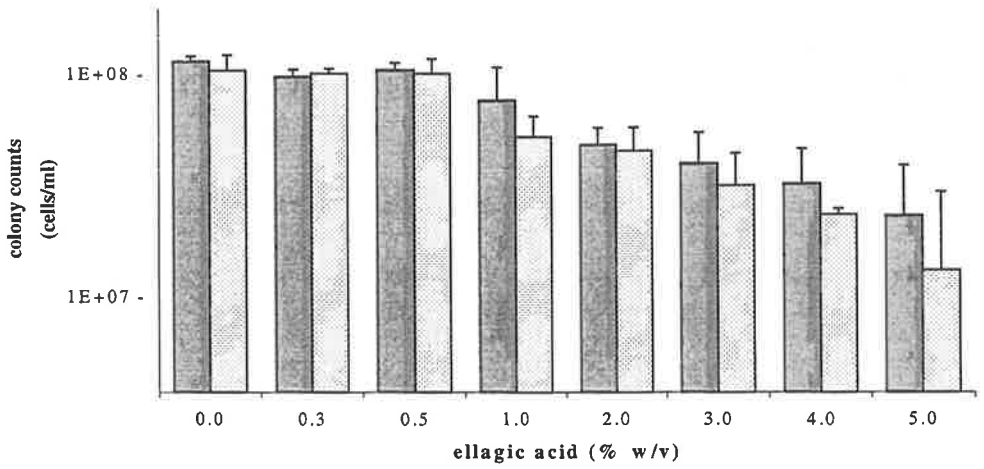
* Significantly different between bacteria, $P<0.05$

Figure 3.9 Growth of *S. caprinus* and *S. bovis* in the presence of phenolic acids

(e) sinapic acid



(f) ellagic acid



S. caprinus ▨ *S. bovis* ⊡

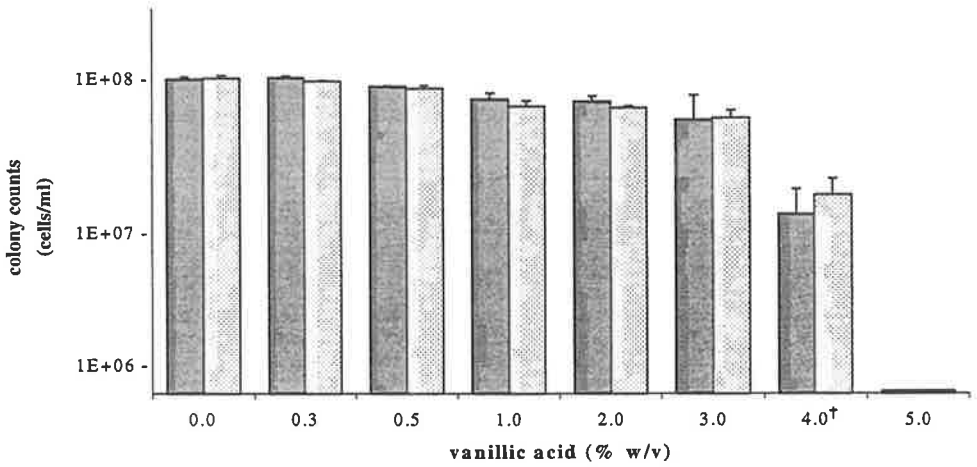
S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with increasing concentrations of phenolic acid. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no phenolic), $P<0.05$

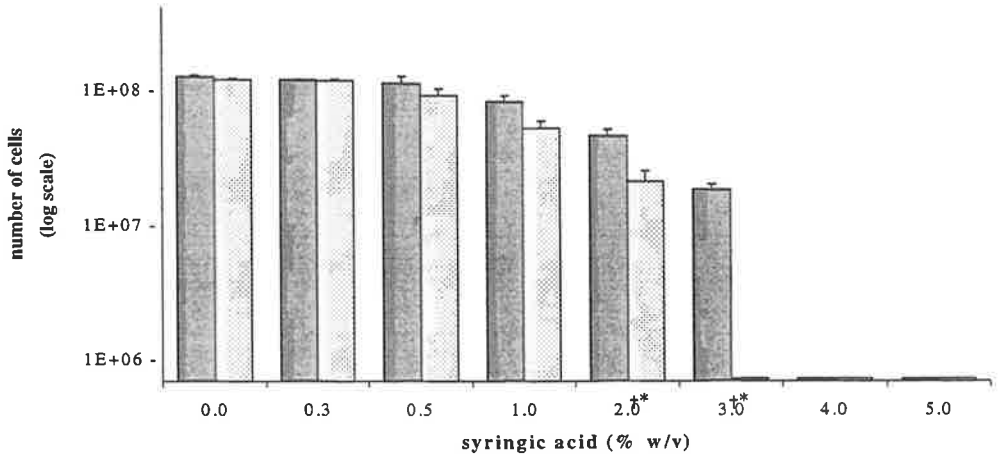
* Significantly different between bacteria, $P<0.05$

Figure 3.9 Growth of *S. caprinus* and *S. bovis* in the presence of phenolic acids

(g) vanillic acid



(h) syringic acid



S. caprinus ▨ *S. bovis* ⊡

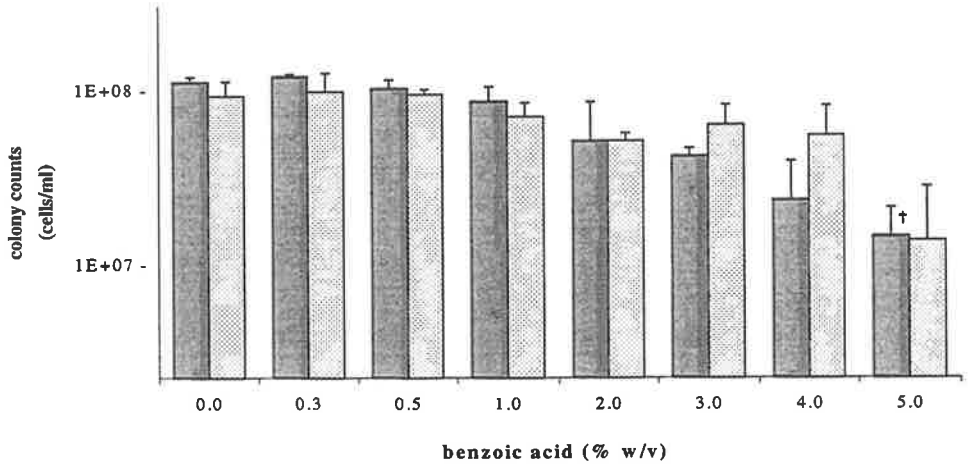
S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with increasing concentrations of phenolic acid. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no phenolic), $P<0.05$

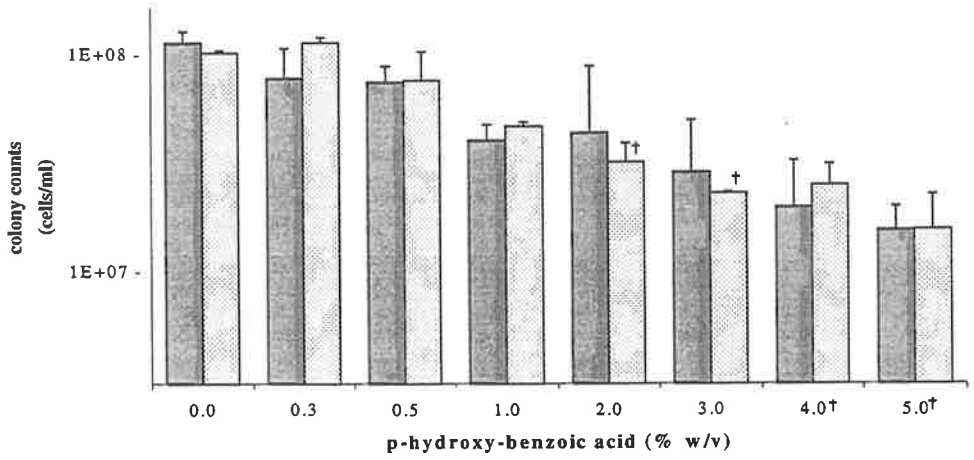
* Significantly different between bacteria, $P<0.05$

Figure 3.9 Growth of *S. caprinus* and *S. bovis* in the presence of phenolic acids

(i) benzoic acid



(j) p-hydroxy-benzoic acid



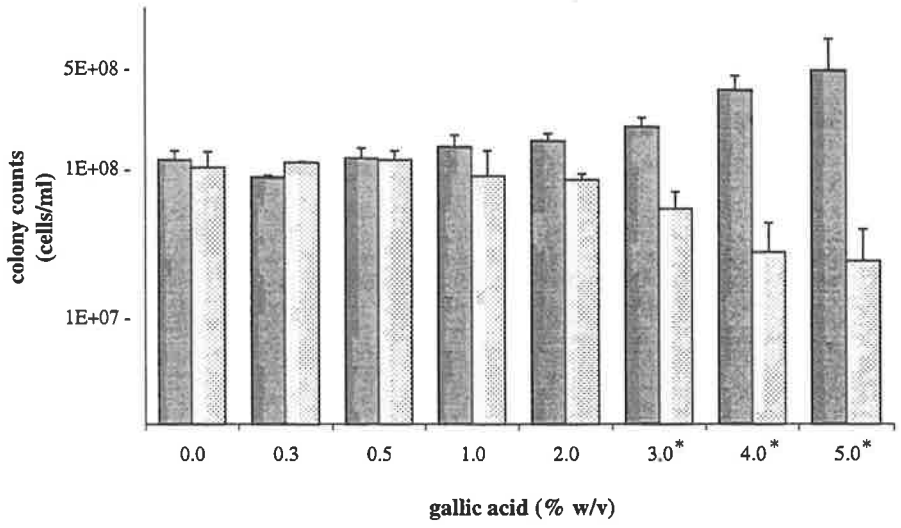
S. caprinus ▨ *S. bovis* ⊡

S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with increasing concentrations of phenolic acid. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no phenolic), $P<0.05$

* Significantly different between bacteria, $P<0.05$

Figure 3.10 Growth of *S. caprinus* and *S. bovis* in the presence of gallic acid

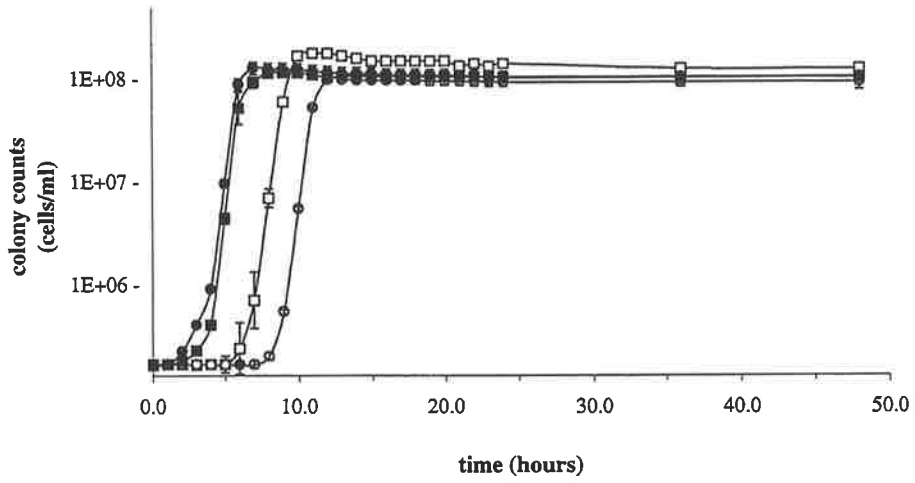


S. caprinus ▨ *S. bovis* ⊡

S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with increasing concentrations of gallic acid. After 24 h incubation at 39 °C, growth was determined on mBHI by serial dilution and viable cell count. Data shown represents the average of triplicate trials; bars represent the standard error.
* Significantly different between bacteria, $P<0.05$? See p 76

Figure 3.11 Effect of phenolic acids on the growth of *S. caprinus* and *S. bovis*

(a) gallic acid



S. caprinus: no phenolic ■ gallic acid†* □

S. bovis: no phenolic ● gallic acid†* ○

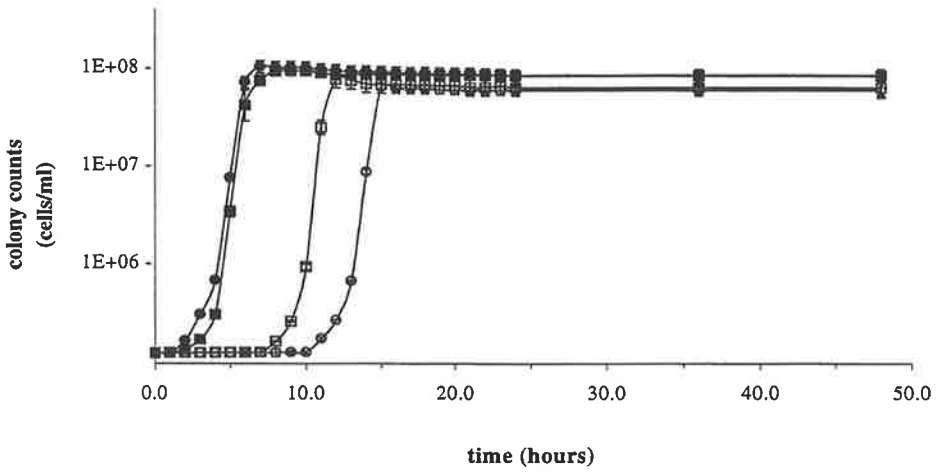
S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with 2% w/v gallic acid. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no phenolic), $P<0.05$

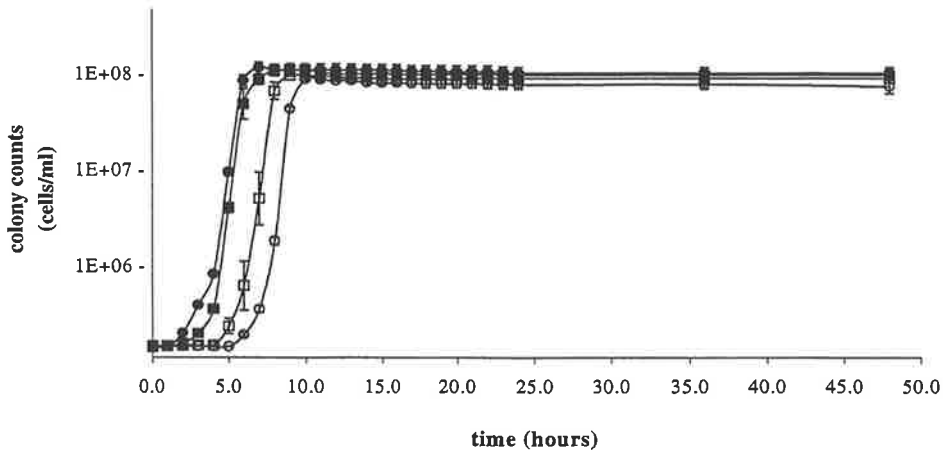
* Significantly different between bacteria, $P<0.05$

Figure 3.11 Effect of phenolic acids on the growth of *S. caprinus* and *S. bovis*

(b) p-coumaric acid



(c) ferulic acid



S. caprinus: no phenolic ■ phenolic acid[†*] □

S. bovis: no phenolic ● phenolic acid[†*] ○

S. caprinus and *S. bovis* were incubated in mBHI medium supplemented with phenolic acid monomers: p-coumaric acid, 0.5% w/v and ferulic acid, 1.0% w/v. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.

[†] Significantly different to control (no phenolic), $P<0.05$

[*] Significantly different between bacteria, $P<0.05$

3.3 Discussion

3.3.1 The effect of tannin on the growth of the bacteria

A number of studies have detailed the effect of tannins on the growth of rumen bacteria (Bae *et al* 1993; Jones *et al* 1994; Sotohy *et al* 1997). Sotohy *et al* (1997) reported that the total reduction of bacterial counts in faecal and ruminal samples obtained from goats fed *Acacia nilotica* was directly proportional to the levels of tannins present in the diet. The level of tannin used in these experiments were higher than those reported previously in the literature (Brooker *et al* 1994; Nelson *et al* 1995) and are higher than levels known to be toxic to bacteria such as *B fibrisolvens*, *R. amylophilus*, *S. bovis* (Jones *et al* 1994), and *F. succinogenes* (Bae *et al* 1993). Carob (*Ceratonia slliqua*) pod extract completely stops growth of *Cellvibrio fulvus* and *Clostridium cellulosolvens* at 15 mg/l (0.0015% w/v), *Sporocytophaga myxococcoides* at 45mg/l, and *Bacillus subtilis* at 75mg/l (Henis *et al* 1964). The same bacteria are unable to grow in medium containing tannic acid at concentrations of 12, 10, 45 and 30 mg/l (ie < 0.0045% w/v). Although the specific concentrations of these phenolic compounds encountered in the rumen have not been documented, a concentration of 3% in the medium has been reported to be equivalent to an animals daily intake of 1 kg of plant material and represents 15% (w/v) tannins in the diet of a ruminant (Nelson *et al* 1995; Brooker *et al* 1994). Mirto (*Mirtus communis*), a forage plant considered to have high levels of hydrolysable tannins, contains 4% hydrolysable tannin on a dry matter basis when measured as gallic acid equivalents (Bae *et al* 1993) and mulga (*Acacia aneura*) is reported to contain 12-15% condensed tannin (Miller *et al* 1997). Thus, *S. caprinus* can tolerate almost four times the hydrolysable tannin and

condensed tannin concentration expected from a forage diet composed exclusively of high tannin plants.

While *S. caprinus* was found to be more tolerant than *S. bovis* to tannic acid and condensed tannins, both bacteria exhibited increased lag periods with increasing concentrations of tannins in the growth medium. Increased lag periods have been found for a number of bacteria that have been subject to sub-optimal or stressful environments *in vitro*. These conditions include decreased incubation temperatures (Schober and Zadoks 1999), nutrient limitation (Ferguson *et al* 1998; Efiuvwevwere *et al* 1999) or the addition of potentially toxic compounds (Nilsson *et al* 1997; Schober and Zadoks 1999; Kleerebezem *et al* 1999). Jones *et al* (1994) showed that lag times of *S. bovis* were extended as concentrations of condensed tannin in the media were increased (0 to 600 µg/ml, equivalent to 0.06% w/v). Nelson *et al* (1995) also reported increased lag times were observed in their Streptococcal isolate when grown in the presence of tannic acid. Unlike the results reported in this study however, their isolate was affected by concentrations of tannic acid as little as 0.1% w/v present in the growth medium. While the addition of increasing concentrations of tannic acid to the growth medium would result in a proportional increase in the number of bacteria bound to the tannic acid, thereby reducing the initial inoculum, it is unlikely that this is the cause of the increased lag periods observed in these experiments. Bacterial lag periods are reported to represent times of intense metabolic activity (Tortora *et al* 1992). This suggests that the extended lag periods observed for both *S. caprinus* and *S. bovis* in response to the presence of tannins in their growth media are representative

of increased periods of DNA transcription and protein synthesis. Thus, this time may be indicative of an active mechanism undertaken by the bacteria in an effort to overcome the detrimental effects of tannins. This possibility will be explored in more detail in the following chapters.

3.3.2 Fractionation of tannic acid

The extraction method used to obtain sufficient quantities of condensed tannin for growth experiments proved difficult and time consuming and as these results indicate, there appears to be no significant differences between the tolerance of *S. caprinus* and *S. bovis* to either condensed tannins or tannic acid. Therefore, due to the comparative ease with which it could be obtained, tannic acid was primarily used in subsequent assays.

Tannic acid is reported to be the best commercially available hydrolysable tannin standard, composed predominantly of β-penta-O-galloyl-D-glucose. However it has also been shown to contain variable amounts of low-molecular weight galloyl esters and non-tannin material (Hagerman and Butler, 1989) and these amounts differ depending on the source of the tannic acid. Characterisation of the tannic acid used in this study by column chromatography was carried out in an attempt to determine its composition and identify a possible growth inhibitory component. Two types of gel chromatography system were utilised which enabled the tannic acid to be separated based on either size or its hydrophobic interactions with Sephadex LH-20. In both cases, two fractions were identified from each chromatograph; size fractionation

produced both low and high molecular weight groups and when determined by hydrophobicity, the fractions were distinguished as 'non-tannin' (those compounds which do not precipitate protein) and 'tannin' components. In my experiments, analysis by GLC-MS showed that the low molecular weight components correlated with those with a reduced capacity to bind protein and the high molecular weight fraction grouped with the tannin components. LH-20 gel is prepared by hydroxypropylation of Sephadex G-25. The hydroxypropyl groups are attached by ether linkages to glucose units of the dextran gel and gives the gel both hydrophilic and lipophilic properties. It has been reported (Strumeyer and Malin, 1975) that the adsorption of phenolic compounds to LH-20 is related to the structure of the phenolic compound and appears to increase with the number of aromatic rings or hydroxyl groups. Hagerman and Robbins (1987) also showed that the interaction of gallotannins with proteins preferentially involves higher molecular weight galloyl esters whereas the low molecular weight species found in tannic acid do not interact strongly with protein. In bacterial growth experiments, the fractions obtained from the two separation methods again produced similar results with only the high molecular weight group or 'tannin' component behaving as the parent tannic acid, and having a growth inhibitory effect on *S. bovis*. Increased lag times were again observed with the addition of both high and low molecular fractions to the growth media.

The results obtained from column chromatography was supported by data obtained from experiments where fractionation of the tannic acid into its soluble and precipitable components was accomplished by centrifugation of the medium/tannic

acid mixture following consolidation of the tannin-protein complex. The isolated tannic acid-protein precipitate inhibited the growth of *S. bovis* and calculation of the amount of bound tannic acid in the complex showed that the equivalent of 0.75% tannic acid was present, an amount known to have an inhibitory effect on *S. bovis*' growth (3.2.1). One interpretation of these results is that the binding of tannic acid to protein and other media components does not reduce the biological activity of the tannic acid, an idea previously proposed by Waterman and Mole (1994). However, for the complexed tannic acid to interact with the bacteria suggests that not all the reactive sites on the tannic acid were saturated by complexing with protein. This scenario also implies that the streptococci interacted with the complex and with the remaining active sites. While there was little evidence to suggest that the bacteria adhered to the complex, further investigation is required to rule out this possibility. A more likely proposal for bacterial growth inhibition under these conditions can be described using the tannic acid-protein equilibrium model which suggests that in an excess of protein the tannic acid would dissociate from the complex back into solution before reacting with the bacteria. A further observation indicates that bacterial growth is still inhibited even in the presence of excess nutrients subsequently added to the precipitable fraction. This implies that growth inhibition is not due simply to the binding up of essential nutrients leading to their apparent removal from the growth medium.

Lag times were increased for both bacteria when grown in the supernatant fraction suggesting the presence of tannic acid and indicating that not all of the tannic acid forms precipitable complexes with medium components. However, lag times were

decreased and normal growth (cell numbers) restored after filtering the supernatant fraction through 0.45 or 0.22 μm filters. This result offers further confirmation of the formation of non-precipitated tannic acid-protein complexes and also implies that there is very little free tannic acid, easily filterable, present in the supernatant fraction. Nevertheless, some phenolic compound remained after filtration as the medium turned brown upon exposure to air. GLC identified this compound as gallic acid.

The addition of specific tannin binding substances, such as PVP or metals, to the bacterial growth medium caused a slight decrease in the inhibitory effect of tannic acid on the growth of *S. bovis*. It is possible that these substances favour irreversible binding to the tannic acid, which would in effect, remove the tannic acid from the media and result in the bacteria being exposed to a lower concentration of tannic acid. This hypothesis is supported by Chung *et al* (1998) who found that the addition of iron to bacterial growth medium decreased the inhibitory effect of tannic acid on *E. coli*. Addition of excess amounts of PVP to the growth medium did not completely remove the tannic acid from the medium suggesting that a saturated level of tannic acid-PVP binding could be obtained. This hypothesis was explored in more detail in the following chapter, however, it is likely that this result is indicative of an equilibrium situation existing between the PVP and the tannic acid, a method previously proposed to describe the action of tannins (Chapter 1). While it is possible that this result may suggest the presence of a component of the tannic acid which does not bind to the PVP and which can cause growth inhibition in *S. bovis*, this explanation is unlikely.

Experiments in this study indicate that in the presence of medium components, it is the bound tannic acid fractions that are responsible for growth inhibition (see above).

3.3.3 The effect of phenolic monomers on the growth of the bacteria

Phenolic monomers, such as the substituted benzoic acids, gallate, p-hydroxybenzoic, vanillic and syringic, and the substituted cinnamic acids, p-coumaric, ferulic, sinapic and caffeic, have been detected either in forage cell walls or identified in the rumen fluid of sheep (Chesson *et al* 1982). In this study, the effect of phenolic monomers was mixed, depending on the compound and the microbial species. However, certain monomers were particularly influential and resulted in a significant alteration in the growth of the bacteria. The descending order of phenolic acid inhibition was: methyl gallate (0.5% w/v) > p-coumaric (1.0% w/v) > ferulic (3.0% w/v), sinapic and syringic (3.0% w/v) > vanillic (4.0% w/v) and > p-hydroxybenzoic acid (5.0% w/v). Benzoic, ellagic and gallic acid appeared to produce no significant inhibitory effect on the growth of either *S. bovis* or *S. caprinus* at the concentrations tested (ie ≤5.0%, compared to the control, no phenolic monomer). The lack of effect of gallic acid on the growth of the bacteria supports the previous results, which suggested that the low molecular weight components of tannic acid, identified by GLC-MS analysis to be gallic acid, were not responsible for growth inhibition.

Ferulic and p-coumaric acids were found to have a significant effect on the growth of *S. caprinus* and *S. bovis* ($P<0.05$). These results support data previously published which report that ruminal bacteria are generally more sensitive to these acids than to

other phenolic monomers (Borneman *et al* 1986; Chesson *et al* 1982; Akin and Barton 1983; Akin *et al* 1993). Ferulic acid and p-coumaric acid are reported to be the most common phenolic monomers in the diet of ruminant animals, and may represent up to 2.5% by weight of the cell walls in temperate grasses (Chesson *et al* 1982). *In vitro* studies have indicated that concentrations of these acids above 1 mM inhibited growth of many species of ruminal bacteria (Borneman *et al* 1986). The most important cellulolytic ruminal bacteria are unable to grow in concentrations of ferulic or p-coumaric acids exceeding 5 mM (Theodorou *et al* 1987; Martin and Akin 1988; Akin *et al* 1993). Chesson *et al* (1982) found that *S. bovis* was tolerant to a number of phenolic acids except 5 mM p-coumaric acid and showed that its cell density declined at the higher concentrations of the more toxic acids. In this study, *S. bovis* and *S. caprinus* were inhibited by similar concentrations of p-coumaric acid (ie. 1.0% ~ 6 mM). Reports have implicated these phenolic monomers as inhibitors of glucose uptake by *Prevotella ruminicola* and *F. sucinogenes* and xylose uptake in *Selenomonas ruminantium* (Martin 1994). The inhibition of glucose and xylose uptake by these compounds may help to partially explain the growth inhibition observed when bacteria in this study were exposed to these compounds.

There is much evidence supporting a toxic effect for phenolic monomers, however, work by Bourneman (1986) and Stack and Hungate (1984) showed that some phenolic acids stimulated growth and cellulolytic activity in some Ruminococcus strains. In their results hydroxy-cinnamic acid and all benzoic acids tested, such as syringic and hydroxy-benzoic acid, produced a stimulatory effect on growth. In the experiments

presented in this chapter, the rate of growth of *S. caprinus* as well as bacterial cell numbers appeared to increase when gallic acid was present in the growth media, suggesting that gallic acid has a stimulatory effect on *S. caprinus* (statistically not significant, P=0.18). None of the other phenolic acids tested produced a stimulatory response on the growth of either *S. caprinus* or *S. bovis*.

The levels of phenolic monomers tested in these experiments were higher than those reported to be present in the rumen (Bourneman *et al* 1986). However, Chesson *et al* (1982) suggested that ruminal microorganisms especially cellulolytic and proteolytic bacteria which are intimately associated with plant material undergoing degradation in the rumen, would encounter levels of phenolic compounds in a microenvironment higher than those found in the ruminal fluid. Data are not available to indicate specific concentrations of phenolic monomers that may be present in such an environment. However, different plant tissues have markedly different levels of phenolic acids, and the levels released in a microenvironment could vary with chemical treatment, plant stress and type of feed. Surprisingly, Chesson *et al* (1982) found that the cellulolytic strains, such as Butyrivibrio and Selenomonas, when examined in pure culture, were found to be no more tolerant of added phenolic acids than rumen bacteria not normally found associated with plant particles. The authors concluded that either these organisms do not regularly encounter toxic levels of phenolic acids in the rumen, or some mechanism for their protection exists. This hypothesis will be addressed in more detail in Chapter 6.

There appears to be a relationship between the growth of the bacteria and chemical structure of the phenolic acids as both *S. caprinus* and *S. bovis* appeared, on the whole, to be more susceptible to the substituted cinnamic acids than those derived from the benzoic acids (Figure 3.12). It has also been reported that the addition of various groups to the phenolic nucleus has been found to increase its antimicrobial activity (Jurd *et al* 1971) and hundreds of isomers and combinations of alkyl, hydroxyl and methoxyl derivatives of phenolics are possible. In this study, the addition of a methyl group via ester bonding to the gallic acid residue (methyl gallate) significantly altered its growth inhibitory effect on both *S. caprinus* and *S. bovis,* rendering both bacteria far more susceptible to the presence of the phenolic acid in the growth medium.

Interestingly, while differences were detected in the concentrations of the individual phenolic acids that each bacteria could tolerate (*S. caprinus* being more tolerant than *S. bovis* to a number of the simpler phenolic acids), none of these acids produced such a large difference in their growth inhibitory effect than that observed between *S. caprinus* and *S. bovis* when grown in the presence of tannic acid or condensed tannin.

Figure 3.12 Classes of phenolic monomers used in this study

(a) (b)

(a) cinnamic acids:

p-coumaric acid ($R_1 = R_2 = H$);

ferulic acid ($R_1 = H$, $R_2 = OCH_3$);

sinapic acid ($R_1 = R_2 = OCH_3$).

(b) benzoic acids:

hydroxybenzoic acid ($R_1 = R_2 = H$);

vanillic acid ($R_1 = H$, $R_2 = OCH_3$);

syringic acid ($R_1 = R_2 = OCH_3$)

Chapter 4

Breakdown of tannin-protein complex

Chapter 4 Breakdown of tannin-protein complex

4.1 Introduction

The ability of tannins to form strong complexes with proteins is one of the most important aspects of their nutritional and toxicological effects (Hagerman and Butler, 1981; Dawra *et al* 1988; Scalbert, 1991; Woodward and Reed 1995, 1997; Sotochy *et al* 1997). The formation of a precipitate upon the addition of tannic acid to bacterial culture media is representative of this ability. *S. caprinus* has been shown to produce zones of clearing when grown on nutrient agar medium containing tannic acid suggesting that the bacteria are capable of hydrolysing the bonds between tannic acid and protein (Figure 1.8). In this chapter, the interaction of tannic acid with protein and other tannin binding substances have been explored, as were ways in which these interactions could be destroyed.

The ability of *S. caprinus* to degrade the tannic acid-protein complex may be indicative of how the bacterium is able to survive the presence of tannins in its environment. Preliminary experiments suggested that the cleared regions surrounding the bacterial colonies were caused by a low molecular weight compound and pH analysis of these zones indicated that the causative agent was an acid (L. O'Donovan, Honours Thesis, Brooker *et al*, 1994). Lactic acid has been reported to be the predominant acid produced by both *S. caprinus* and *S. bovis* and previous results indicate that the production of this acid by *S. bovis* is reduced in the presence of tannic acid (Brooker *et al* 1994). In this study, further work was carried out to determine the concentration of lactate produced by both *S. caprinus* and *S. bovis* in the presence of

increasing concentrations of tannic acid. The effect of tannic acid on the end step enzyme responsible for lactate production, lactate dehydrogenase (LDH) was also determined.

Several mechanisms have been proposed to account for the antimicrobial properties of tannins, including the inhibition of bacterial enzymes such as proteases (Attwood and Reilly 1996; Jones *et al* 1994; Messman *et al* 1996) and cellulases (Bae *et al* 1993). The inhibition of these enzymes resulted in decreased microbial growth. Wallace and Brammel (1985) have reported that *S. bovis*, along with *Selenomonas ruminantium* and Butyrivibrio spp. are the most important bacteria in the hydrolysis of protein in the rumen. These bacteria have also been reported to be susceptible to the presence of tannins in their growth medium (*in vitro*) (Jones *et al* 1994; Brooker *et al* 1994). Conversely, *S. caprinus'* ability to tolerate tannins in the growth medium may reflect the presence of tannin tolerant enzymes, such as proteinases that would provide the bacteria with a selective advantage over the more common rumen inhabitants and enable it to compete in the rumen environment. Thus, proteinase activity of *S. caprinus* was determined in the presence of increasing concentrations of tannic acid and compared with that of the tannin sensitive bacterium, *S. bovis*.

In the previous chapter, bacterial lag times were observed to increase when both *S. caprinus* and *S. bovis* were grown in the presence of tannic acid and it was proposed that this may be indicative of upregulation of gene transcription and/or protein synthesis. In this chapter, these possibilities were explored in more detail. Polyacrylamide gel electrophoresis (PAGE) was used to create profiles of bacterial

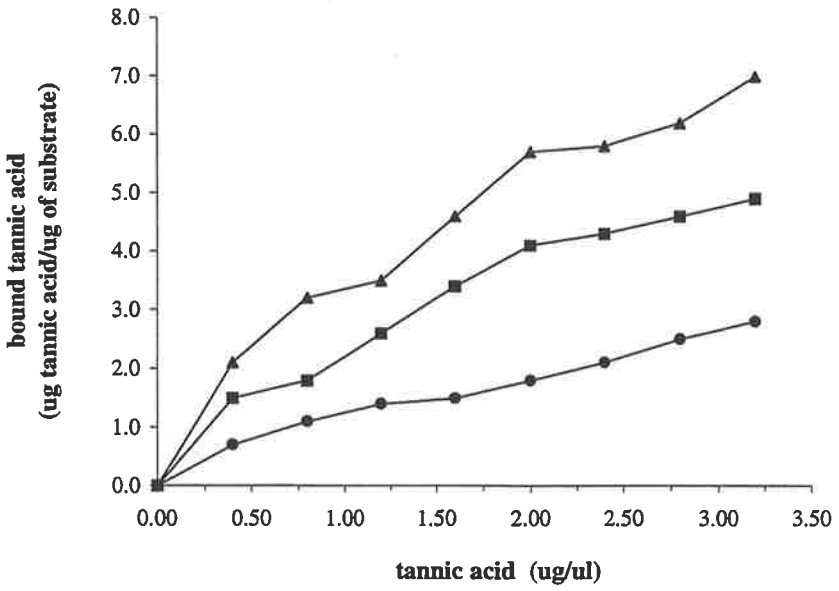
cell proteins following growth in the presence and absence of tannic acid. Isolation of the gene(s) responsible for tannin tolerance was also attempted through the transformation of *E. coli* and *S. bovis* with plasmid vectors containing *S. caprinus* DNA. The zone of clearing surrounding bacterial colonies following growth on tannic acid nutrient agar medium provided a relatively simple, visual method which was used to screen the libraries.

4.2 Results

4.2.1 Binding properties of tannic acid used in this study

The addition of tannic acid (aqueous) to a solution containing protein (bovine serum albumin (BSA), gelatin) or a known tannin binding compound, polyvinylpyrrolidone (PVP) caused the precipitation of a tannic acid-substrate complex. Increasing the concentration of tannic acid resulted in a proportional increase in the amount of precipitate formed. However, the amount of tannic acid present in the complex depended on both the type of substrate as well as on the pH of the mixture. At pH 6.8, PVP exhibited the highest capacity among the three substrates to bind tannic acid, with gelatin precipitating only slightly less. BSA, however appeared to bind significantly less tannic acid over the range of tannic acid used, ie at a concentration of 2 μg/μl tannic acid, PVP, gelatin and BSA precipitated approximately 5.5, 4 and 1.5 μg tannic acid/μg substrate respectively ($P<0.05$, Figure 4.1). When either acidic (pH 3.5) or alkaline (pH 9.0) conditions were used, a significant reduction in tannic acid-substrate precipitate was observed ($P<0.05$, Figure 4.2). Upon re-establishing neutral pH, more tannic acid-substrate precipitation was detected, ie a concentration of

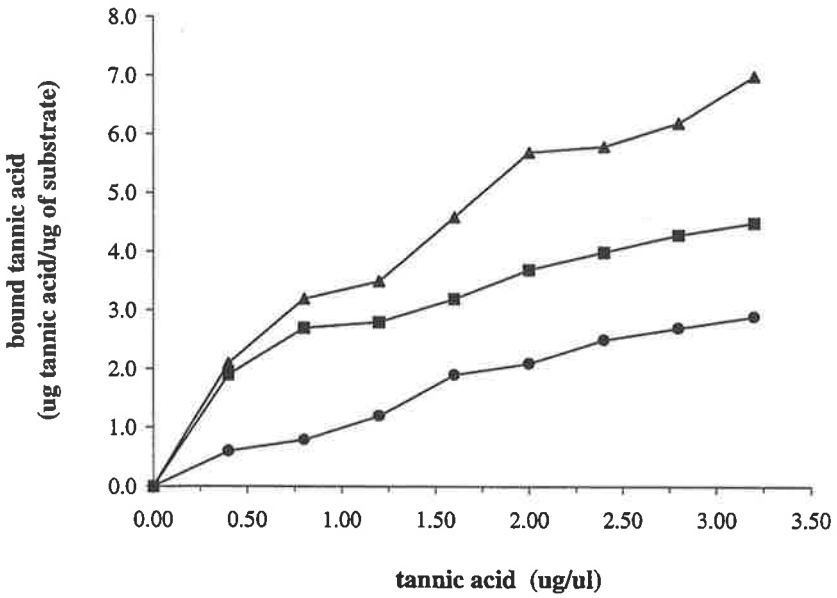
Figure 4.1 Effect of substrate on tannic acid binding properties



Substrate PVP ▲ gelatin ■ BSA ●

Tannic acid was added in increasing amounts to solutions (pH 6.8) of BSA, gelatin or PVP (1 mg/ml). The mixture was incubated under anaerobic conditions for 1 h at 37 °C and the amount of tannic acid in the precipitate was determined as described (2.5).

Figure 4.2 Effect of pH on tannic acid binding properties



Sodium citrate pH 3.5 ■ sodium phosphate pH 6.8 ▲ Tris pH 9.0 ●

Tannic acid was added in increasing amounts to PVP (1 mg/ml) at a pH of 3.5, 6.8 or 9.0. The mixture was incubated under anaerobic conditions for 1 h at 37 °C and the amount of tannic acid in the precipitate was determined as described (2.5).

2.0 µg/µl tannic acid precipitated 5.5 µg tannic acid/µg substrate at pH 6.8 compared to 3.5 at pH 3.5 and 2.0 at pH 9.

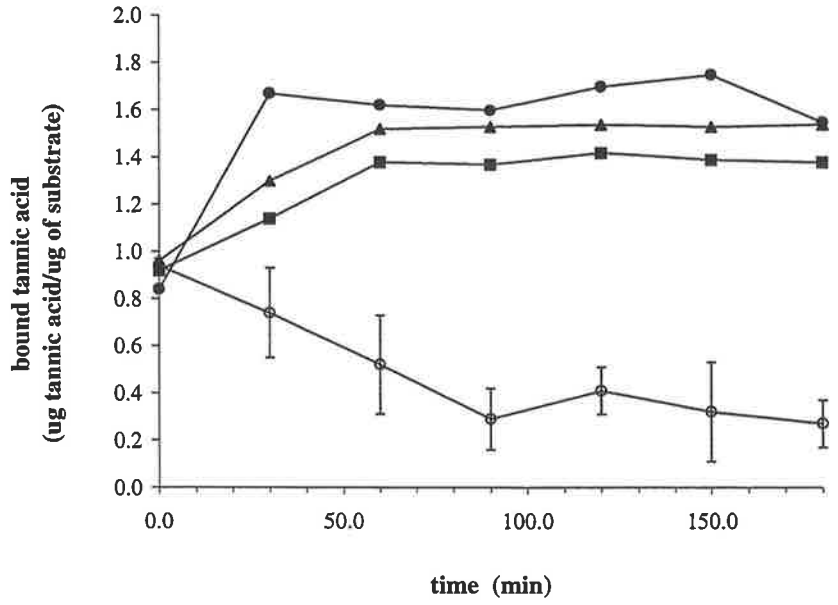
Over a period of time, the amount of tannic acid present in the precipitate appeared to increase until a plateau was reached within a 60 minute period (Figure 4.3). At this time approximately 75% of the tannic acid appeared bound in the complex. No further change (increase or decrease) in the amount of bound tannic acid was detected. Increasing the temperature at which the complex was formed, ie 25 to 60 °C appeared to result in a slight increase in the amount of tannic acid bound to PVP (not significant, P=0.22, Figure 4.3).

Incubating the tubes aerobically caused a blackening of the tannic acid-PVP precipitate due to oxidation of the tannic acid. Determination of the amount of tannic acid present in the precipitate was difficult as results varied. However, it appeared that incubation of the tannic acid protein complex in the presence of air decreased the amount of tannic acid precipitated, ie 0.9 to 0.3 µg tannic acid/µg of substrate over a 15 minute period (significant, $P<0.05$, Figure 4.3).

4.2.2 The effect of tannic acid on lactate production and LDH activity

Lactate production by *S. caprinus* and *S. bovis* was determined during growth in the presence of increasing concentrations of tannic acid (Figure 4.4 and Figure 4.5 respectively). Both *S. caprinus* and *S. bovis* were found to produce similar quantities of lactate, ie 18.2 and 19.7 mM respectively and this concentration did not alter significantly when tannic acid was added to the growth media. With both bacteria,

Figure 4.3 **Effect of time, temperature and air on tannic acid binding properties**

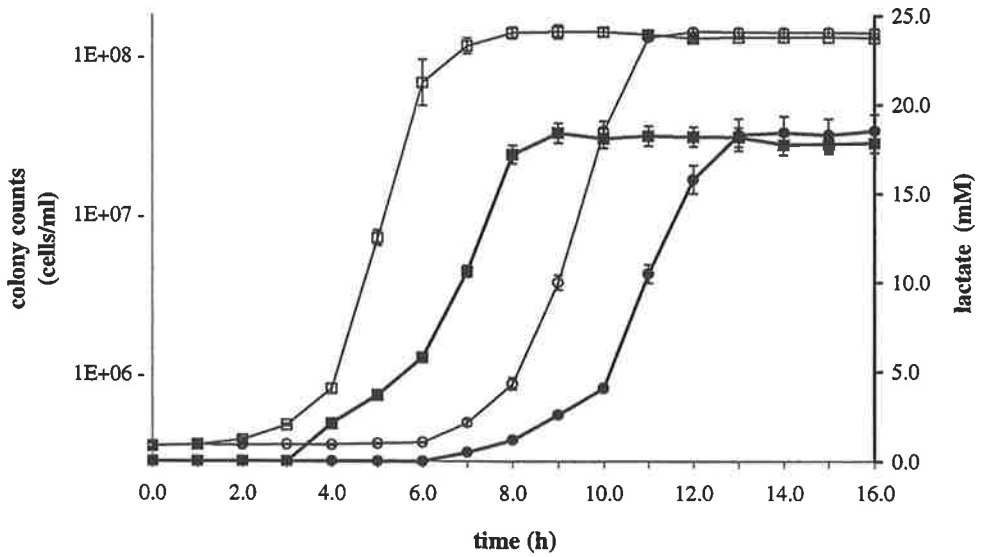


Temperature (°C) 25 ■ 39 ▲ 60 ●

Air o

Equal volumes of tannic acid (4 mg/ml) and PVP (1 mg/ml) were combined and incubated anaerobically at the temperatures stated. The tannic acid/PVP mixture was also incubated in air at 25 °C. The amount of tannic acid in the precipitate was determined over time (2.5).

Figure 4.4 Effect of tannic acid on lactate production by *S. caprinus*

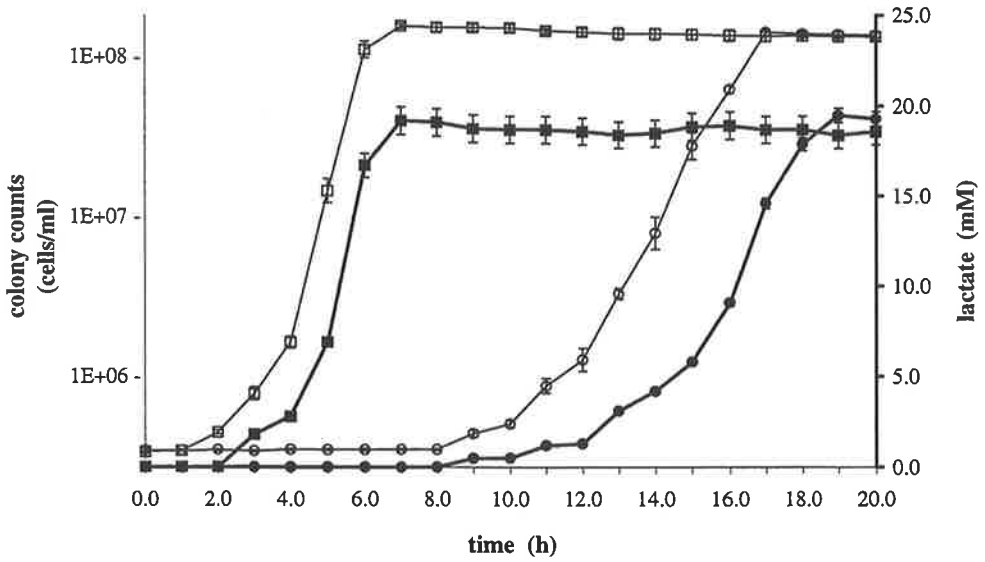


Growth on mBHI (colony counts) □ corresponding lactate production ■

Growth on mBHI plus tannic acid (2% w/v) ○ corresponding lactate production ●

S. caprinus was incubated in mBHI medium supplemented with tannic acid (2% w/v). At regular time intervals aliquots (1 ml) were removed and growth was quantified on mBHI by serial dilution and viable cell count. At these times, the amount of lactate present in the medium was determined by GLC analysis (2.16). Points represent the average of triplicate trials; bars represent the standard error.

Figure 4.5 Effect of tannic acid on lactate production by *S. bovis*



Growth on mBHI (colony counts) □ corresponding lactate production ■

Growth on mBHI plus tannic acid (0.5% w/v) ○ corresponding lactate production ●

S. bovis was incubated in mBHI medium supplemented with tannic acid (0.5% w/v). At regular time intervals aliquots (1 ml) were removed and growth was quantified on mBHI by serial dilution and viable cell count. At these times, the amount of lactate present in the medium was determined by GLC analysis (2.16). Points represent the average of triplicate trials; bars represent the standard error.

lactate was found to accumulate at the end of the logarithmic growth phase of the bacteria.

Analysis of cell fractions showed that the LDH activity in both bacterial species was predominantly found in the cell cytosol (Figure 4.6). Maximal activity was determined with respect to time (10 min), temperature (39 °C), pH (6.8) and protein concentration (3 mg/ml). The presence of tannic acid in the assay mix was found to inhibit LDH activity in both Streptococcal species (significant, $P<0.05$, Figure 4.7).

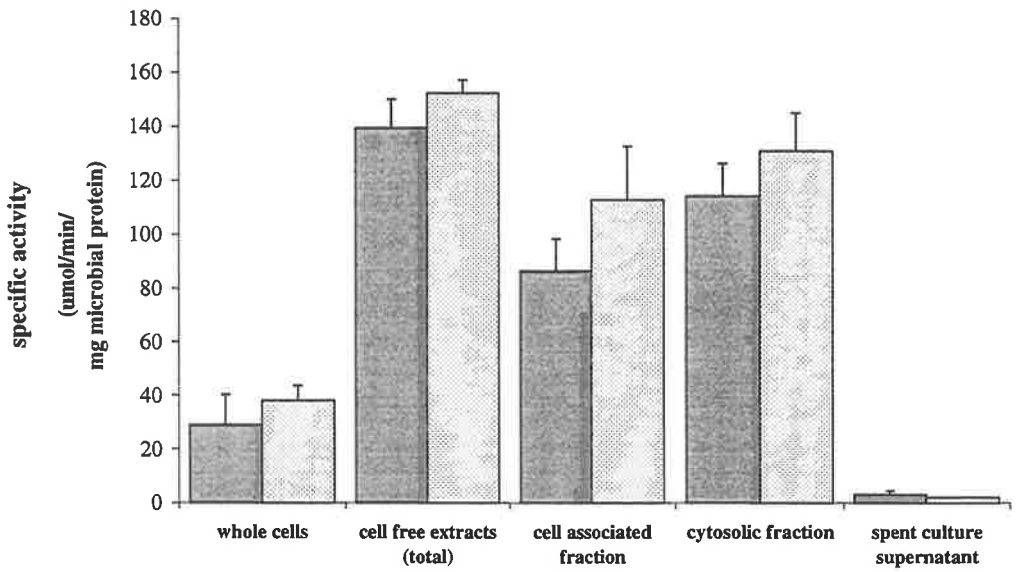
4.2.3 The effect of tannic acid on proteinase activity

Proteinase activity in *S. bovis* and *S. caprinus* was found to be predominantly cell associated (Figure 4.8). Maximal activity was determined with respect to time (3 h), temperature (39 °C), pH (6.8) and protein concentration (3 mg/ml).

The presence of tannic acid in the assay mixture significantly reduced the proteinase activity in whole cells and cell free extracts prepared from both *S. caprinus* and *S. bovis* ($P<0.05$, Figure 4.9). However, *S. bovis* appeared to be more affected by the presence of tannic acid than *S. caprinus*, ie. a concentration of 75 µg/ml tannic acid resulted in a decrease in protease activity in cell free extracts by approximately 76% in *S. bovis* compared with 47% in *S. caprinus* (significant, $P<0.05$).

The addition of PVP to the proteinase assay system caused a slight decrease in the inhibitory effect of tannic acid on proteolytic activity in cell free extracts prepared from both *S. caprinus* and *S. bovis* (Figure 4. 10a and b). For example, the presence

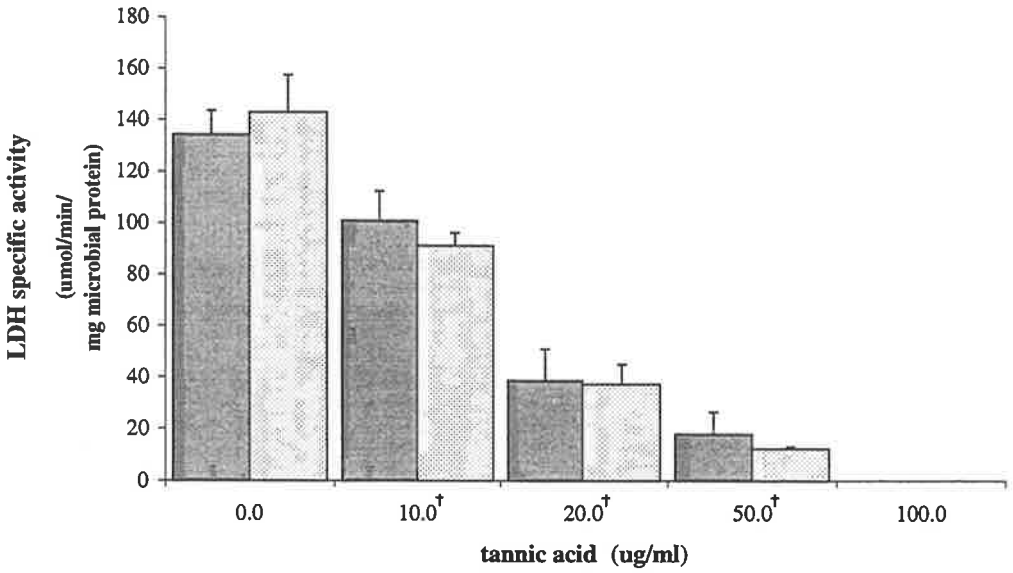
Figure 4.6 Localisation of lactate dehydrogenase activity



S. caprinus ▨ *S. bovis* ⊡

Lactate dehydrogenase activity was determined spectrophotometrically under optimal conditions in whole cells and cell free extracts prepared from *S. caprinus* and *S. bovis*. Specific activity was expressed as µmol per minute per milligram of microbial protein. In the case of whole cells, specific activity was expressed per 3.0E+06 cells. Data shown represents the average of triplicate trials; bars represent the standard error.

Figure 4.7 Effect of tannic acid on lactate dehydrogenase activity

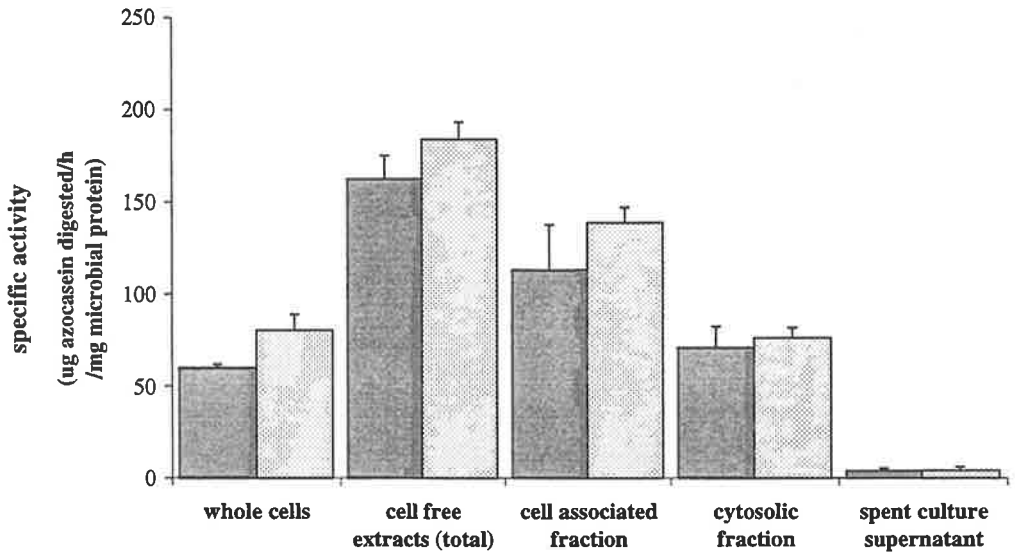


S. caprinus ▨ *S. bovis* ⊡

Cell free extracts prepared from *S. caprinus* and *S. bovis* (3 mg bacterial protein/ml) were incubated with increasing concentrations of tannic acid at room temperature for 30 min. Lactate dehydrogenase activity was determined spectrophotometrically under optimal conditions. Specific activity was expressed as µmol per minute per milligram microbial protein. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no tannic acid), $P<0.05$

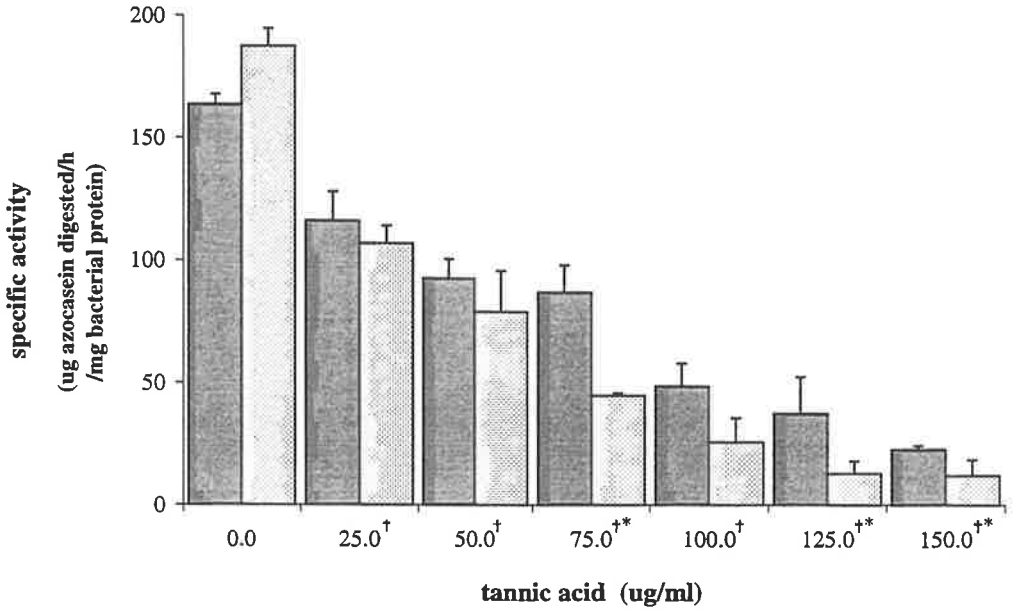
Figure 4.8 Localisation of proteinase activity



S. caprinus ▨ S. bovis ⊡

Proteinase activity was determined spectrophotometrically under optimal conditions in whole cells and cell free extracts prepared from *S. caprinus* and *S. bovis*. Specific activity was expressed as μg azocasein hydrolysed per hour per milligram of microbial protein. In the case of whole cells, specific activity was expressed per 3.0E+06 cells. Data shown represents the average of triplicate trials; bars represent the standard error.

Figure 4.9 Effect of tannic acid on proteinase activity



S. caprinus ▨ *S. bovis* ⊡

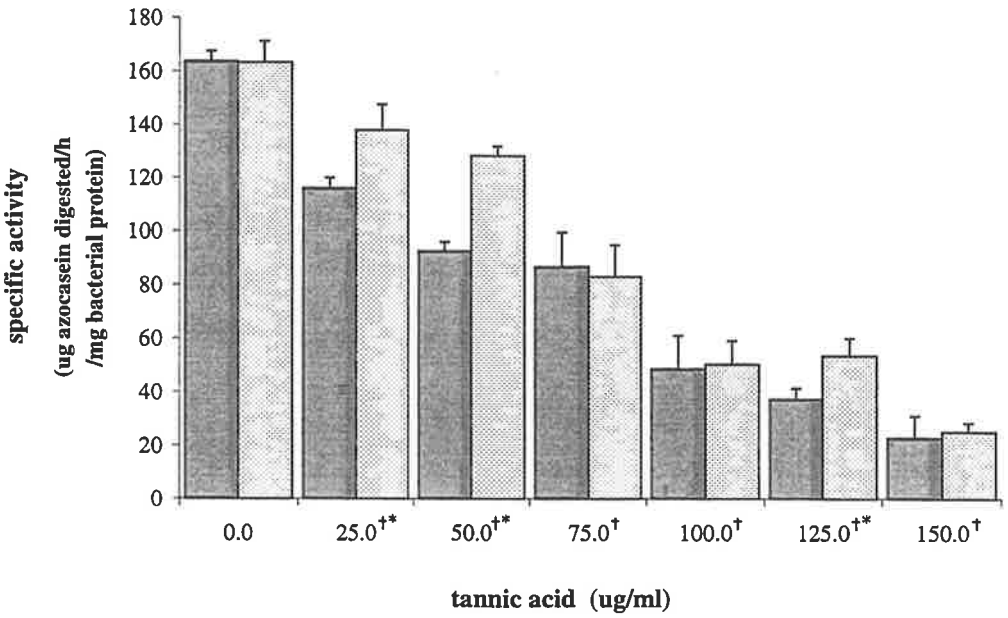
Cell free extracts prepared from *S. caprinus* and *S. bovis* (3 mg bacterial protein/ml) were incubated with increasing concentrations of tannic acid at room temperature for 30 min. Proteinase activity was determined spectrophotometrically under optimal conditions. Specific activity was expressed as µg azocasein digested per hour per milligram microbial protein. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no tannic acid), $P<0.05$

* Significantly different between bacteria, $P<0.05$

Figure 4.10 **Effect of tannic acid on proteinase activity following addition of polyvinyl pyrrolidone**

(a) *S. caprinus*



S. caprinus ▨ *S. caprinus* and PVP ⊡

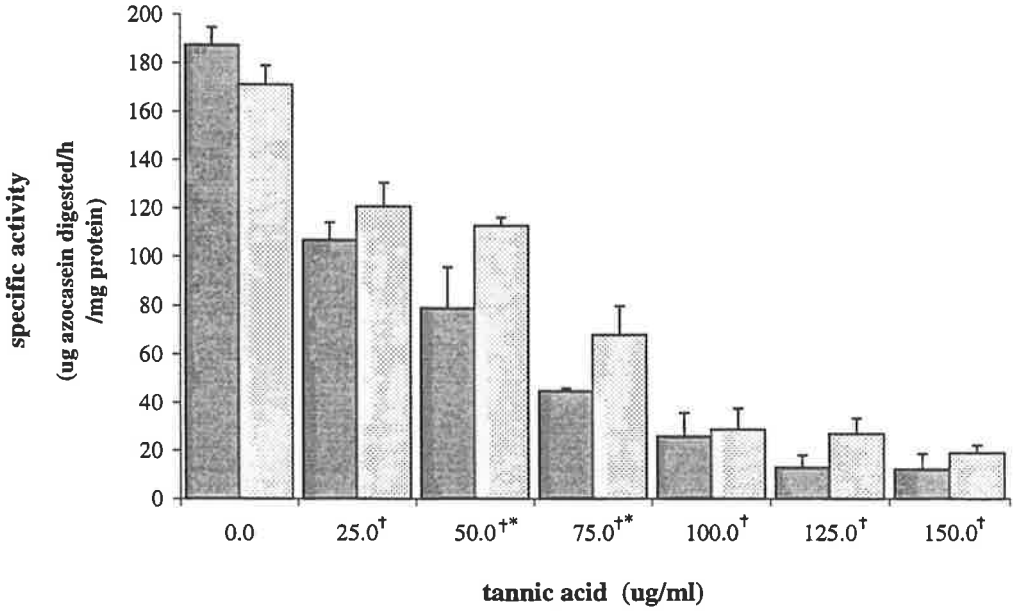
Cell free extracts prepared from *S. caprinus* (3 mg bacterial protein/ml) were incubated with polyvinyl pyrrolidone (PVP, 1 mg/ml) followed by increasing concentrations of tannic acid at room temperature for 30 min. Proteinase activity was determined spectrophotometrically under optimal conditions. Specific activity was expressed as µg azocasein digested per hour per milligram microbial protein. data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no tannic acid), $P<0.05$

* Significantly different between treatments (PVP), $P<0.05$

Figure 4.10 **Effect of tannic acid on proteinase activity following addition of polyvinyl pyrrolidone**

(b) *S. bovis*



S. bovis ▨ *S. bovis* and PVP ⊡

Cell free extracts prepared from *S. bovis* (3 mg bacterial protein/ml) were incubated with polyvinyl pyrrolidone (PVP, 1 mg/ml) followed by increasing concentrations of tannic acid at room temperature for 30 min. Proteinase activity was determined spectrophotometrically under optimal conditions. Specific activity was expressed as μg azocasein digested per hour per milligram microbial protein. Data shown represents the average of triplicate trials; bars represent the standard error.

† Significantly different to control (no tannic acid), $P<0.05$

* Significantly different between treatments (PVP), $P<0.05$

of PVP in the assay mixture resulted in ~67% decrease in proteinase activity of *S. caprinus* following incubation with tannic acid (125 µg/ml) compared with 77% when no PVP was included (significant, $P<0.05$).

Proteinase activity could also be decreased in a dose dependent manner by the inclusion of a mixture of commercially obtained protease inhibitors (Table 4.1). Results are displayed in Figure 4.11.

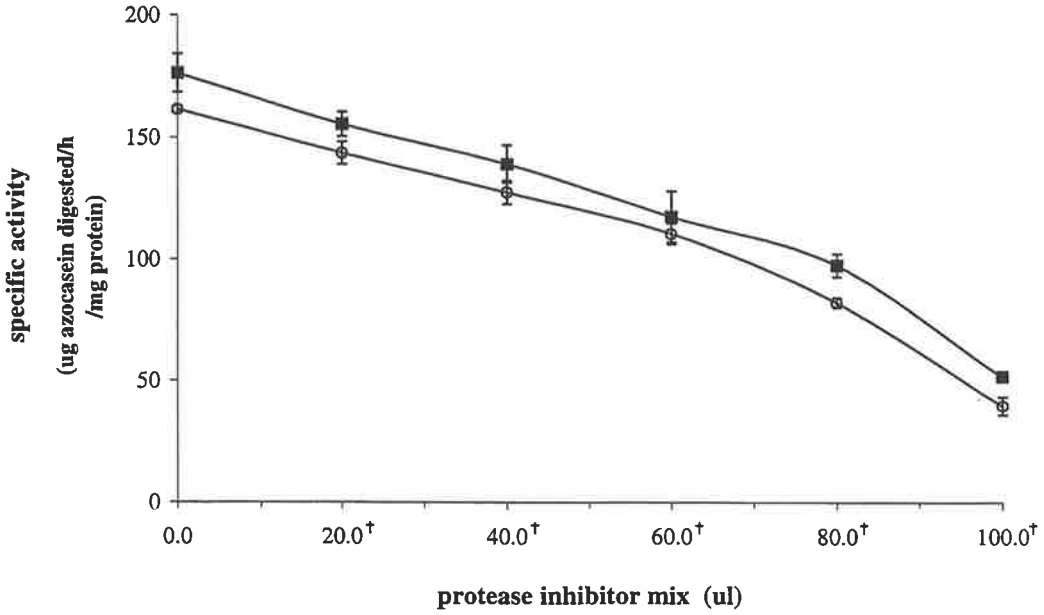
Table 4.1 Protease inhibitor mix

Protease Inhibitor	**Concentration used**	
Chymostatin	0.1	µg/ml
Aprotinin	2.0	µg/ml
Pepstatin A	1.0	µg/ml
Leupeptin	0.5	µg/ml
Antipain	0.25	µg/ml

4.2.4 The effect of tannic acid on bacterial protein profiles

Polyacrylamide gel electrophoresis of cell free extracts prepared from both *S. caprinus* and *S. bovis* enabled visualisation of the proteins expressed by the bacteria. The results indicated that while *S. bovis* and *S. caprinus* have a number of distinctly different protein bands, growth of the bacteria in the presence of tannic acid did not

Figure 4.11 Effect of protease inhibitor mix on proteinase activity



S. caprinus ○ *S. bovis* ■

Proteinase activity was determined in cell free extracts (3 mg bacterial protein/ml) prepared from *S. caprinus* and *S. bovis* under optimal conditions. Increasing concentrations of known protease inhibitors (Table 4.1) were included in the proteinase assay mixture. Specific activity was expressed as μg azocasein digested per hour per milligram microbial protein. Points represent the mean of triplicate trials; bars represent the standard error.

† Significantly different to control (no proteinase inhibitor), $P<0.05$

alter this pattern (Figure 4. 12). NB, the differences discerned in lane D (*S. caprinus* plus 0.5% tannic acid) compared with lane C (*S. caprinus*, no tannic acid) and E (*S. caprinus* plus 0.5% tannic acid) are due to excess protein in the lane. Repeated PAGE of cell free extracts prepared from the bacteria grown in the absence and presence of tannins indicated no difference.

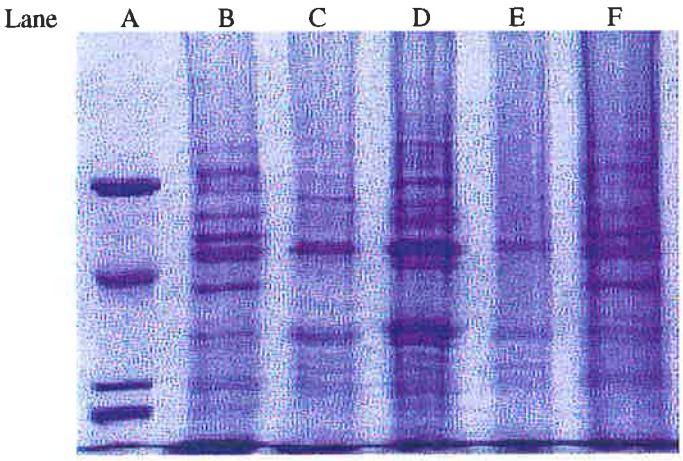
4.2.5 Genetic characterisation of tannin tolerance

Isolation of the genes responsible for tannin tolerance from *S. caprinus* met with little success. Initially, a plasmid library of *S. caprinus* genomic DNA was constructed in the *E. coli* vector, pUC19. Construction of a representative library proved successful with approximately 4 x 10^4 insert-containing transformants. The fragments selected for this library were between 0.5 and 10 kb, which suggested that this library contained between 20 and 400 mb of *S. caprinus* DNA. Assuming the size of the Streptococcal genome to be similar in size to that of *E. coli* ($\cong$ 4 mb), this represents approximately 5 to 100 genomes. However, when this library was screened for clearing activity and an increased tolerance to tannic acid, no positive clones were detected.

A library of *S. caprinus* genomic DNA was also made in the *E. coli*/*S. bovis* shuttle vector, pMU1328. This plasmid vector has an origin of replication for both *E. coli* and *S. bovis*. Clones were electroporated into *E. coli* and *S. bovis* electrocompetent hosts. From the *E. coli* transformation, 1.2 x 10^5 insert-containing colonies were obtained. However, transformation of *S. bovis* proved less successful, resulting in approximately

4 x 10^3 transformants. Screening the library for clearing activity or an increased tolerance to tannic acid again proved unsuccessful.

Figure 4.12 SDS-PAGE of cell free extracts prepared from *S. caprinus*



Lane A molecular weight markers

B *S. bovis*

C *S. caprinus*

D, E *S. caprinus* plus 0.5% w/v tannic acid

F *S. bovis* plus 0.5% w/v tannic acid

Cell free extracts were prepared from *S. caprinus* and *S. bovis* grown in mBHI medium in the presence and absence of tannic acid. PAGE was performed as described in the text (2.17).

4.3 Discussion

4.3.1 Binding properties of tannic acid

Variable results have been reported on the binding capacity of tannic acid obtained from different sources and this necessitated the determination of the properties of the tannic acid that was used in this study (Sigma, batch #11F-0559).

The ability of tannins to interact with proteins and other substrates is dependent on the size, conformation and charge of the substrate molecule. For example, proteins are amino acid polymers in the form of either an α-helix or a β-sheet stabilised by hydrogen bonds. In order to precipitate proteins, the tannins must alter the hydrogen bonds responsible for the helix or sheet form. This can be done through interaction of the protein with exposed -OH molecules on the phenolic units. Enough polymers of tannin must be available to bind with the protein before actual precipitation takes place (Dawra *et al* 1988; Van Hoven and Furstenburg 1992).

In the experiments presented in this chapter, increasing the concentration of tannic acid in a protein or PVP solution resulted in a proportional increase in the amount of tannic acid bound to substrate and forming a precipitable complex. However, not all of the added tannic acid was found in the precipitate, suggesting that a proportion of tannic acid remained in solution either as free tannin, bound to the substrate in a soluble complex or existing in equilibrium with the tannic acid-protein precipitate.

Hagerman and Butler (1981) showed that some proteins have an extremely high affinity for tannins and that other proteins have a much lower affinity. Those proteins

with a high affinity for tannic acid appear to be rich in the amino acid, proline (Mehansho *et al* 1985; Asquith *et al* 1987; Mehansho *et al* 1992). Proline has a secondary amine nitrogen and it has been reported that the carbonyl oxygen adjacent to this nitrogen is a very good hydrogen bond acceptor. Hydrogen bonding between phenolic hydroxyl and peptide carbonyl is a major force stabilising tannin-protein complexes and this makes proline rich proteins form especially strong hydrogen bonds with tannin (VanHoven and Furstenburg 1992). The presence of proline also restricts the protein molecular structure to some random coils giving them open extended structures as opposed to the regular firm helix/sheet formation, thus increasing the accessibility of the peptide backbone for hydrogen bonding (Murray *et al* 1994). In the experiments conducted in this study, PVP and gelatin had the strongest affinity for tannic acid. While PVP is not a protein, it contains heterocyclic vinyl pyrrolidone subunits that bear some structural resemblance to the pyrrolidone ring of proline and gelatin contains 18 mol % proline plus hydroxyproline. BSA however was not so effective in forming complexes with tannic acid, even though it too is reported to be of open configuration.

The complexes formed through hydrogen bonding can be dissociated by high and low pH, which results in ionisation of the phenolic hydroxyl, thus destroying its hydrogen bonding ability (Hagerman and Butler 1981, 1989, Perez-Maldonado *et al* 1995). In the experiments reported in this chapter, increasing the pH of the tannic acid-substrate reactions had a significant effect on the amount of tannic acid bound to the substrate, thereby producing a decrease in the amount of tannic acid precipitated. Contrary to this, decreasing the pH appeared to have no significant effect on the binding capacity

of tannic acid. These data are in agreement with those reported by Yan and Bennick (1995) and Makker *et al* (1995) who found respectively that proline rich proteins isolated from human saliva and polyethylene glycol were able to bind tannins effectively at neutral pH. In contrast to this study however, they determined that lowering the pH led to increased binding capacity of these proteins for tannins. Osawa and Walsh (1993) have also explored the effect of pH on tannic acid interactions in an attempt to determine whether the tannins may survive unaltered in conditions resembling those of the stomach. Their results suggest that tannic acid is hydrolysed to yield gallic acid under these conditions (low pH), which causes a reduction in the amount of tannic acid bound to protein. These conflicting results are indicative of the complex nature of tannic acid and provide evidence for the necessary inclusion of reporting on the tannic acid source and a more detailed examination of variation in different tannins.

The oxidation state also appeared to effect the binding capacity of tannic acid with less tannic acid found in complexed form following exposure to air rather than those kept under anoxic conditions. It can be assumed from these results that the age and storage environment of the tannic acid would also be a factor in effecting its binding capacity. In our laboratory, oxidation of the tannic acid was kept to a minimum by keeping the compound under anaerobic conditions and making up the tannic acid solutions immediately prior to use.

4.3.2 The effect of tannic acid on lactate production

Preliminary experiments suggested that bacterial secretion of lactic acid was responsible for the dissociation of the tannic acid-protein bonds resulting in a cleared region surrounding the bacterial colonies (Brooker *et al* 1994). However, in this study, GLC analysis described the amount of lactate produced by both *S. caprinus* and *S. bovis* and found them to be similar. Lactate levels remained unchanged with the addition of tannic acid (0.5% w/v) to the growth medium in both cases. As this protein sparing effect is only observed around colonies of *S. caprinus*, the results presented in this chapter indicate that the cleared zones were not due to lactic acid-mediated hydrolysis of the tannic acid-protein complex. No other acid was detected by GLC analysis in quantities sufficient to produce a cleared region. Thus at this stage, the mechanism(s) or substance(s) responsible for the cleared regions observed in the protein-tannin nutrient agar medium remain elusive. However, a scenario capable of explaining the breakdown of tannin-protein complexes by *S. caprinus* will be put forward in the concluding chapter of this thesis.

4.3.3 The effect of tannic acid on proteinase activity

Preliminary experiments utilising casein-containing agar failed to indicate proteinase activity in either *S. caprinus* or *S. bovis* cells. Wallace and Brammel (1985) suggested that the failure of some proteolytic species to produce the characteristic cleared regions indicative of casein degradation were due to the proteinase activity being predominantly cell bound and not released into the medium. Subsequent experiments localised proteolytic activity in *S. caprinus* and *S. bovis* to be primarily cell associated. The measurement of proteinase activity was successfully described for both bacteria

using diazotised protein as substrates. This procedure has been reported to be a convenient, reliable method that does not have the disadvantage of the loss of hydrolysis products, such as amino acids, due to microbial utilisation (Brock *et al* 1982). Experiments in this study indicated that under optimal conditions, *S. caprinus* and *S. bovis* had similar proteinase activities. Upon the addition of tannic acid to the assay system however, a significant decrease in proteolytic activity in both bacteria was observed although *S. caprinus* showed an increased tolerance to tannic acid when compared to *S. bovis*.

The inhibition of proteinase activity by tannic acid is in agreement with previous studies detailing the effect of tannins on enzyme systems, although condensed tannins have primarily been used in these experiments. Jones *et al* (1994) reported the effect of condensed tannins on proteolysis by four strains of ruminal bacteria, including *S. bovis* and determined that the presence of as little as 25 μg of condensed tannins/ml decreased total protease activity in *S. bovis* by more than 90%. In experiments reported in this chapter, tannic acid concentrations greater than 100 μg/ml (0.01% w/v) were necessary to reduce protease activity in *S. bovis* (type strain 2B) by this amount. The differences in tannin tolerance between these experiments are likely to reflect the different structure and chemical composition of tannins used in these experiments although intra-bacterial strain variations may also play a role. Proteolysis was also inhibited in a diverse group of proteolytic rumen bacteria by condensed tannins of *Calliandra calothyrsus* following binding of the tannin to protein (McSweeney *et al* 1999). Other enzyme systems known to be affected by tannins include extracellular endoglucanases of *F. succinogenes* S85 (inhibited by condensed

tannins from birdsfoot trefoil at concentrations greater than 44 μg/ml (0.0044% w/v), (Bae *et al* 1993), salivary α-amylase (Yan and Bennick 1995), and pancreatic amylase (Ahmed *et al* 1991).

The addition of PVP to the assay system resulted in an increase in the proteinase activity of *S. caprinus* and to a lesser extent, *S. bovis*. Thus it appears that the binding of tannic acid to other substrates results in a decrease in its inhibitory effect and enables the bacteria to degrade the free protein present more effectively. However, supporting previously obtained results (Table 3.1), tannic acid is still capable of causing bacterial inhibition in bound form.

The ability of *S. caprinus* to continue to degrade protein in the presence of, or when complexed with tannic acid provides the bacterium with an increased advantage over those bacteria incapable of this activity in the competitive rumen environment.

4.3.4 Identification of a protein involved in tannin tolerance

Growth studies (3.2) revealed an increase in the lag times of bacteria grown in the presence of tannic acid, suggesting that upregulation of protein synthesis or gene transcription was occurring at this time. These results, combined with the suggestion of a tannin tolerant proteinase(s) in *S. caprinus*, instigated the use of SDS-PAGE to visualise bacterial proteins. PAGE of cell extracts prepared from *S. caprinus* and *S. bovis* cells revealed a number of unique bands particular to each bacterial species, providing further evidence of their genetic differences. However, PAGE of cell extracts prepared from the bacteria grown in the presence or absence of tannic acid

showed no unique protein band in response to the presence of tannic acid which could have indicated how the bacteria were able to tolerate tannins. This result cannot rule out the possibility of a protein involved in the ability of *S. caprinus* to tolerate tannins. Should the protein involved be constitutively expressed or present in very low concentrations, it would not be detected by this method. Two-dimensional polyacrylamide gel electrophoresis may be more useful in identifying the protein(s) involved.

4.3.5 Genetic characterisation of tannin tolerance

In an attempt to isolate a gene involved in tannin tolerance, *E. coli* and *S. bovis*, host cells were transformed with recombinant plasmids containing regions of *S. caprinus* DNA. The selection criteria employed utilised two unique aspects of *S. caprinus*' tannin-tolerating ability. That is:

1 an increased resistance to tannic acid

2 the production of cleared regions around the bacterial colonies following growth on tannic acid-nutrient agar medium.

The plate assay used to screen recombinant libraries of *S. caprinus* DNA for either clearing activity or an increased resistance to tannic acid was found to be a rapid method which allowed a large number of recombinant clones to be screened. However, the successful screening of recombinant libraries relied firstly on the construction of a library in which every possible DNA sequence of the organism of interest was represented at least once; and secondly, that the cloned genes must have been expressed in the chosen host, either from their own promoters or from host

encoded promoters. In this study, construction of a representative library of *S. caprinus* DNA was successful in both of the plasmids used (pUC19 and pMU1328), however screening of the libraries for increased tolerance to tannic acid and clearing activity produced no positive results. As mentioned previously, in order for replication and expression of genes in the host bacteria, appropriate replicon and promoter elements are fundamental. When the promoter elements are not cloned in association with the gene, it is possible that the gene of interest may be driven by the host cell promoter. However, the gene must be inserted in the correct orientation and reading frame for this to occur and this has a very low probability of incidence. Detection of gene expression is more likely if the gene of interest is driven by its own promoter, which has been cloned with the gene. While this method has a higher probability of success in detecting a functional gene, it relies on the recognition of foreign promoter elements by the host's transcription machinery. A number of studies have indicated that consensus promoter sequences of rumen bacteria, such as in Ruminococcus sp (Vercoe *et al* 1995) were different to those found in *E. coli*. This lack of promoter function has been given as a major reason for a number of genes isolated from prokaryotic and eukaryotic sources that will not express in *E. coli* (Brooker *et al* 1992). In this case, the bacteria also display different cell wall structures, compounding the differences between the two species: *S. caprinus* is Gram positive, whereas *E. coli* stains Gram negative. It is therefore possible that the failure to detect increased tannic acid tolerance or clearing ability from the cloning of *S. caprinus* genes into an *E. coli* host was due to a lack of sequence recognition.

Transformation of a more closely related species, such as *S. bovis* may circumvent this problem. Unfortunately problems were encountered in the transformation of *S. bovis* which resulted in the marginal representation of the entire *S. caprinus* genome, a criteria essential for the successful screening and detection of cloned genes. Altering the electroporation conditions resulted in reduced transformation frequencies. Transformation of cells by treatment with calcium to encourage DNA uptake (Ausubel *et al* 1991) was also unsuccessful. The reason(s) for inadequate transformation frequencies of *S. bovis* in this study are unclear although transformation frequencies of *S. bovis* using pMU1328 have proven to give variable results (J. D. Brooker, personal communication). Further work beyond the time frame of this project is required to improve this system.

Another reason for the lack of success in locating a gene involved in tannin tolerance may be due to the possibility that the information coding for tannin tolerance is not a simple gene effect but is located on more than one gene, ie is a multi gene effect. Two scenarios are possible in this case.

1. If the genes are present as a cluster in one region of the genome, as in the lac operon of *E. coli* (Tortora *et al* 1992), it is conceivable that the cluster would exceed the size that could be cloned into the plasmid (ie >10 kb). Cloning a portion of the operon would render it inoperable and hence expression and subsequent detection would not occur.
2. If the genes were arranged throughout the genome, the likelihood of expression is even more remote. Even if all the genes governing the operation of tannin tolerance were successfully cloned, it is probable that the

transcription of one would rely on the transcription and translation of another, as again exampled in the lac system where the regulator gene coding for the lac repressor lies upstream of the lac operon. Spacial isolation of the genes, in different vector/host cells, would inhibit these elements and again render each gene inoperable.

Further experiments suggested that the ability of *S. caprinus* to tolerate tannins was not due to a single gene effect and it was decided that continuation along this path of analysis would be suspended at this point. Isolation of the genes responsible for tannin tolerance by mutation analysis is an ongoing project by another member of the laboratory (P. Muslera) and was therefore not presented in this study.

Chapter 5

Breakdown of tannins

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5.1 Introduction

The continuous operation of the carbon cycle depends to a large extent upon the rapid fusion of the benzene ring and several energy-yielding processes permit the anaerobic degradation of such compounds by various microorganisms. A number of ruminal microorganisms that have the capacity to detoxify hydrolysable tannins and phenolic monomers have been described (Krumholz *et al* 1986; Krumholz *et al* 1987; Haddock and Ferry 1993; Osawa *et al* 1993; Bhat *et al* 1996; Brooker *et al* 1994; Nelson *et al* 1995; Nelson *et al* 1998; Odenyo and Osuji 1998; Reichenbecher *et al* 1994; Brune and Schink 1990; Philipp and Schink 1998). Osawa (1990), Brooker *et al* (1994) and Nelson *et al* (1995, 1998) have described Streptococcal species capable of producing cleared regions following growth on tannic acid containing nutrient agar and suggest that this activity is indicative of tannic acid degradation. While Osawa and Walsh (1993b) and Nelson *et al* (1995) claim this degradation occurs via enzymatic means, no evidence for these claims have been given thus far. The first evidence of the presence of a 'tannase' (tannin acylhydrolase) in a rumen bacterium (*Selenomonas ruminantium* subsp. *ruminantium*) was described in detail by Skene and Brooker (1995).

In the previous chapters, *S. caprinus* was shown to be able to tolerate high concentrations of tannic acid and condensed tannins in its growth medium. However, determination of how the bacteria survived in the presence of these phenolic compounds was not resolved. Although it has previously been determined that *S.*

caprinus cannot utilise either tannic acid or condensed tannin as a sole carbon source (L. O'Donovan, Honours thesis; Brooker *et al* 1994), it was suggested that the bacteria were able to degrade the phenolic compounds which would enable the bacteria to

(a) utilise the phenolic compounds and/or their breakdown products or,

(b) produce a form which is less toxic to the bacteria than the parent phenolic compound.

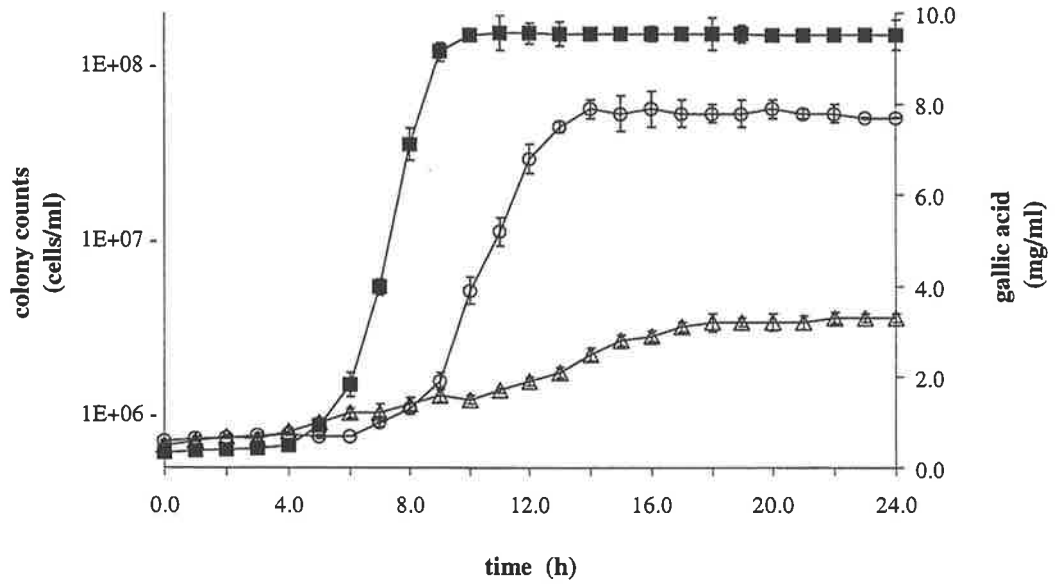
To this end, the ability of *S. caprinus*' to breakdown tannic acid was investigated.

5.2 Results

5.2.1 Breakdown of tannic acid

In a number of microbial systems, the initial breakdown product of tannic acid is gallic acid. Using a spectrophotometric method described by Inoue and Hagerman (1988), the concentration of gallic acid in the bacterial growth media was determined during the growth of *S. caprinus* in the presence of tannic acid (1.0% w/v, Figure 5.1). Residual gallic acid at a concentration of approximately 0.6 mg/ml (0.06%) was detected in the growth media prior to inoculation with bacteria. Following incubation, the concentration of gallic acid in the media increased. This increase was observed in the uninoculated control as well as after inoculation with *S. caprinus*. In the uninoculated controls, the amount of gallic acid increased from 0.6 to 3.2 mg/ml, an increase comparable to that obtained following incubation with *S. bovis*. However, a significant increase in the amount of gallic acid was detected in the growth media after inoculation and incubation with *S. caprinus,* ie. 0.6 to 7.8 mg/ml ($P<0.05$). The increase in gallic acid occurred upon completion of active growth of the bacteria (ie. stationary phase) and appeared to reach a plateau following ~ 16 to 20 hours of

Figure 5.1 **Production of gallic acid by S. *caprinus* during growth in mBHI media supplemented with tannic acid**



Growth in mBHI media plus tannic acid ■

Corresponding gallic acid production ○

Uninoculated control △

S. caprinus was incubated in mBHI medium supplemented with 1.0% tannic acid. At regular time intervals, aliquots (1 ml) were removed and growth was quantified on mBHI by serial dilution and viable cell count. At these times, the amount of gallic acid present in the media was determined spectrophotometrically (2.20). mBHI medium supplemented with tannic acid was incubated as a control. Points represent the average of triplicate trials; bars represent the standard error.

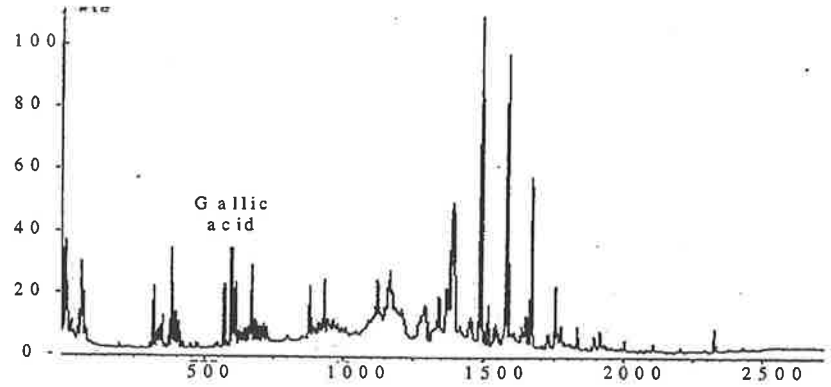
incubation. These results suggested that *S. caprinus* was capable of hydrolysing tannic acid to release gallic acid into the growth media.

Confirmation of the tannic acid breakdown products was undertaken using gas liquid chromatography - mass spectrometry (GLC-MS). Figure 5.2 shows the gas chromatograph of trimethylsilyl-derivatised culture supernatant samples obtained (a) prior to and (b) following overnight growth of *S. caprinus* in mBHI media supplemented with tannic acid (1.0% w/v). While the tannic acid chromatograph revealed a number of peaks corresponding to its many constituents, no increase in the peak corresponding to gallic acid was observed. Conversely, it appeared that a disappearance in the peaks corresponding to the low molecular weight components, such as gallic acid occurred following incubation with *S. caprinus* (not quantified). No new peaks, or increases in peak size corresponding to tannic acid breakdown products were detected at this time although slight modification of the chromatography conditions enabled detection of these products (see later).

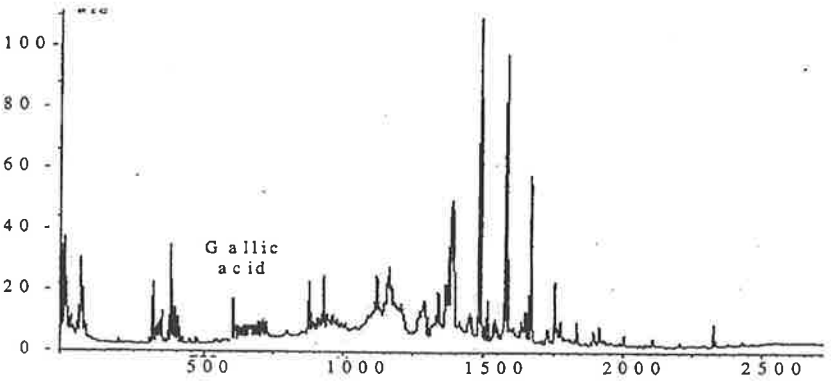
GLC-MS analysis of supernatant samples obtained prior to and following incubation of *S. caprinus* in the presence of gallic acid (1.0% w/v) revealed a disappearance of the peak corresponding to gallic acid and the emergence of a new peak (Figure 5.3). Comparison with phenolic acid standards identified the new peak as pyrogallol. Pyrogallol could also be detected in the medium following growth of the bacteria in the presence of tannic acid (1.0%). Further degradation of pyrogallol was not detected. Similar incubation of uninoculated media and that inoculated with *S. bovis* revealed no change in the chromatographs.

Figure 5.2 **GLC of TMS-derivatised culture supernatant samples of *S. caprinus* following growth in the presence of tannic acid**

(a) Prior to inoculation with *S. caprinus*

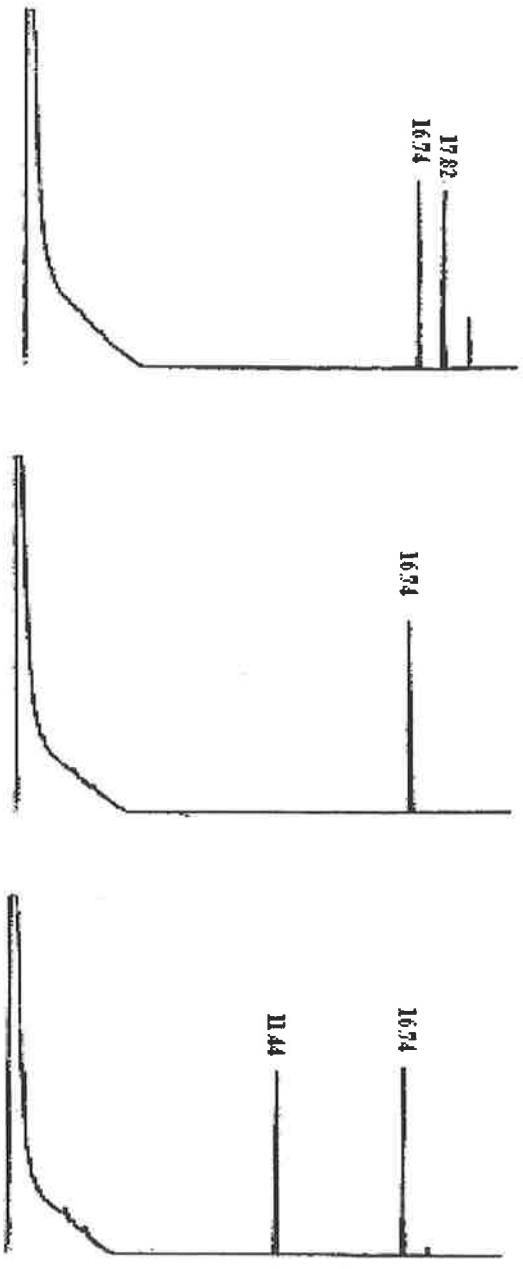


(b) Following incubation with *S. caprinus*



S. caprinus was incubated in mBHI medium supplemented with tannic acid (1.0% w/v). Prior to inoculation with and following overnight growth of bacteria, aliquots of culture supernatant samples were removed, TMS derivatives were prepared and the samples analysed by GLC.

Figure 5.3 **GLC of TMS derivatised culture supernatant samples from *S. caprinus* grown in mBHI medium supplemented with gallic acid**



S. caprinus was incubated in mBHI medium supplemented with 1.0% gallic acid. At 3, 6 and 12 h, aliquots were removed, TMS derivatives of the culture supernatant were prepared and the samples analysed by GLC. Breakdown products were identified by comparison with authentic standards. Numbers indicate retention times. 17.82: gallic acid; 11.44: pyrogallol; 16.74: methyl gallate internal standard.

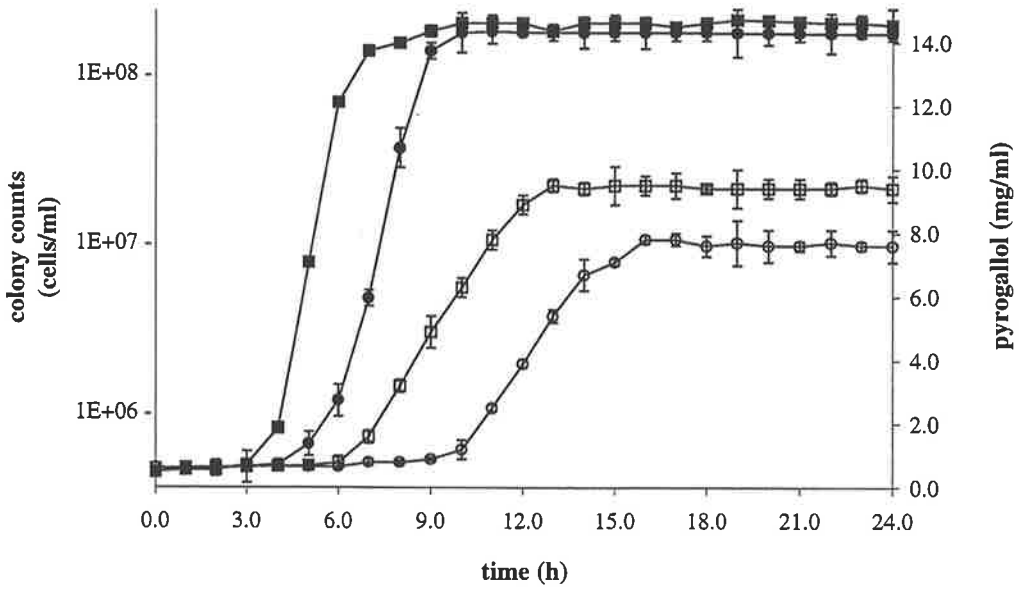
Time course experiments of *S. caprinus* grown in the presence of gallic acid or tannic acid indicated that the phenolic acids disappeared within 3 hours of bacterial inoculation. Pyrogallol remained undetected during bacterial growth until stationary phase was reached (Figure 5.4). Gallic acid was not detected as an intermediary breakdown product after incubation with tannic acid at any stage of bacterial growth.

GLC analysis was also used to determine whether *S. caprinus* or *S. bovis* could degrade other phenolic compounds present in their growth media. However, disappearance of peaks which corresponded to the phenolic monomers tested (listed in Figure 2.1) did not occur. Determination of the degradation products of condensed tannin was also attempted using this method however no change in the chromatograph was observed and hence no breakdown products were identified.

5.2.2 Characterisation of tannin acyl hydrolase activity in *S. caprinus*

The results obtained suggested that *S. caprinus* possessed tannin acyl hydrolase (TAH) activity and was capable of hydrolysing tannic acid and gallic acid to produce pyrogallol. Experiments were then carried out to determine whether *S. caprinus* employed the same mechanism as *S. ruminantium* subsp. *ruminantium* to degrade tannic acid. This activity was assayed in *S. caprinus* by measuring the release of gallic acid following incubation of the bacteria in a solution of gallic acid methyl ester (GAME). Data presented in Figure 5.5 shows that no gallic acid could be detected upon incubation of the bacteria with GAME. This result suggested that *S. caprinus* did not degrade tannic acid in a way analogous to that of *S. ruminantium* subsp. *ruminantium* (used as a control).

Figure 5.4 **Production of pyrogallol during the growth of *S. caprinus* in the presence of tannic acid and gallic acid**

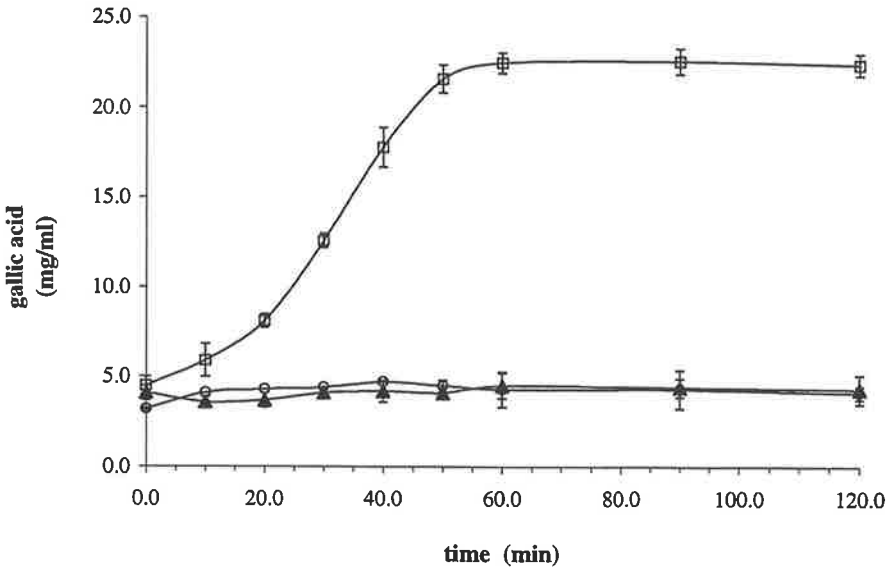


Growth in mBHI medium plus gallic acid ■ corresponding pyrogallol production □

Growth in mBHI medium plus tannic acid ● corresponding pyrogallol production ○

S. caprinus was incubated in mBHI medium supplemented with 1.0% w/v (10 mg/ml) gallic acid or tannic acid. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count (left axis). Corresponding pyrogallol production was determined by GLC analysis (right axis). Points represent the average of triplicate trials; bars represent the standard error.

Figure 5.5 **Tannin acylhydrolase activity by *S. caprinus***



S. caprinus ○ *S. ruminantium* □ *S. ruminantium* (boiled) ▲

TAH activity in *S. caprinus* was determined in whole cell suspensions following incubation in GAME (0.5% w/v, pH 6.8). At various time intervals, aliquots (1 ml) were removed and the concentration of gallic acid in the supernatant was determined spectrophotometrically. *S. ruminantium* subsp. *ruminantium* was included as a control. Points represent the average of triplicate trials; bars represent the standard error.

5.2.3 Characterisation of gallate decarboxylase activity in *S. caprinus*

Degradation of gallic acid by the enzyme gallate decarboxylase resulting in the production of pyrogallol has been demonstrated for a number of bacterial systems (Krumholz and Bryant 1986; Krumholz *et al* 1987; Brune and Schink, 1992). Gallate decarboxylase activity was initially assayed spectrophotometrically using washed whole cells and cell free extracts prepared from *S. caprinus* grown in mBHI. However, enzyme activity was only detected in whole cell preparations under strictly anaerobic conditions in the presence of Mg^{2+} (Table 5.1). The inclusion of tannic acid in the bacterial growth medium prior to the assay appeared to decrease gallate decarboxylase activity. However, results varied considerably in these experiments.

Successful characterisation of enzyme activity was accomplished in a media-free system by incubation of bacterial cells in a solution of gallic acid or tannic acid under anaerobic conditions and determining the amount of pyrogallol present by GLC analysis. Optimum enzyme activity was determined with respect to gallic acid concentration (Km = 50 mM, Vmax = 5.1 mmoles pyrogallol produced/min/5.0E+05 cells, Figure 5.6 and 5.7) or in the case of the combined activity of tannin acyl hydrolase and gallate decarboxylase, tannic acid concentration (0.5 to 1.0% w/v, Figure 5.8), time (20 min, Figure 5.9), bacterial cell numbers (10^4 to 10^6 cells/ml, Figure 5.10); pH (6.8, Figure 5.11) and temperature (15 to 40 °C, Figure 5.12).

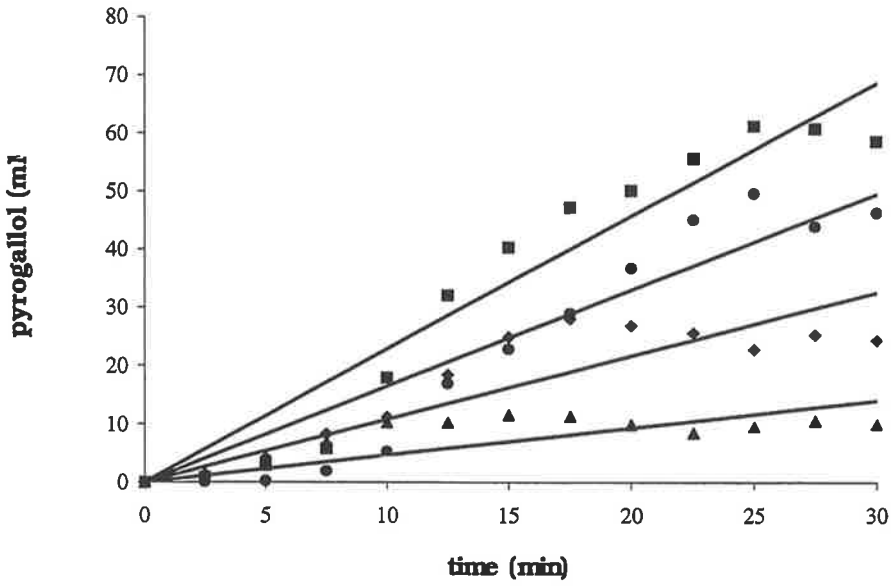
The correlation between growth of *S. caprinus*, gallic acid decomposition and pyrogallol production is shown in Figure 5.13. Stoichiometric analysis detailed that conversion of gallic acid to pyrogallol was complete as the amount of pyrogallol

Table 5.1 **Specific activity of gallate decarboxylase activity by *S. caprinus***

Cell fraction	Gallate decarboxylase activity (ρmoles gallic acid decarboxylated/min/ bacterial cell)
Whole cells	0.41 ± 0.22
Boiled cells	0.01 ± 0.0013
Cell free extract (total)	0.03 ± 0.0012

Gallate decarboxylase activity was assayed spectrophotometrically under anaerobic conditions in whole cells (5 x 10^6 cells/ml) and in cell free extracts (1 mg/ml) prepared from *S. caprinus* following growth in mBHI medium (2.). Specific gallate decarboxylase activity was expressed as ρmoles phenolic acid decarboxylated/min/cell or in the case of cell free extract, ρmoles/min/mg bacterial cell protein. Results represent the mean of triplicate assays ± standard error.

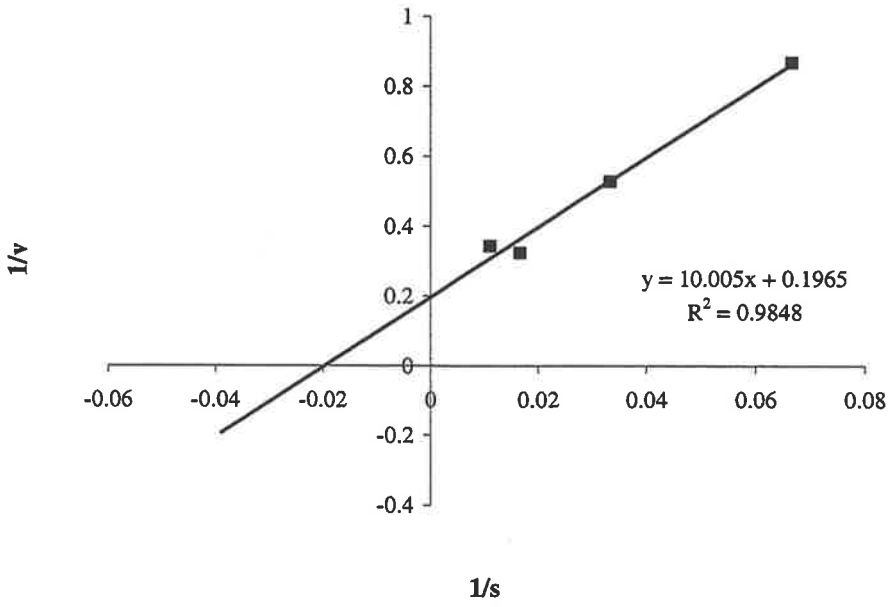
Figure 5.6 **Effect of gallic acid concentration on gallate decarboxylase activity by *S. caprinus***



Gallic acid concentration (mM) 15 ▲ 30 ◆ 60 ■ 90 ●

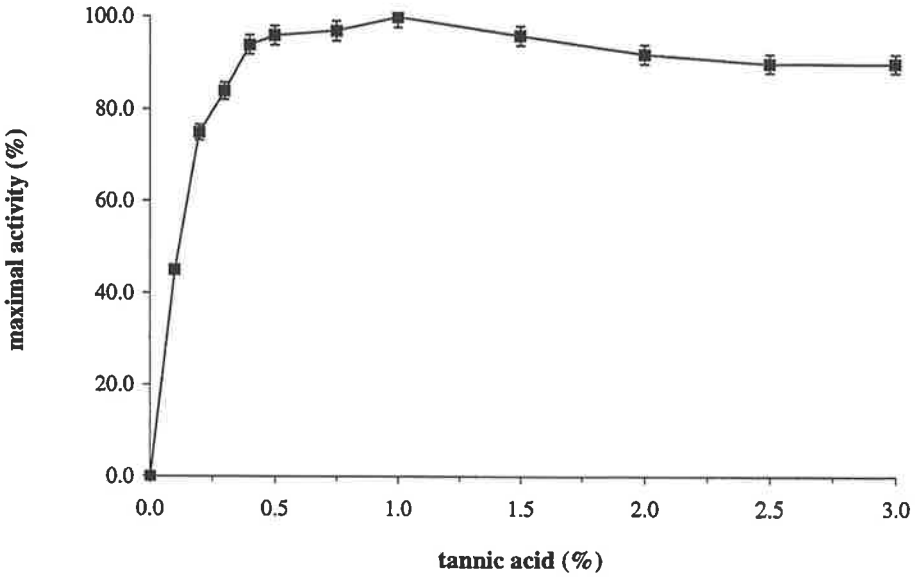
S. caprinus (5.0×10^5 cells/ml) were incubated in increasing concentrations of gallic acid (as stated) at pH 6.8 and 25 °C. At various time intervals, aliquots (0.1 ml) were removed from the assay system and the amount of pyrogallol determined by GLC analysis. Points represent the average of triplicate trials. Line represents regression analysis of the assay.

Figure 5.7 **Lineweaver-Burk plot of gallate decarboxylase activity by *S. caprinus***



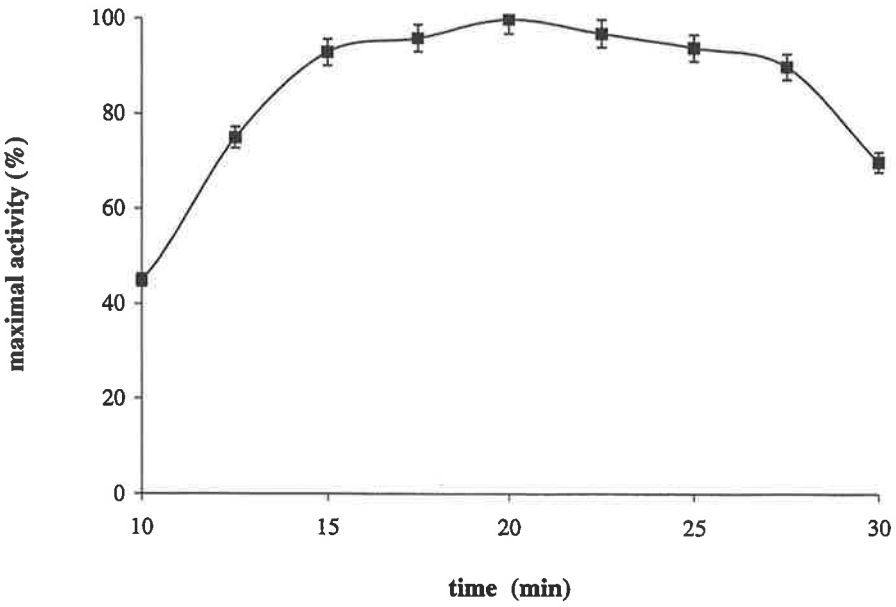
The reciprocal reaction velocity (v) was calculated from the slopes of the linear regression analysis represented in Figure 5.6 and plotted against the reciprocal of gallic acid substrate concentration (s). The line represents a linear regression analysis. Km (mM) and Vmax values as determined from the graph are 50 and 5.1 respectively.

Figure 5.8 **Effect of tannic acid concentration on gallate decarboxylase activity by *S. caprinus***



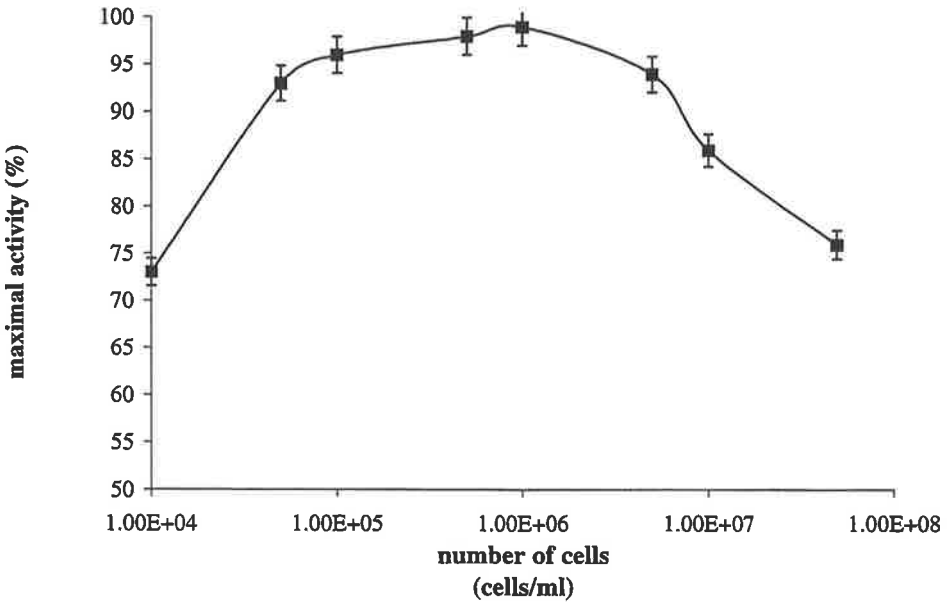
S. caprinus (5.0×10^5 cells/ml) was incubated in increasing concentrations of tannic acid (as stated) at pH 6.8 for 20 min at 25 °C. The amount of pyrogallol was determined by GLC analysis. Points represent the average of triplicate trials; bars represent the standard error.

Figure 5.9 Effect of time on gallate decarboxylase activity by *S. caprinus*



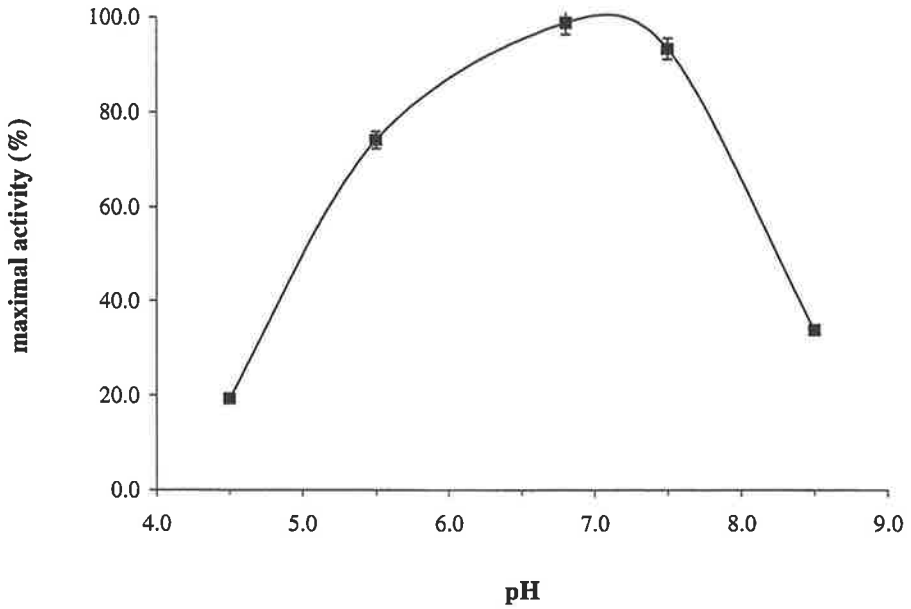
S. caprinus (5.0 x 10^5 cells/ml) was incubated at 25°C in 50 mM gallic acid, pH 6.8, for the times shown. The amount of pyrogallol produced was determined by GLC analysis. Points represent the average of triplicate trials; bars represent the standard error.

Figure 5.10 **Effect of cell number on gallate decarboxylase activity by *S. caprinus***



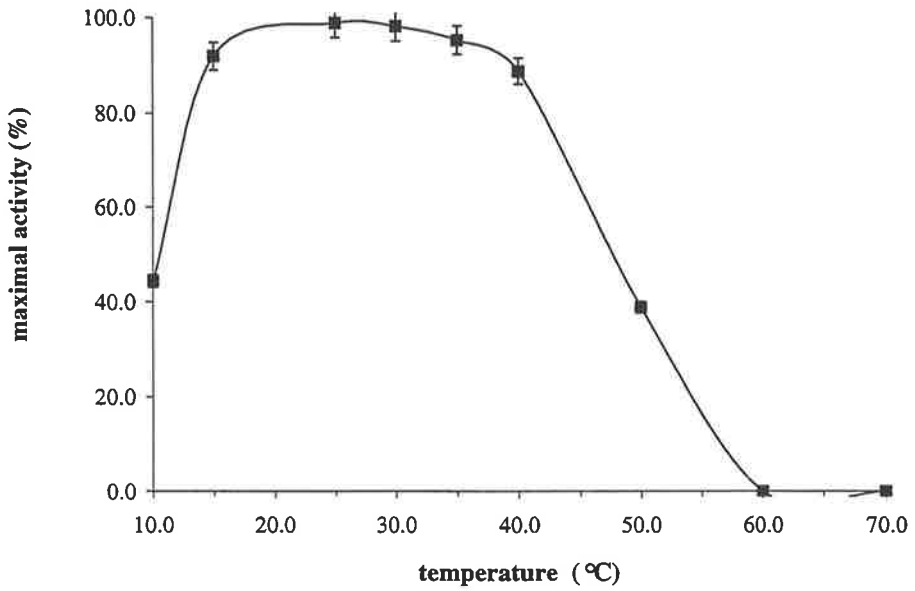
S. caprinus (cell numbers as shown) was incubated in 50 mM gallic acid, pH 6.8, for 20 min at 25 °C. The amount of pyrogallol produced was determined by GLC analysis. Points represent the average of triplicate trials; bars represent the standard error.

Figure 5.11 Effect of pH on gallate decarboxylase activity by *S. caprinus*



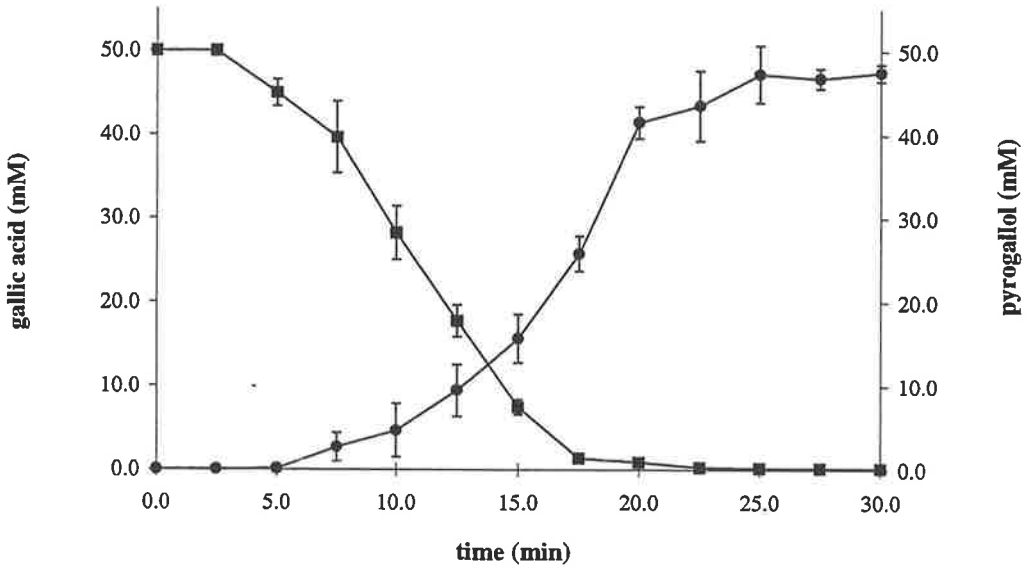
Whole cell suspensions of *S. caprinus* were washed and resuspended in buffer of pH as shown. The cells were incubated in 50mM gallic acid dissolved in the same buffer system. Pyrogallol production after 15 min incubation at 25 °C was determined by GLC analysis. The activity at each pH value is expressed as a percentage of the activity at pH 6.8. Points represent the average of triplicate trials; bars represent the standard error.

Figure 5.12 **Effect of temperature on gallate decarboxylase activity by *S. caprinus***



Whole cell suspensions of *S. caprinus* were washed and resuspended in phosphate buffer (pH 6.8). The cells were incubated in 0.5% gallic acid at the temperatures shown. Pyrogallol production after 15 min incubation was determined by GLC analysis. The activity at each temperature is expressed as a percentage of the activity at 25 °C. Points represent the average of triplicate trials; bars represent the standard error.

Figure 5.13 **Degradation of gallic acid and corresponding pyrogallol production by *S. caprinus***



Gallic acid degradation ■ corresponding pyrogallol production ●

Whole bacterial cells were incubated with 50mM gallic acid at 25 °C for 30 min. Aliquots (0.1 ml) were removed at various time intervals, extracted with ethyl acetate and derivatised with TMS for GLC analysis as described in the text. Points represent the average of triplicate trials. Bars represent the standard error.

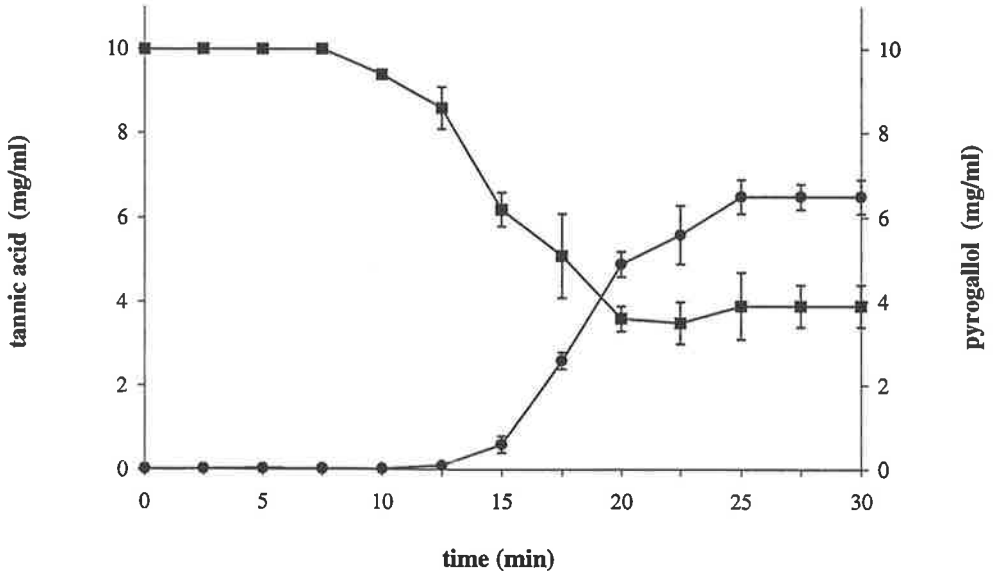
produced by *S. caprinus* equalled that of the gallic acid consumed by the bacteria, ie 50 mM and 47 mM respectively. In contrast, total breakdown of tannic acid to form pyrogallol did not appear to occur following incubation of the bacteria with this acid (Figure 5.14).

The decarboxylation of gallic acid results in the liberation of CO_2 into the growth medium. Measurement of the amount of gas produced by *S. caprinus* following growth in the presence of increasing concentrations of gallic acid or tannic acid revealed a corresponding increase in the amount of gas produced (Figure 5.15). The gas was identified by GLC analysis to be carbon dioxide. Time course experiments indicated that CO_2 was produced maximally at the end of the bacterial growth phase and coincided with the production of pyrogallol by *S. caprinus* (Figure 5.16).

Gallate decarboxylase activity appeared to be upregulated following growth of the bacteria in mBHI media in the presence of gallic acid (4-fold increase) or tannic acid (2.5-fold) compared with those grown in mBHI medium (significant $P<0.05$, Table 5.2). However, significant upregulation of enzyme activity did not appear to occur following growth of the bacteria in the presence of condensed tannin or any of the other phenolic acids tested.

Gallate decarboxylase appeared to be specific for gallic acid as incubation of the bacteria in similar carboxylated compounds, such as protocatechuic acid, 3,5-dihydroxy benzoic acid or hydroxybenzoic acid failed to produce their decarboxylated counterparts; catechol, resorcinol and phenol respectively.

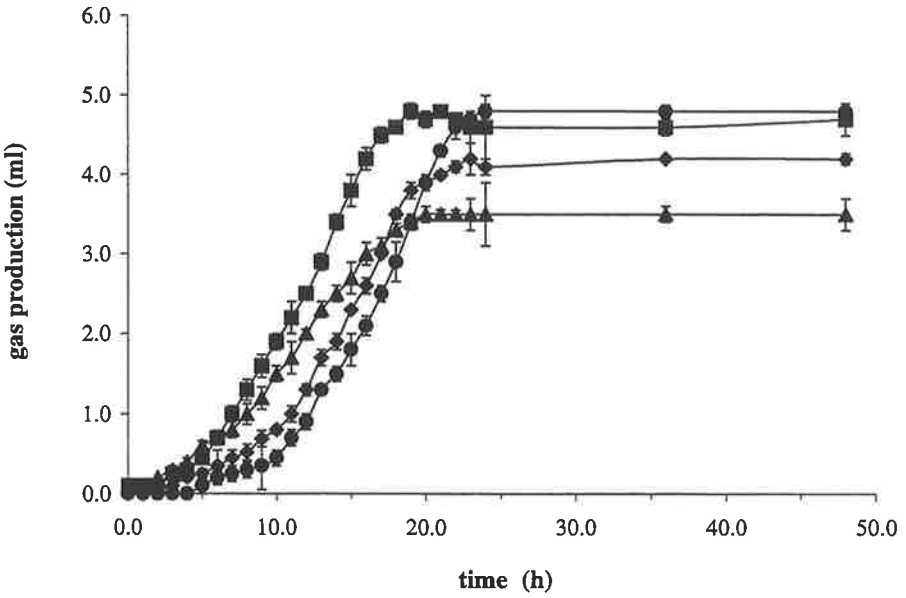
Figure 5.14 **Degradation of tannic acid and corresponding pyrogallol production by *S. caprinus***



Tannic acid degradation ■ corresponding pyrogallol production ●

Whole bacterial cells were incubated with 10 mg/ml tannic acid at 25 °C for 30 min. Aliquots (0.1 ml) were removed at various time intervals, extracted with ethyl acetate and derivatised with TMS for GLC analysis as described in the text. Points represent the average of triplicate trials. Bars represent the standard error.

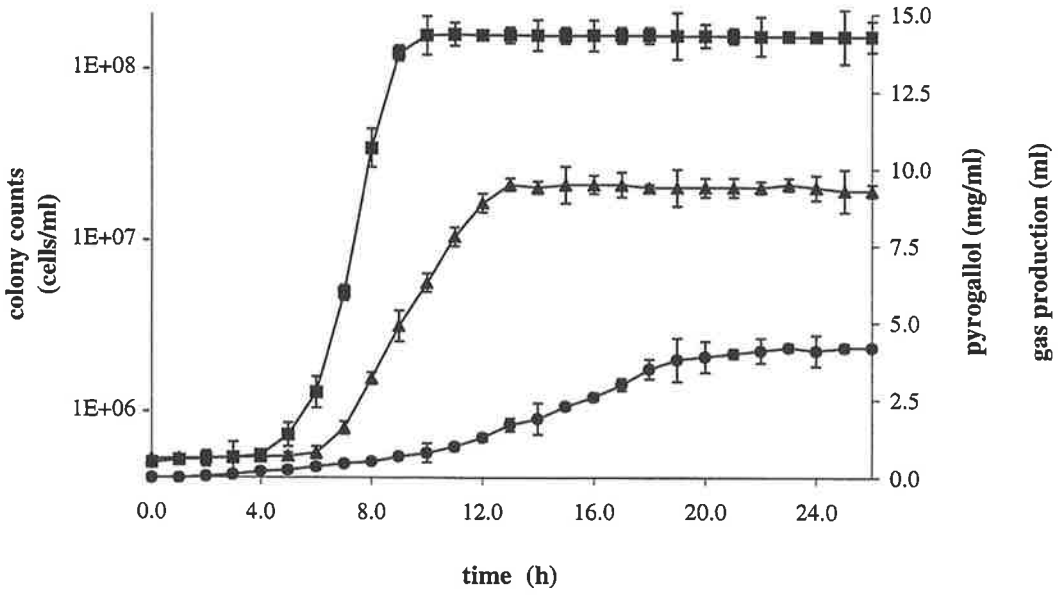
Figure 5.15 **Gas production by *S. caprinus* in the presence of increasing concentrations of tannic acid**



Tannic acid concentration (%) 0 ▲ 1.0 ■ 2.0 ◆ 4.0 ●

S. caprinus was incubated in mBHI medium supplemented with increasing concentrations of tannic acid. The amount of gas produced by the culture was determined over a 48 h period by displacement of a syringe plunger inserted into the top of the tube. The nature of the gas was then analysed by GLC and identified by comparison with standards.

Figure 5.16 **Pyrogallol production and gas production by *S. caprinus* when grown in the presence of tannic acid**



Growth in mBHI medium plus tannic acid ■

Corresponding pyrogallol production ▲

Corresponding gas production ●

S. caprinus was incubated in mBHI medium supplemented with 10 mg/ml tannic acid. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count (left axis). Corresponding pyrogallol production was determined by GLC analysis (right axis). The amount of gas produced by the culture was determined by displacement of a syringe plunger inserted into the top of the tube and identified by GLC analysis (right axis). Points represent the average of triplicate trials; bars represent the standard error.

Table 5.2 **Specific activity of gallate decarboxylase by *S. caprinus* following growth in the presence of phenolic compounds**

medium	Specific activity (nmoles pyrogallol produced/min/cell)
mBHI	14.2 ± 0.12
mBHI plus 1.0% tannic acid	34.8 ± 0.95[†]
mBHI plus 1.0% gallic acid	59.2 ± 1.21[†]
mBHI plus 0.5% condensed tannin	17.7 ± 0.21
mBHI plus 0.5% ferulic acid	12.9 ± 0.26
mBHI plus 0.5% p-coumaric acid	13.8 ± 0.17
Boiled cells	0.78 ± 1.4 x 10^{-2}

The specific activity of gallate decarboxylase activity was determined in whole cell suspensions prepared from *S. caprinus* grown in mBHI and mBHI supplemented with the phenolic acids listed. The cells were washed in CE buffer (pH 10) and phosphate buffer (pH 6.8) and then incubated (5.0 x 10^5 cells/ml) in 50 mM gallic acid for 20 min at 25 °C. The amount of pyrogallol was determined by GLC analysis. Boiled cells were included as a control. Results are the averages of triplicate trials ± the standard error.

[†] Significantly different to control (no phenolic), $P<0.05$

5.3 Discussion

5.3.1 Breakdown of tannic acid

A number of reports have described the anaerobic breakdown of tannic acid and the liberation of gallic acid by different bacterial species (Krumholz and Bryant 1986, Brune and Schink, 1992, Skene and Brooker 1995). Experiments undertaken as part of this study have shown that the first indication of the hydrolysis of tannic acid was an apparent increase in the amount of gallic acid in the growth medium after incubation of *S. caprinus* in the presence of tannic acid. In these initial experiments, gallic acid was measured using a spectrophotometric assay utilising rhodanine, a substance capable of reacting with the vicinal hydroxyl groups of gallic acid to give a red complex (Inoue and Hagerman 1988). This assay system is reported to be specific for the detection of free gallic acid and not gallic acid esters, ellagic acid or other phenolics (Inoue and Hagerman 1988). However, confirmation of the tannic acid breakdown product(s) by GLC analysis revealed pyrogallol rather than gallic acid, to be present in the growth medium following incubation with *S. caprinus* suggesting that rhodanine cannot distinguish between gallic acid and its decarboxylated counterpart, pyrogallol. These results indicated that *S. caprinus* was capable of degrading tannic acid to gallic acid which was then hydrolysed to pyrogallol, a phenolic compound previously ascertained to be far less toxic to a number of ruminal bacteria than tannic acid (3.1.2; Haslam and Lilley 1986; Field and Lettinga 1992, Ahn *et al* 1998, Chung *et al* 1998). *S. bovis* was unable to degrade either tannic acid or gallic acid and thus these results may be indicative of *S. caprinus*' ability to tolerate tannins in its growth environment.

5.3.2 Tannin acyl hydrolase activity

The breakdown of tannic acid to gallic acid requires the hydrolysis of the intergallate depside linkages and the ester bonds between gallate and glucose by the enzyme tannin acylhydrolase (TAH, EC 3.1.1.20). TAH is common to some fungal strains belonging to the genera Aspergillus and Candida (Aoki *et al* 1976; Jean *et al* 1981; Barthomeuf *et al* 1994; Bajpai and Patil 1996) and has recently been described in the rumen bacteria, *S. ruminantium* subsp. *ruminantium* (Skene and Brooker 1995). In this bacterium, enzymatic degradation of tannic acid occurs by breaking the ester bonds present in the tannic acid and removing the gallate groups attached to the central carbohydrate core to liberate glucose, which the bacterium then utilises for growth. Experiments in this study however suggested that while *S. caprinus* also appeared to possess TAH activity, as was evident from the degradation of tannic acid, it was incapable of degrading methyl gallate, the simplest gallic acid ester consisting of a molecule of methyl alcohol esterified to a molecule of gallic acid, to produce gallic acid. These results suggest that the esterase enzymes in *S. caprinus* do not appear to have the same substrate range as the esterase in *S. ruminantium*. This result was supported by *S. caprinus*' inability to utilise tannic acid as a sole carbon source (Brooker *et al* 1994) and the growth inhibition that occurs due to the presence of methyl gallate in the bacterial growth medium (Figure 3.11c). Further experiments utilising other phenolic acid ester substrates is required to fully characterise TAH activity in *S. caprinus*. However, a possible scenario describing degradation of tannic acid by *S. caprinus* will be discussed towards the end of this chapter.

5.3.3 Gallate decarboxylase activity

Degradation of gallic acid to pyrogallol by the enzyme gallate decarboxylase has been described in a number of bacteria (Krumholz and Bryant 1986; Krumholz *et al* 1987; Brune and Shink 1992). Initially, experiments in this study attempted to define gallate decarboxylase activity using an assay described by Krumholz *et al* (1987). The assay is based on the premise that phenolic carboxylic acids have an absorbance maximum between 250 and 300 nm which is not present in their decarboxylated counterparts. Thus the presence of the enzyme can be determined by recording the absorbance change observed as the phenolic carboxylic acid is decarboxylated. In experiments presented in this study, gallate decarboxylase activity could only be determined in whole cells of *S. caprinus* in the presence of Mg^{2+} and under strictly anaerobic conditions. However, measurement of enzyme activity proved variable and as the differences obtained in absorbance were small, the specific activity of the enzyme was difficult to ascertain with any accuracy. Inconsistent measurements have previously been described for this enzyme (Krumholz and Bryant 1986; Brune and Shink 1992) and may explain some of the problems encountered in these experiments. In *E. oxidoreducens*, gallate decarboxylase activity was reported in the cytosolic cell fraction using this method (Krumholz and Bryant 1986). However, the authors also found that measurements were unreliable when compared with those of other enzymes involved in aromatic metabolism and suggested that the low activity of was due to the instability and oxygen sensitivity of the enzyme.

Gallate decarboxylase activity was described with more accuracy when correlated to the amount of pyrogallol produced by the bacteria after incubation in gallic acid. GLC

of breakdown products has previously been used to correlate enzyme activity. Jean *et al* (1981) developed a gas chromatographic method, which determined the production of gallic acid after enzymatic hydrolysis of methyl gallate by fungal tannase. In this study, while pyrogallol was again not detected in cell-free extracts prepared from *S. caprinus* following anaerobic incubation with gallic acid, incubation of washed whole cells resulted in hydrolysis of the substrate and thus enabled enzyme activity to be described.

The pH and temperature optima of the *S. caprinus* gallate decarboxylase were consistent with an enzyme derived from a rumen bacterium that grows in an environment of neutral pH and 39 °C temperature and correlated well with other bacterial systems describing gallate decarboxylase activity (Brune and Schink 1992) The Km/Vmax of gallate decarboxylase activity by *S. caprinus* could not be compared to other bacterial systems due to the different procedures employed to determine enzyme activity.

An increase in enzyme activity was observed following growth of *S. caprinus* in the presence of either gallic acid or tannic acid suggesting that gallate decarboxylase activity is upregulated by these phenolic compounds. However, as upregulation of activity was less following incubation with tannic acid, it may be the gallic acid component present in tannic acid that is responsible, rather that the tannic acid molecule itself. The extended lag periods that were observed following incubation of *S. caprinus* in the presence of tannic acid or gallic acid (3.2) might be a reflection of the switching on of genes associated with this enzyme (or TAH) or in the production

of the protein. This increase in activity was not observed following growth of *S. caprinus* in the presence of other phenolic compounds, such as condensed tannin, p-coumaric or ferulic acids which suggests that upregulation of gallate decarboxylase activity was specific for gallic acid and possibly tannic acid.

Gallate decarboxylase also appeared to be substrate specific (gallic acid) as no decarboxylated counterparts were detected upon incubation of the bacteria with alternative phenolic acid substrates, such as protocatechuic acid (following decarboxylation gives catechol), 3,5-dihydroxy benzoic acid (resorcinol) or hydroxybenzoic acid (phenol). Specificity of the decarboxylase enzyme was also observed in *E. oxidoreducens*, (Krumholz *et al* 1987). However, in Citrobacter sp. (Yoshida *et al* 1982) and in cells of *P. acidigallici* (Brune and Schink, 1992), the decarboxylase reaction was not specific and facilitated growth of the organisms on a number of phenolic compounds, such as the aforementioned compounds tested in this study. The difference in substrate specificity of gallate decarboxylase suggests a non-specific decarboxylase or the presence of isomers of this enzyme.

5.3.4 Cellular uptake of gallic acid and tannic acid

Experiments in this study indicated that disappearance of the gallic acid substrate from the growth media was found to occur during early log phase of bacterial growth, whereas pyrogallol was not detected in the media until stationary phase. This suggests that gallic acid was taken up by *S. caprinus* and degraded intercellularly. In the experiments presented in this study, localisation of gallate decarboxylase could not be determined due to the lability of the enzyme in cell free extracts. However, previous

reports in other bacterial systems have determined gallate decarboxylase activity can be detected in the cytosolic fraction of cell free extracts (Brune and Schink 1992; Krumholz *et al* 1987). In these studies the authors also propose that gallate degradation occurs internally and that uptake of gallic acid by the cells occurs via simple diffusion. Gallic acid has not been found to complex with proteins and other media components and this, along with its small size would facilitate its passage through the bacterial cell membrane.

Incubation of *S. caprinus* with tannic acid also resulted in the disappearance of tannic acid from the growth medium during the early stages of bacterial growth, followed by pyrogallol production during stationary phase. This result suggested that the bacterium was capable of degrading tannic acid to gallic acid before pyrogallol was subsequently produced. Gallic acid, however, was not detected as an intermediary breakdown product and this suggests that degradation of tannic acid through the breakdown of intergallate depside linkages and the ester bonds between gallate and glucose are both readily hydrolysed and that gallate decarboxylation takes place rapidly.

The non-detection of gallic acid as an intermediary suggests that degradation of tannic acid also occurs intracellularly. However, while the lower molecular weight components may pass through the bacterial cell membrane, the high molecular weight components of tannic acid, ie approximately 789-1027 kDa and ranging from 3.5 - 4.7 nm in diameter, have been shown to bind to protein and other media components to form large complexes (3.2.2; Hagerman *et al* 1992). These complexes are unlikely to

be able to enter the cell through simple diffusion. Thus, in order for *S. caprinus* to take up the tannic acid and degrade it intracellularly, the bonds between the tannic acid and protein must first be destroyed. There is evidence to suggest that *S. caprinus* possesses the ability to degrade the tannic acid-protein complex by acid hydrolysis of the bonds between the two compounds (4.2; Brooker *et al* 1994).

Pulse chase experiments with radio-labelled tannic acid and gallic acid may determine whether or not the phenolic acids are internalised before their degradation, however, these compounds are not commercially available and would have to be specifically made. It was decided that these experiments were beyond the scope of this project at this time.

5.3.5 Stoichiometric considerations

The amount of pyrogallol detected from the breakdown of gallic acid was more than that obtained from the breakdown of the same amount of tannic acid. This result is not totally unexpected considering the variability of the composition of tannic acid, however, it may also suggest that *S. caprinus* can only degrade a proportion of the tannic acid, for example the lower molecular weight gallic acid components. The tannic acid used in this study was determined to contain ~ 8% gallic acid (Chapter 3.), thus a concentration of 1.0% tannic acid in the assay correlates to approximately 0.08% or 0.8 mg/ml gallic acid. As the conversion of gallic acid to pyrogallol appeared to be complete, breakdown of this amount of gallic acid would result in the production of an equivalent amount of pyrogallol, ie 0.8 mg/ml. This amount was significantly less than the amount of pyrogallol detected in this study following

growth of *S. caprinus* in media containing 1.0% tannic acid (ie 7.8 mg/ml). This result therefore provides further evidence that *S. caprinus* is capable of hydrolysing the tannic acid molecule itself.

Preliminary experiments in this study revealed that tannic acid can also be broken down via non-enzymatic means (5.2.1). In these experiments, incubation of media containing tannic acid resulted in an increase in the amount of gallic acid detected suggesting that tannic acid is unstable under these conditions. After 25-30 h incubation, a plateau in the amount of gallate detected in the media was reached. However, further tannic acid degradation could be induced by the removal of the supernatant containing gallic acid, followed by the addition of fresh media to the tannic acid-protein precipitate. This suggests that a state of equilibrium exists between gallic acid and tannic acid. In the bacterial system therefore, the ability of *S. caprinus* to degrade gallic acid to produce pyrogallol would effectively remove gallic acid from the media, and facilitate the natural hydrolysis of tannic acid. The spontaneous degradation of tannic acid over time has previously been proposed (Brune and Schink 1992). In this report, the authors also describe a similar equilibrium scenario for gallic acid degradation in *E. oxidoreducens*.

5.3.6 Further phenolic degradation

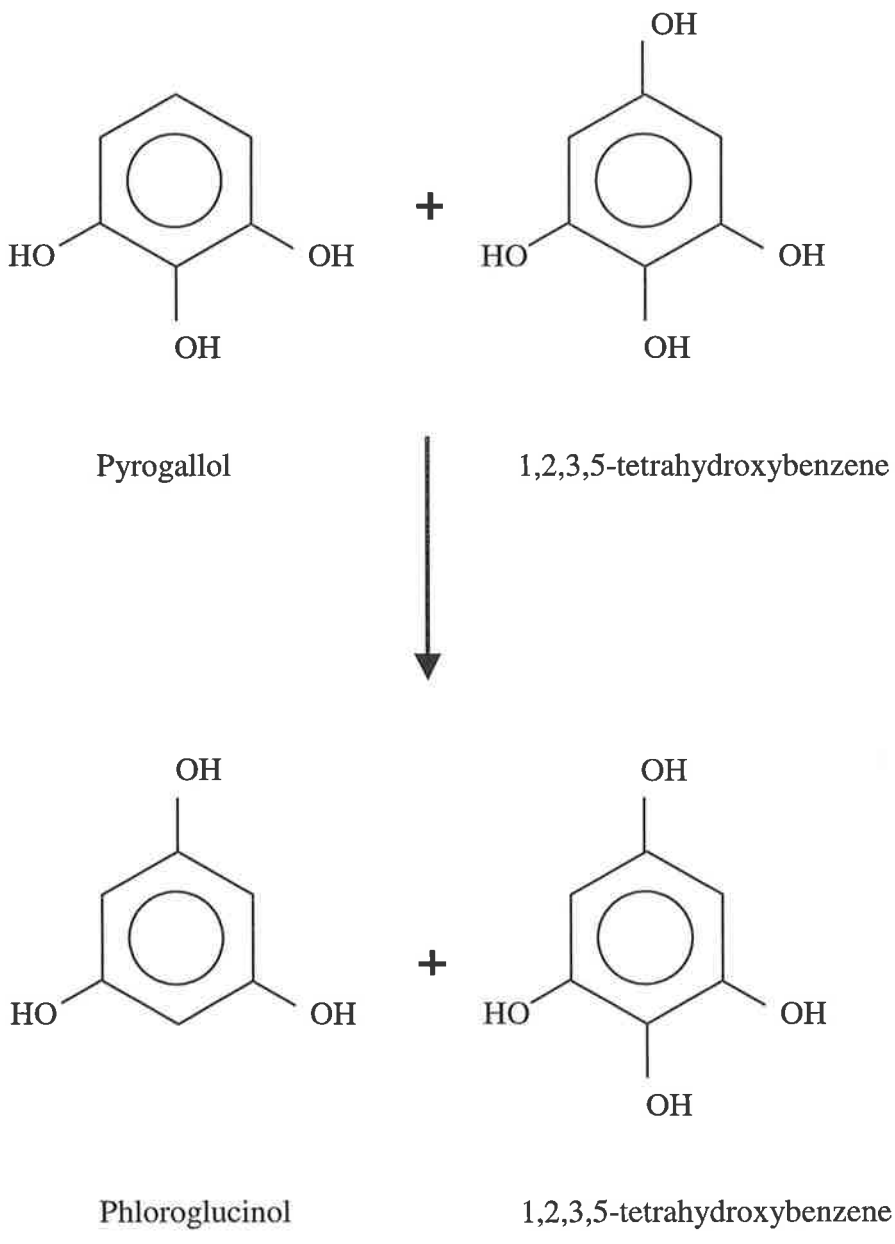
Degradation of pyrogallol has been reported to occur in a number of bacterial systems. *E. oxidoreducens* has been shown to employ a consecutive dehydroxylation and hydroxylation reaction that utilises the enzyme pyrogallol-phloroglucinol isomerase to metabolise pyrogallol to phloroglucinol which is reported to be easily reduced in the

rumen, to form dihyrophloroglucinol, which is no longer aromatic (Krumholz and Bryant 1988; Tsai and Jones 1975; Schink and Pfening 1982; Osawa *et al* 1993; Haddock and Ferry 1993; Kohler *et al* 1993; Reichenbecher 1994). In *P. acidigallici*, a transhydroxylation reaction occurs following decarboxylation of gallic acid. This transfers a hydroxyl group from a hydroxyl donor (1,2,3,5-tetrahydroxybenzene) to pyrogallol to form the product phloroglucinol and results in the regeneration of the cosubstrate (Figure 5.17, Krumholz and Bryant 1988, Brune and Schink 1990; Reichenbecher *et al* 1994; Reichenbecher and Schink 1999; Haddock and Ferry 1993). In experiments carried out in this study, addition of 1,2,3,5-tetrahydroxybenzene to the bacterial growth media did not appear to promote degradation of pyrogallol by *S. caprinus'*. No growth inhibition was observed when either *S. caprinus* or *S. bovis* were grown in the presence of pyrogallol indicating that the monomeric product of the breakdown of tannic acid is far less toxic than the intact hydrolysable tannin, a result which is in agreement with the findings of Field and Lettinga (1992) and Haslam and Lilley (1986).

5.3.7 The condensed tannin conundrum

No breakdown products were detected by GLC analysis of supernatant samples of *S. caprinus* grown in the presence of condensed tannin. This suggested that the mechanism(s) by which *S. caprinus* hydrolysed tannic acid and gallic acid, was not necessarily the same as those used to degrade/tolerate the condensed tannins. This result may reflect the fundamental differences in chemical structure between the two classes of tannins. Hydrolysable tannins, such as tannic acid, consist of a central carbohydrate core such as glucose esterified to a number of phenolic acid residues,

Figure 5.17 Transhydoxylase-catalysed reaction in *P. acidigallici*



The transfer of a hydroxyl group from a hydroxyl donor (1,2,3,5-tetra-hydroxybenzene) to pyrogallol results in the regeneration of the co-substrate (Haddock and Ferry 1993).

whereas condensed tannins contain no central carbohydrate core nor ester bonds and consists of polymers of flavonols linked by carbon-carbon bonds. The ability of *S. caprinus* to tolerate the presence of high concentrations of condensed tannins in its growth medium (Chapter 3) suggested that there were other mechanisms employed by the bacterium to overcome the detrimental effects of tannins.

Chapter 6

Protection against tannins

Chapter 6 Protection against tannins

6.1 Introduction

Experiments reported in the previous chapter used GLC analysis to demonstrate *S. caprinus'* ability to hydrolyse tannic acid and gallic acid to produce pyrogallol. However, no breakdown products were detected following growth of the bacteria in the presence of condensed tannin. As in the case of tannic acid, *S. caprinus* has been shown to tolerate high concentrations of condensed tannin in its growth medium (Chapter 3). These results suggested that *S. caprinus* might possess another mechanism that enables it to cope with the presence of condensed tannin in its environment.

In a study by Jones *et al* (1994), microscopic examination of bacterial cells grown in the presence of condensed tannins, showed that at higher concentrations of condensed tannins, the appearance of the cells changed. The bacteria were more gram variable and some lysis and escape of cell contents was visible. Stack and Hungate (1984) demonstrated that hydroxy-cinnamic acid (HCA)-grown *R. albus* 8 cells showed a surface structure different from that of cells grown without the phenolic acid. HCA appears to increase capsular size concomitant with increased cellulolytic activity and the authors suggested that phenolic monomers may play a role in altering cell metabolism. These reports suggest that while little is known of the affinity of microbial cell surfaces for condensed tannins, they clearly represent potential binding sites.

In this chapter, morphological characterisation of *S. caprinus* after incubation in the presence of condensed tannin or tannic acid was explored in detail. Initially, phase-contrast microscopy was used to monitor changes in cellular morphology as tannin concentrations were increased, followed by electron microscopy to examine the ultrastructure of the cells. *S. bovis* was included as a control.

For the sake of expediency in presenting this data, where no differences in morphological changes were observed between tannic acid and condensed tannin, the term 'tannin' is used to define both the hydrolysable and the condensed tannin. Any differences observed between the two tannins are stated.

6.2 Results

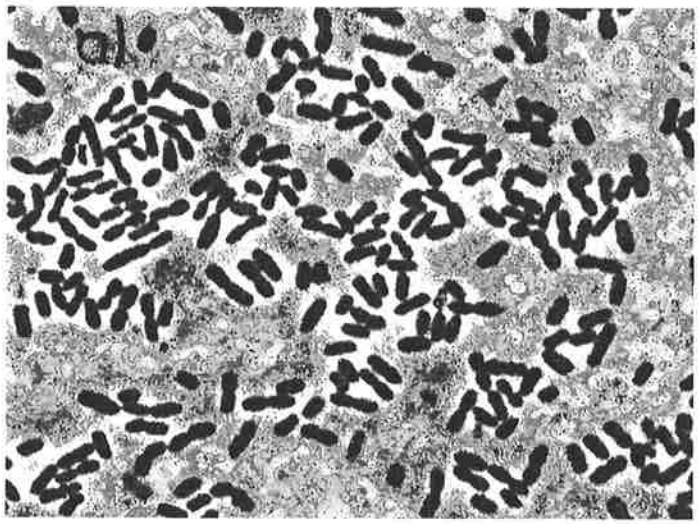
6.2.1 Light microscopy

Phase contrast microscopic examination of purified colonies of *S. caprinus* and *S. bovis* grown in mBHI medium, showed homogeneous cocci, occurring mainly in pairs or short chains (Figure 6.1a). Both bacteria stained Gram positive.

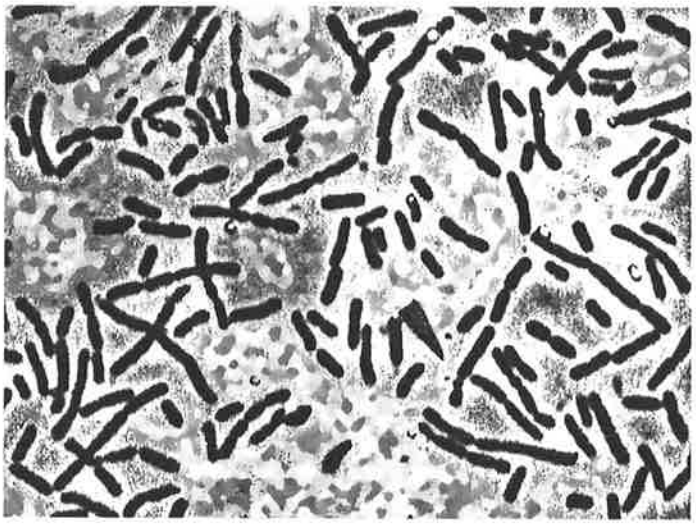
A change in morphology was visualised when the bacteria were grown in the presence of either condensed tannin or tannic acid. An increase in chain formation was detected in cultures of *S. bovis* when the concentration of tannin in the medium was greater than 0.2% w/v. In cultures of *S. caprinus*, an increase in chain formation was not observed until the concentration of tannin exceeded 1.5% w/v (Figure 6.1b). *S. bovis* cells also tended to form clumps in the presence of the tannins. In both cases, Gram stains became variable with the addition of tannin. Neither bacteria displayed

Figure 6.1 **Light micrographs of *S. caprinus***

(a) no tannic acid



(b) 2.0% tannic acid



Light micrographs of *S. caprinus* following growth in mBHI medium containing (a) no tannic acid and (b) 2% w/v tannic acid. Note the increased chain formation in the presence of tannic acid. Magnification x 400.

any capsular material in the presence or absence of tannin when stained with India ink or using the Anthony method.

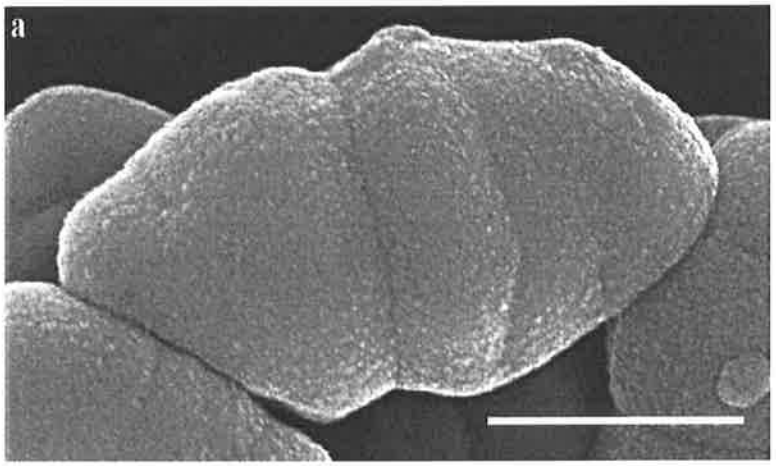
The presence of simple phenolic acids in the growth medium appeared to have no effect on the morphology of either *S. caprinus* or *S. bovis* when viewed at the light microscopy level.

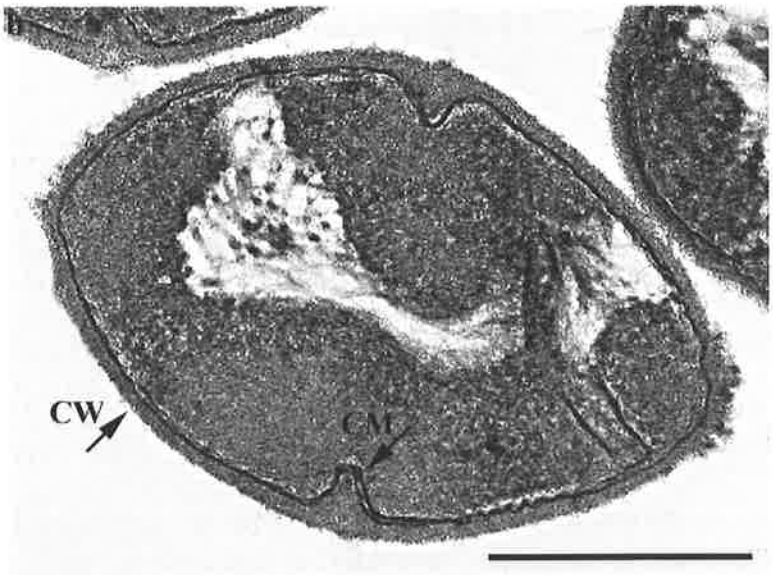
6.2.2 Electron microscopy

Transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) of *S. caprinus* and *S. bovis* grown in mBHI medium in the absence of tannin showed both bacterial species to be ovoid (elongated) cocci. Dimensions were approximately 1.8 μm long by 1.0 μm wide (Figure 6.2a and b). The bacteria possessed the typical Gram positive morphology with a bilayered cytoplasmic membrane and the cell wall consisting of a single, think, continuous layer of approximately 50 nm thick (Figure 6.2b).

As observed under light microscopy, an increase in chain formation and clumping was observed in TEM preparations of *S. bovis* cells when levels of tannin exceeded 0.2% w/v. However, differences where also detected in the size and the shape of the cells. With an increase in the concentration of tannin up to 0.75% w/v, an increase in abnormally shaped cells was observed (Figure 6.3a) and cells appeared more susceptible to lysing, with a greater number of lysed cells in any field of view. Some cells also appeared to be undergoing unusual or incomplete cell division, with multiple divisional planes observed at the same time (Figure 6.3b). Measurement of

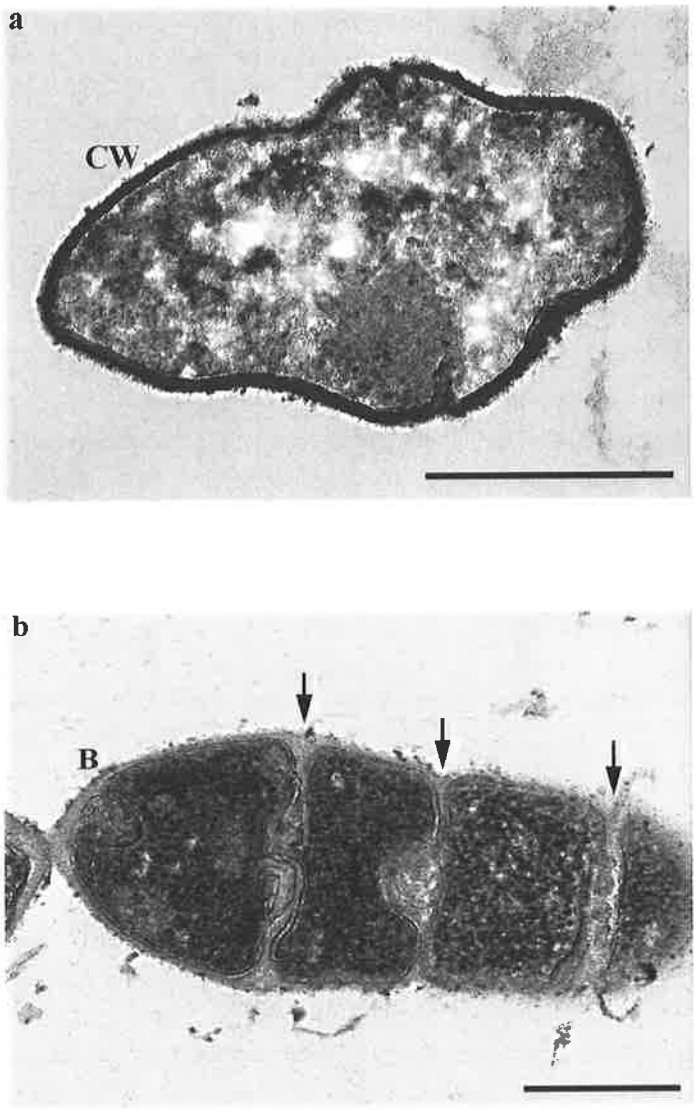
Figure 6.2 Electron micrographs showing size and shape of *S. caprinus*





Field emission electron micrograph and transmission electron micrograph of *S. caprinus* following growth in mBHI medium. 50 bacteria were selected and their cross sectional dimensions determined using Adobe Photoshop. CW: cell wall; CM: cell membrane. Bar = 0.5 µm.

Figure 6.3 **Transmission electron micrographs of *S. bovis* following growth in mBHI medium containing 0.5% tannic acid**



Transmission electron micrographs of *S. bovis* following growth in the presence of 0.5% w/v tannic acid showing (a) abnormal shaped cell and (b) multiple divisional planes. Bacterial cells were fixed in glutaraldehyde and dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. CW: cell wall; B: blebbing on the surface of the cell. Arrows indicate divisional planes. Bar = 0.5 μm.

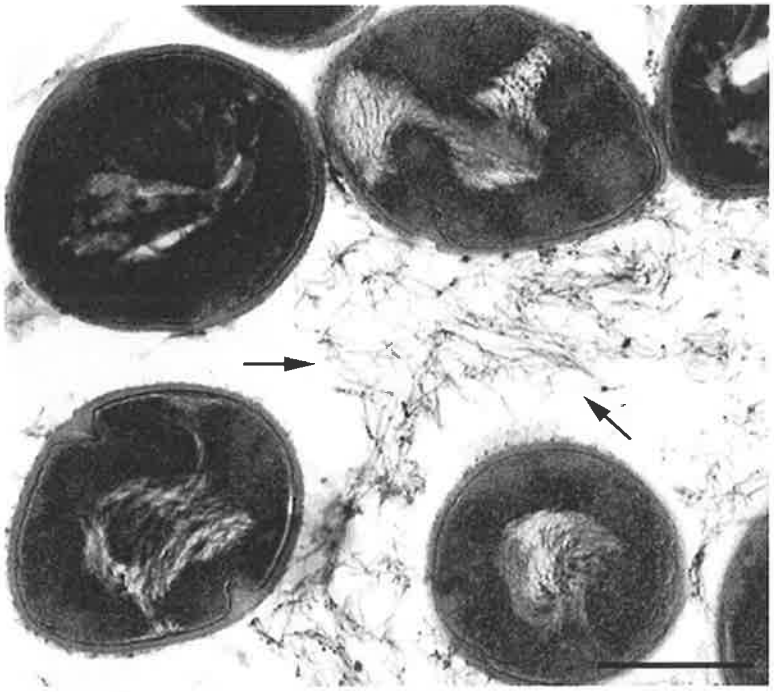
the diameter and length of cells, indicated that the presence of tannin caused a proportional reduction in their size. At a tannin concentration of 0.5% w/v, *S. bovis* cells measured, on average, 1.4 μm x 0.5 μm. The cell wall structure also appeared to change and become thinner with an increase in tannin (approximately 15 to 25 nm). Blebs were also visible on the cell surface in the presence of tannins (Figure 6.3b)

The size and shape of *S. caprinus* cells appeared unaffected by the presence of condensed tannin or tannic acid until concentrations reached 2.5 and 3.0% w/v respectively. Concentrations greater than these amounts produced similar changes to those observed in *S. bovis* cells, although no abnormal cell division was detected and minimal cell lysis was observed. TEM also revealed the presence of what appeared to be fibrous material extending from the surface of *S. caprinus* following growth of the bacteria in the presence of tannin (Figure 6.4a to d). The addition of ruthenium red to the EM preparation procedure visually enhanced the extracellular material and exposed its presence surrounding *S. caprinus* in the absence of tannic acid (Figure 6.5a and b) and presence of condensed tannin (Figure 6.6). In contrast, TEM preparations containing ruthenium red of cells grown in the presence of tannins showed little fibrous extracellular material surrounding *S. bovis* (Figures 6.7 and 6.8).

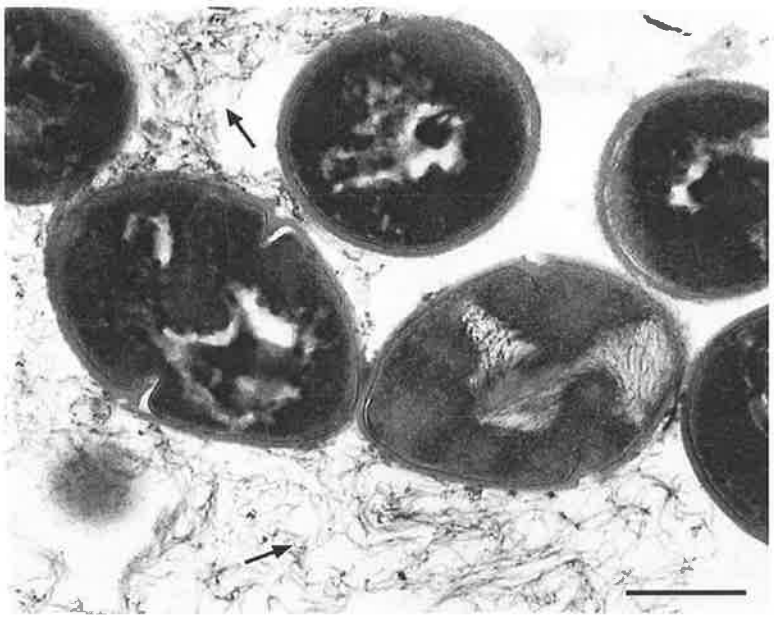
FESEM analysis demonstrated the extracellular material to be globular in structure. In the absence of tannin and in the presence of low concentrations of tannin (<0.5% w/v), this material appeared to be present in patches on the surface of *S. caprinus* (Figure 6.9a and b). No surface material was evident in preparation of *S. bovis* (Figure 6.10). However, with the addition of 0.5% w/v tannin to the growth medium,

Figure 6.4 Transmission electron micrographs of *S. caprinus*

(a) 0.5% tannic acid



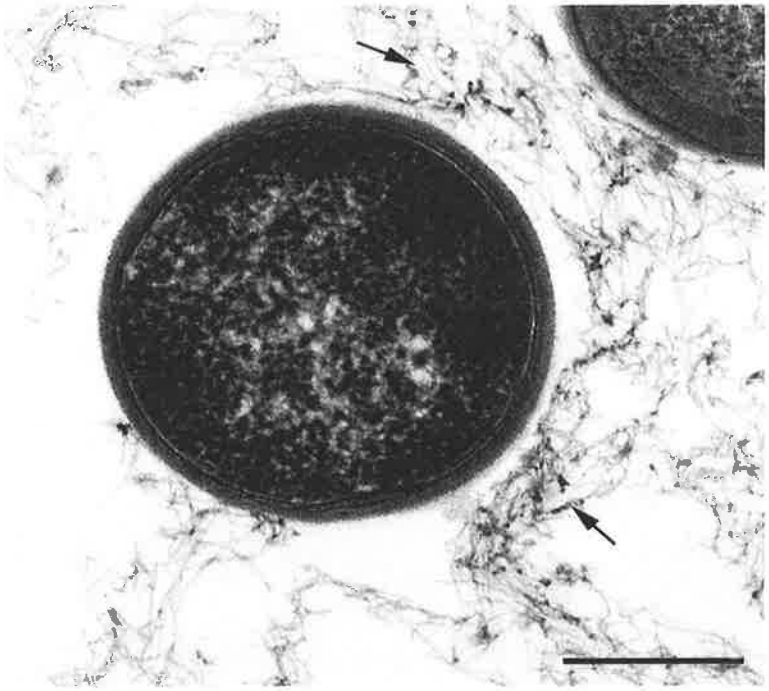
(b) 2.0% tannic acid



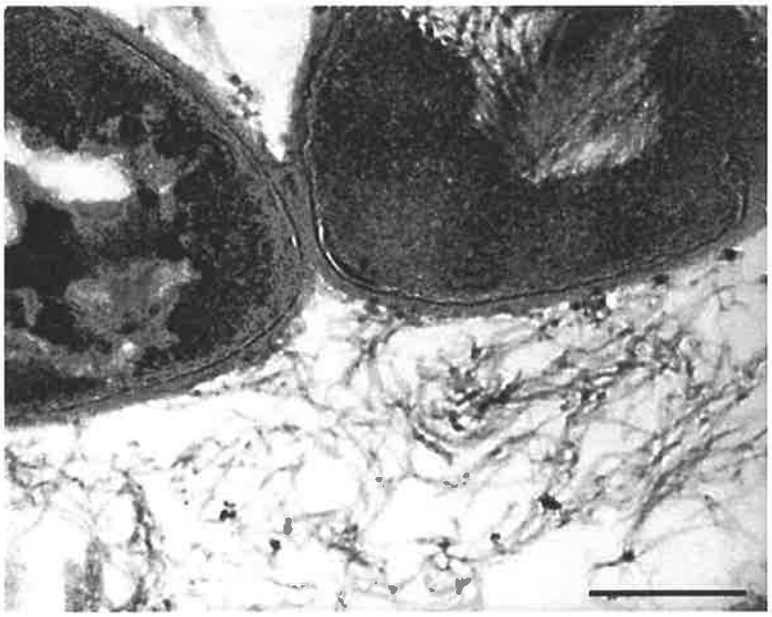
Transmission electron micrographs showing *S. caprinus* following overnight growth in mBHI medium containing (a) 0.5% and (b) 2.0% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. Arrows indicate extracellular material. Bar = 1.0 μm.

Figure 6.4 Transmission electron micrographs of *S. caprinus*

(c) 3.0% tannic acid



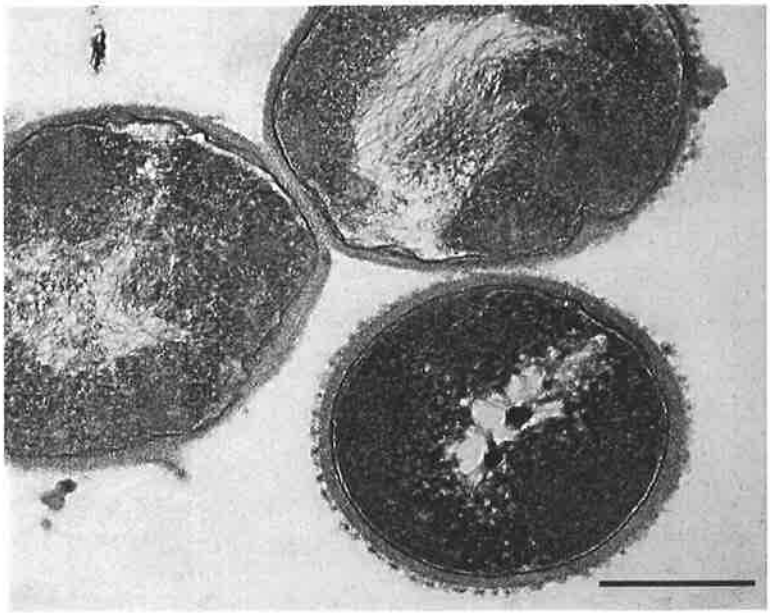
(d) 4.0% tannic acid



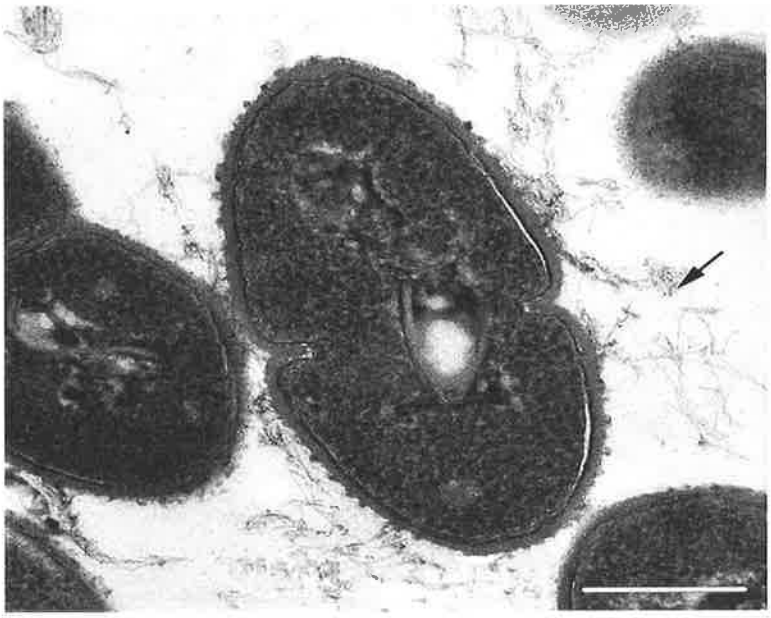
Transmission electron micrographs showing *S. caprinus* following overnight growth in mBHI medium containing (c) 3.0% and (d) 4.0% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. Arrows indicate extracellular material. Bar = 0.5 and 0.2 μm respectively.

Figure 6.5 **TEM showing the effect of ruthenium red addition on extracellular material surrounding *S. caprinus***

(a) no ruthenium red

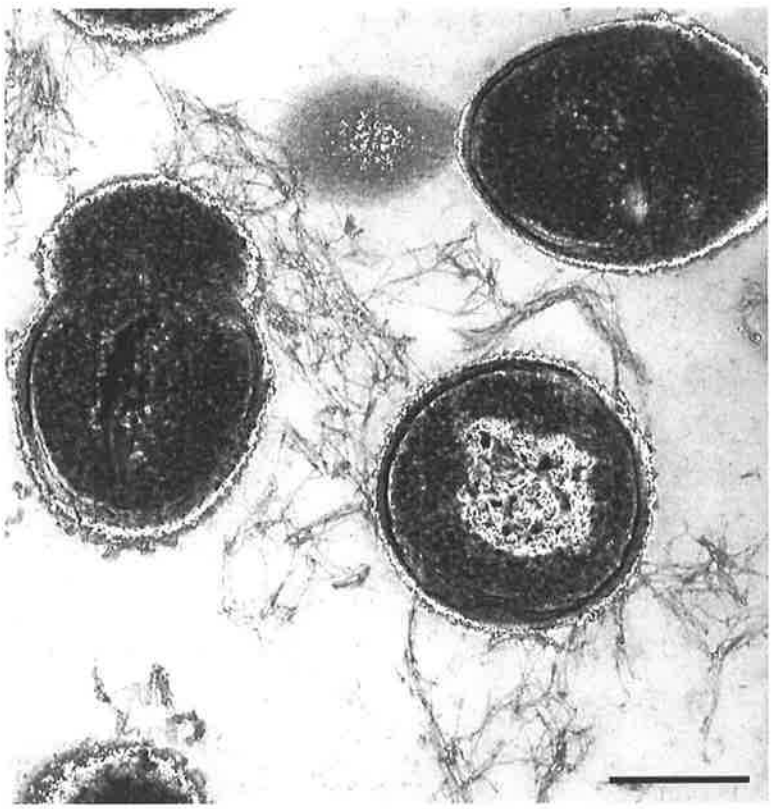


(b) ruthenium red



Transmission electron micrographs showing *S. caprinus* following overnight growth in mBHI medium. Bacterial cells were fixed in (a) glutaraldehyde and (b) glutaraldehyde containing ruthenium red. The cells were dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. Arrows indicate extracellular material. Bar = 1.0 μm.

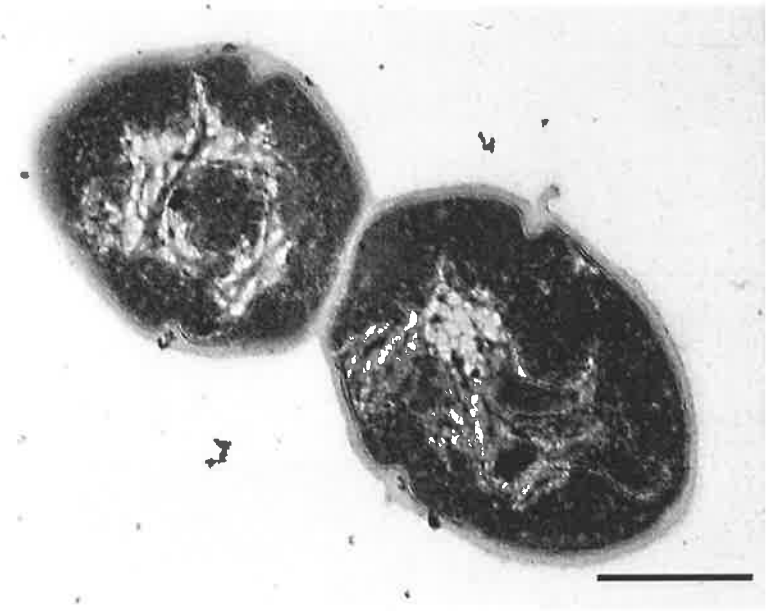
Figure 6.6 **Transmission electron micrograph of *S. caprinus* following growth in mBHI medium containing 1.0% w/v condensed tannin**



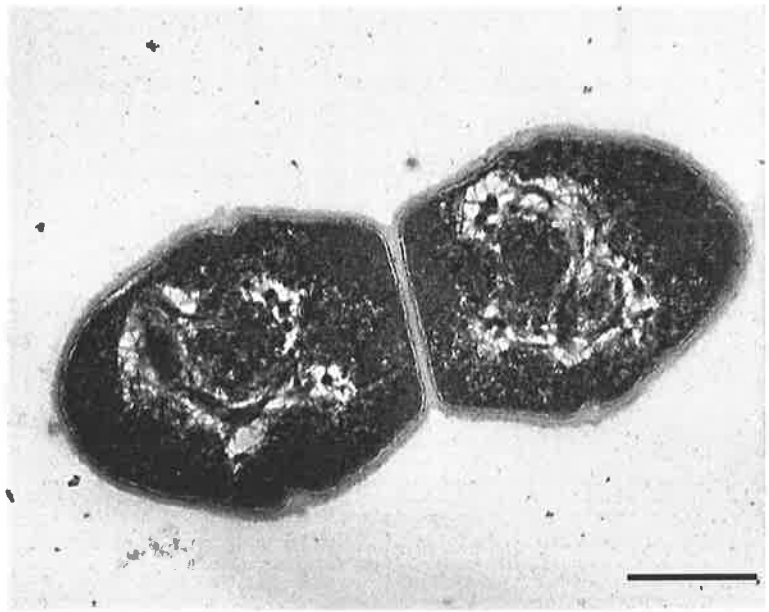
Transmission electron micrograph showing *S. caprinus* following overnight growth in mBHI medium containing 1.0% w/v condensed tannin. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. Bar = 0.5 μm.

Figure 6.7 Transmission electron micrographs of *S. bovis*

(a) no tannic acid

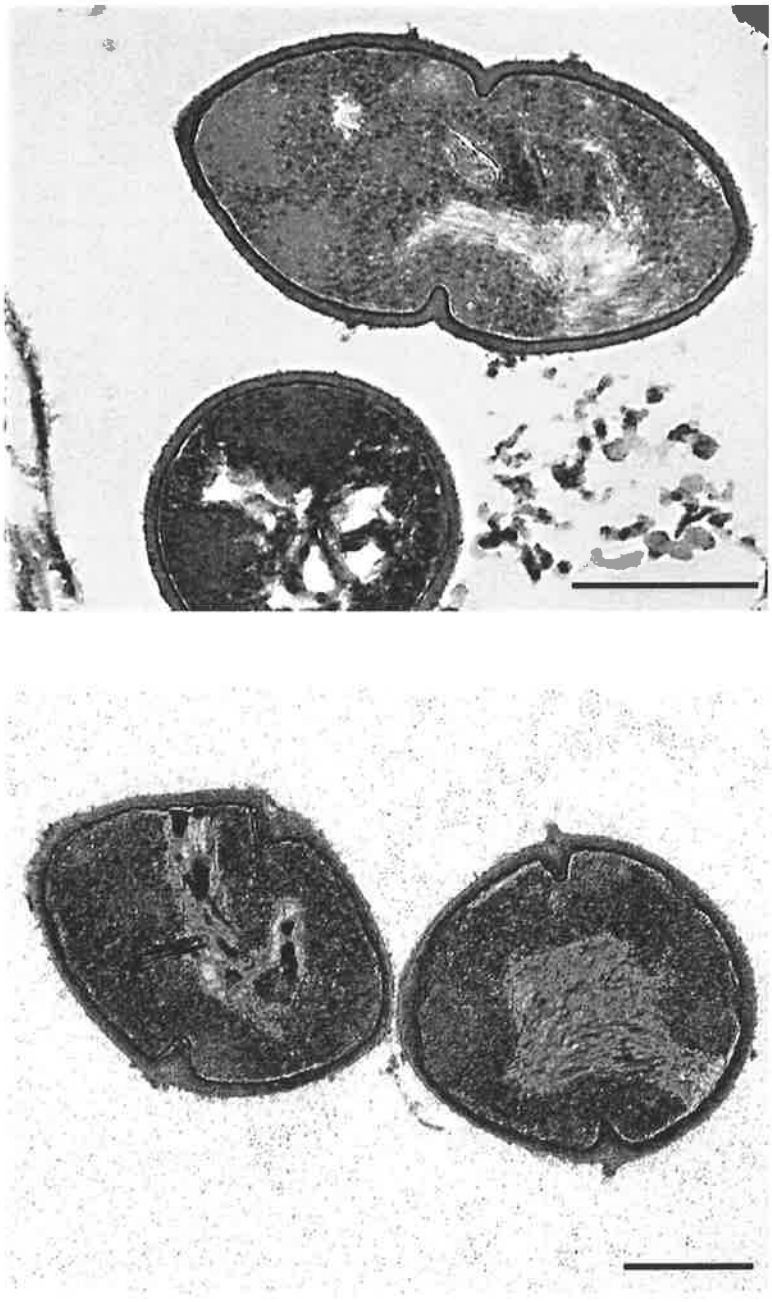


(b) 0.2% tannic acid



Transmission electron micrographs showing *S. bovis* following overnight growth in mBHI medium containg (a) no tannic acid and (b) 0.2% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. Bar = 1.0 μm.

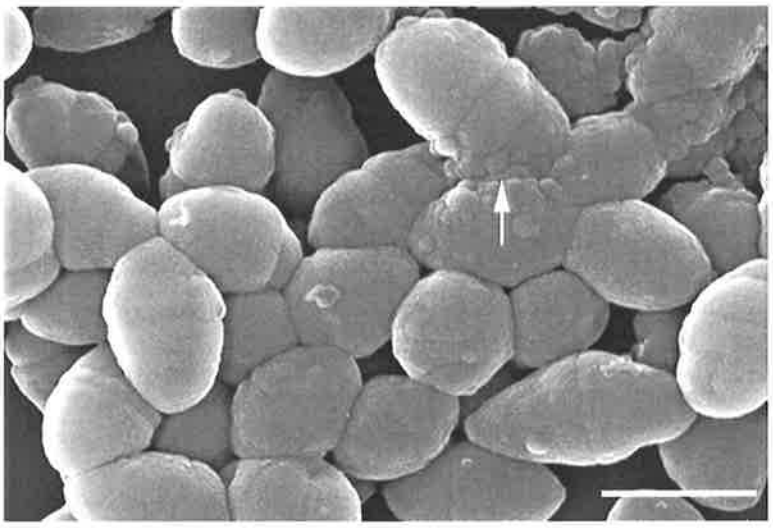
Figure 6.8 **Transmission electron micrographs of *S. bovis* following growth in mBHI medium containing 0.5% w/v tannic acid**



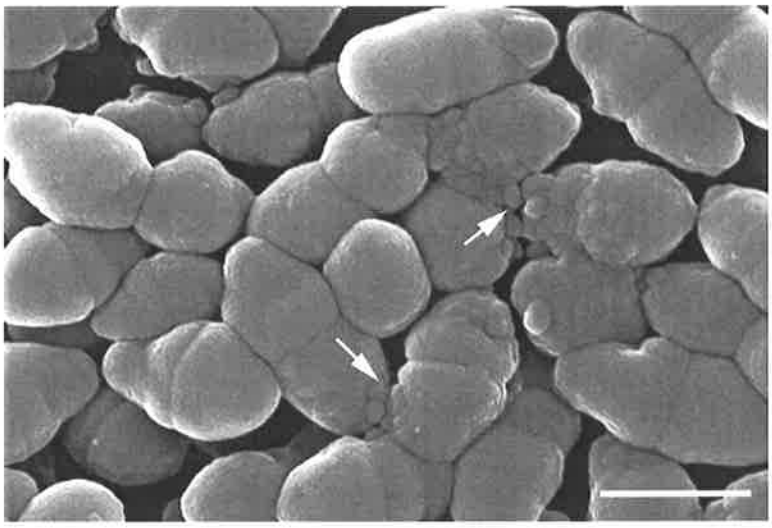
Transmission electron micrographs showing *S. bovis* following overnight growth in mBHI medium containing 0.5% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before embedding in Spurrs resin. Thin sections were cut and stained with uranyl acetate and lead citrate and viewed under a Phillips CM100 TEM. Bar = 0.5 μm.

Figure 6.9 **Field emission scanning electron micrographs of *S. caprinus* following growth in the presence of tannic acid**

(a) no tannic acid



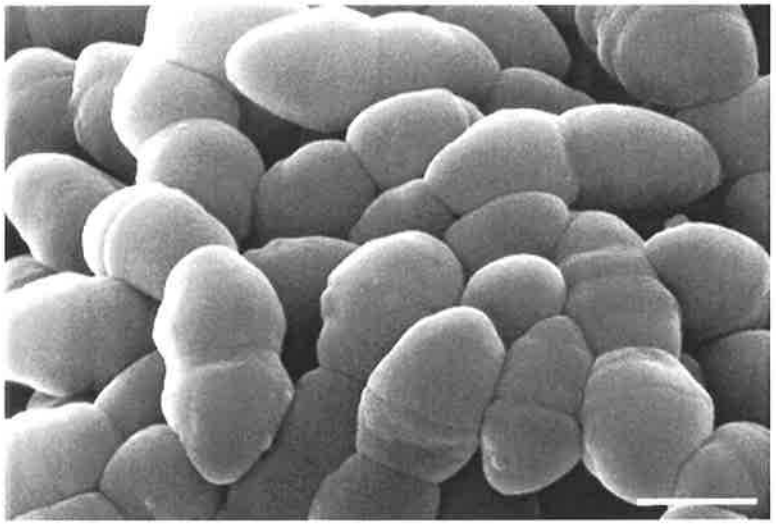
(b) 0.5% tannic acid

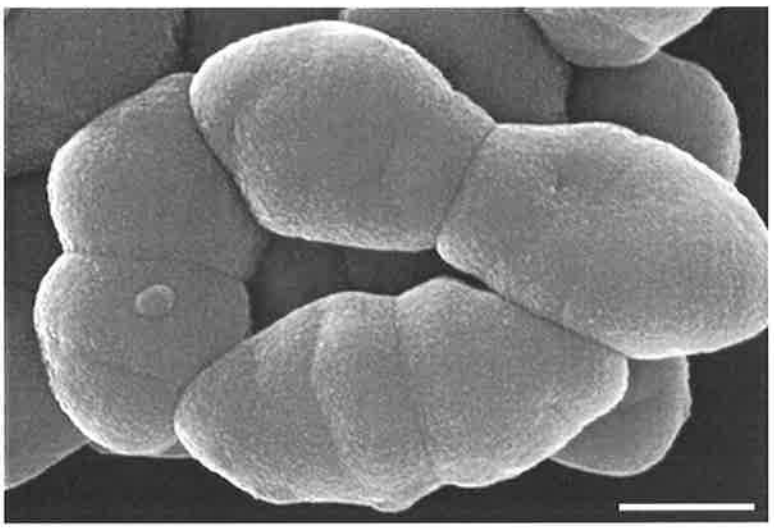


Field emission scanning electron micrographs showing *S. caprinus* following overnight growth in mBHI medium containing (a) no tannic acid and (b) 0.5% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before infiltration with Peldri II and gold-palladium-carbon coating. Samples were viewed under a Phillips XL30 FESEM. Arrows indicate extracellular material. Bar = 1.0 μm.

Figure 6.10 **Field emission scanning electron micrographs of *S. bovis* following growth in the presence of tannic acid**

(a) no tannic acid

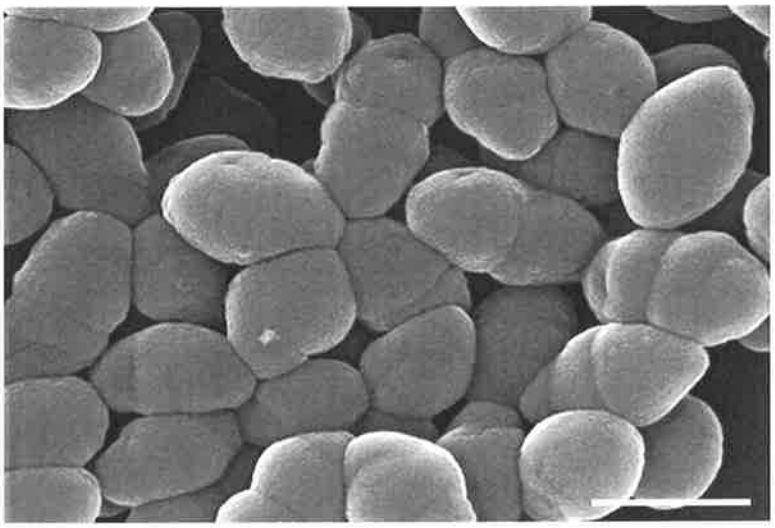




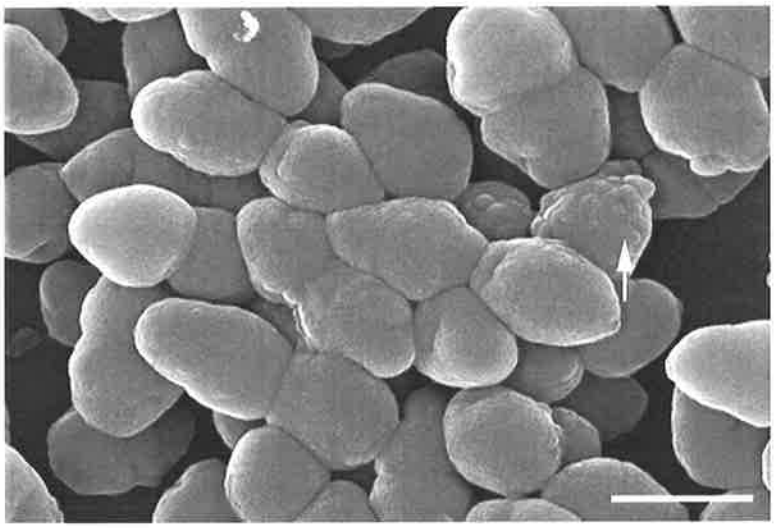
Field emission scanning electron micrographs showing *S. bovis* following overnight growth in mBHI medium. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before infiltration with Peldri II and gold-palladium-carbon coating. Samples were viewed under a Phillips XL30 FESEM. Bar = 1.0 and 0.5 μm respectively.

Figure 6.10 **Field emission scanning electron micrographs of *S. bovis* following growth in the presence of tannic acid**

(b) 0.2% tannic acid



(c) 0.5% tannic acid



Field emission scanning electron micrographs showing *S. bovis* following overnight growth in mBHI medium containing (b) 0.2% and (c) 0.5% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before infiltration with Peldri II and gold-palladium-carbon coating. Samples were viewed under a Phillips XL30 FESEM. Arrow indicates extracellular material.
Bar = 1.0 μm.

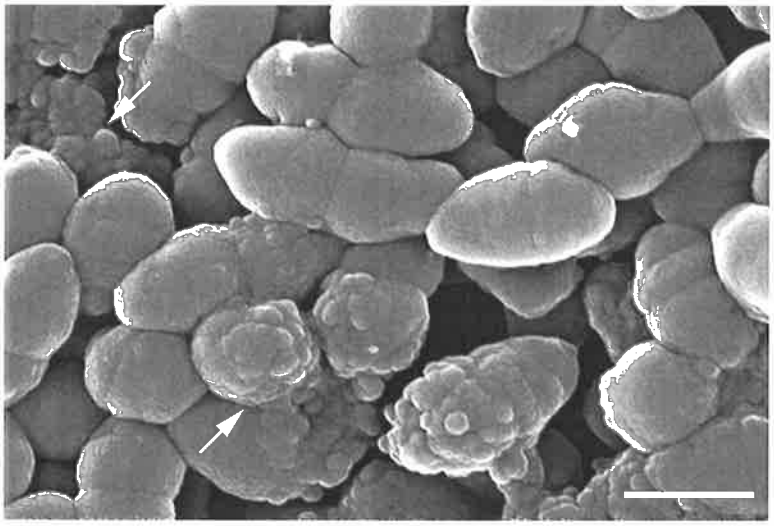
a small amount of extracellular material was observed in patches on the surface of *S. bovis* (Figure 6.10a to c). The addition of increasing concentrations of tannin to *S. caprinus'* growth medium resulted in a substantial increase in extracellular material surrounding the bacteria and at concentrations of tannin exceeding 2% w/v, the extracellular material completely encased *S. caprinus* (Figure 6.11a to d). At these levels of tannin, *S. bovis* could not grow.

6.2.3 Characterisation of extracellular material

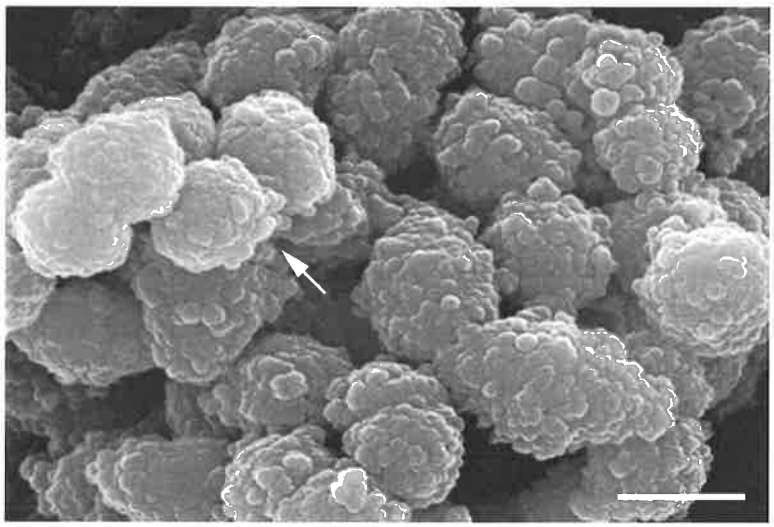
Removal of the extracellular material surrounding the bacteria was achieved through sodium acetate extraction (confirmed by EM). Surprisingly, in light of the electron microscopic analysis, the results indicated that both bacterial species produced extracellular material in similar amounts following growth in the absence of tannins. The average yield of crude extracellular material isolated from *S. caprinus* was approximately 0.95 ± 0.12 mg/mg cells (dry weight, following removal of extracellular material) compared with 0.8 ± 0.06 mg/mg cells isolated from cultures of *S. bovis*. In both bacterial species the amount of material isolated from cells growing on solid media was greater than that isolated from those grown in liquid cultures. The extracellular material had a molecular weight similar to that of blue dextran (2×10^6) and was principally found to be closely associated with the bacterial cell surface (approximately 74.6%) although a portion was secreted into the media. A comparison of the extracellular material obtained from *S. caprinus* and *S. bovis* is displayed in Table 6.1. Colorimetric assays indicated that the material was composed predominantly of carbohydrate. However, analysis of the alditol acetate derivatives of the hydrolysates by GLC and GLC-MS indicated that the neutral sugar composition of

Figure 6.11 **Field emission scanning electron micrograph of *S. caprinus* following growth in the presence of high concentrations of tannic acid**

(a) 1.0% tannic acid



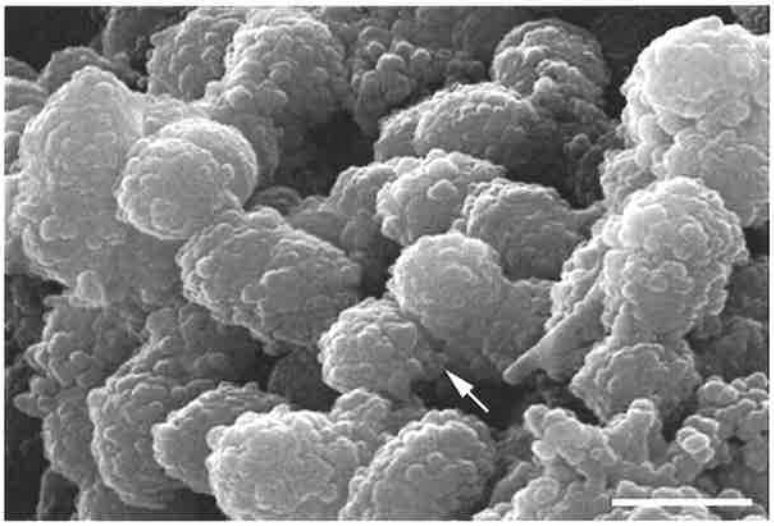
(b) 2.0% tannic acid



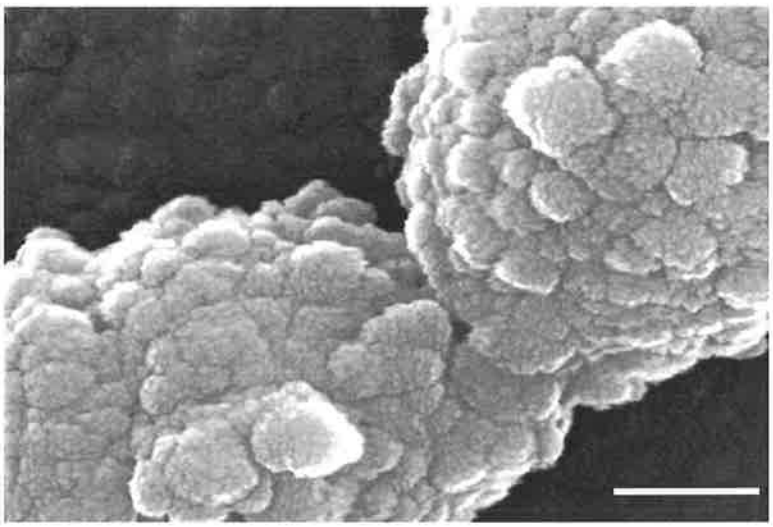
Field emission scanning electron micrographs showing *S. caprinus* following overnight growth in mBHI medium containing (a) 1.0% and (b) 2.0% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before infiltration with Peldri II and gold-palladium-carbon coating. Samples were viewed under a Phillips XL30 FESEM. Arrows indicate extracellular material. Bar = 1.0 μm.

Figure 6.11 **Field emission scanning electron micrograph of *S. caprinus* following growth in the presence of high concentrations of tannic acid**

(c) 3.0% tannic acid



(d) 4.0% tannic acid



Field emission scanning electron micrograph showing *S. caprinus* following overnight growth in mBHI medium containing (c) 3.0% and (d) 4.0% w/v tannic acid. Bacterial cells were washed in CE buffer, fixed in glutaraldehyde containing ruthenium red and dehydrated through a graded ethanol series before infiltration with Peldri II and gold-palladium-carbon coating. Samples were viewed under a Phillips XL30 FESEM. Arrow indicates extracellular material. Bar = 1.0 and 0.2 μm respectively.

Table 6.1 **Quantitative analysis of extracellular material isolated from *S. caprinus* and *S. bovis***

Component	Composition of extracellular material (% w/v)	
	S. caprinus	*S. bovis*
Carbohydrate (total)	93.7 ± 5.56	87.3 ± 9.29
Uronic acids	nd (2.1 ± 0.9)[a]	2.7 ± 1.2 (5.4 ± 1.5)[a]
Acyl/N-acyl residues	0 - 4.2[b]	0 - 7.3[b]
Hexosamines	nd	nd
Protein	nd	nd
Pyruvate	nd	nd

Extracellular material was extracted from bacterial cells grown in mBHI medium or mBHI medium containing 0.5% w/v tannic acid. The material was purified and the composition determined as described in the text (2.33). The numbers are the means of triplicate assays ± standard error and are expressed as a percentage of the total.

[a] determined following growth of the bacteria in the presence of 0.5% tannic acid

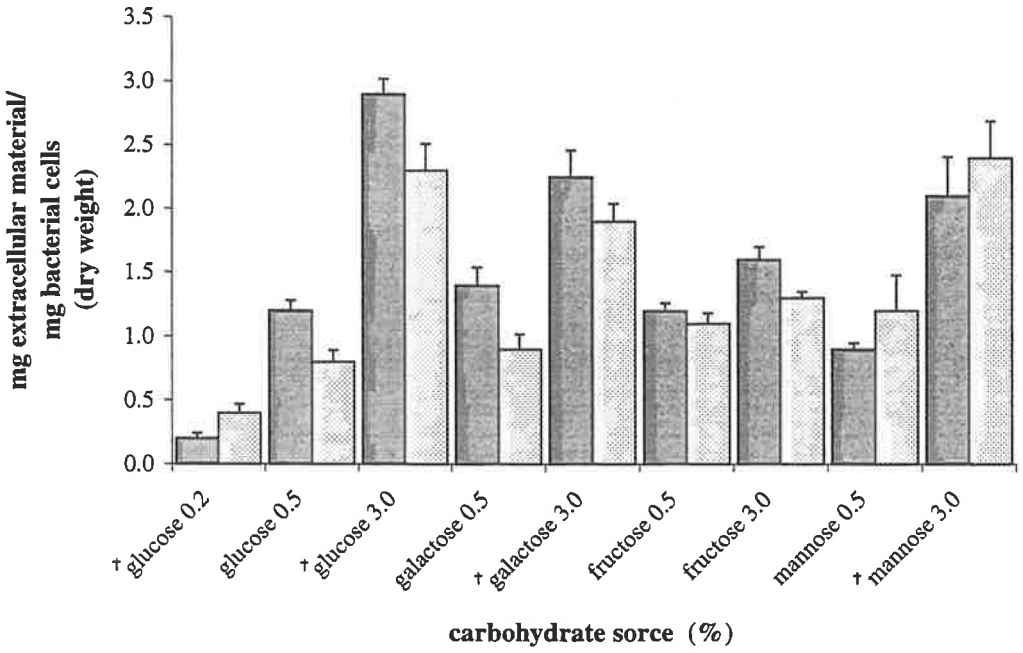
[b] variable

nd: not detected

the material surrounding the *S. caprinus* and *S. bovis* was different. In *S. caprinus*, the neutral sugar composition was primarily glucose with trace amounts of mannose (ratio glucose:mannose 1:0.2). Variable amounts of acyl and N-acyl residues were also detected (0 to 4.2%). No uronic acids, proteins or hexosamines were found. In *S. bovis*, the neutral sugar composition was shown to consist of mannose, glucose and galactose in the ratio of 1: 0.7: 0.2. Larger amounts of acyl and N-acyl groups were detected (0 to 7.3%) and uronic acids were also found (2.7 ± 1.2%). When the bacteria were grown in the presence of tannin, no change in the carbohydrate composition was determined although the amount of uronic acids appeared to increase in both *S. caprinus* and *S. bovis*, ie 2.1 ± 0.9% and 5.4 ± 1.5% respectively (Table 6.1).

The effect of increasing or altering the carbon source on the production of extracellular material by *S. caprinus* and *S. bovis* is shown in Figure 6.12. An increase in glucose concentration from 0.5% to 3.0% w/v in NB medium resulted in greater yields of extracellular material harvested from both bacteria following 24 h growth (significant, $P<0.05$). Conversely, limiting the carbon source (glucose, 0.2%) resulted in minimal polysaccharide production. No significant differences were detected when galactose, fructose or mannose replaced glucose at a concentration of 0.5% w/v. Increasing the concentration of these carbon sources to 3% w/v resulted in a proportional increase in the amount of polysaccharide produced by the cells (significant, $P<0.05$). The composition of extracellular material isolated from the bacteria was not affected by the change in carbon source.

Figure 6.12 **Effect of carbon source on the production of extracellular material by *S. caprinus* and *S. bovis***



S. caprinus ▨ *S. bovis* ⊡

EPS was isolated from *S. caprinus* and *S. bovis* following 48 h growth on NB agar plates containing the carbon source listed. The amount of EPS produced by the cells was expressed as glucose equivalents and correlated to the dry weight of the cells. Data shown represents the average of triplicate trials; bars represent the standard error. † significantly different to control (0.5% glucose), $P<0.05$.

Time course experiments revealed that the extracellular material was produced at the end of the logarithmic phase and during the stationary phase of bacterial growth (Figure 6.13).

6.2.4 Effect of tannin on the production of extracellular material

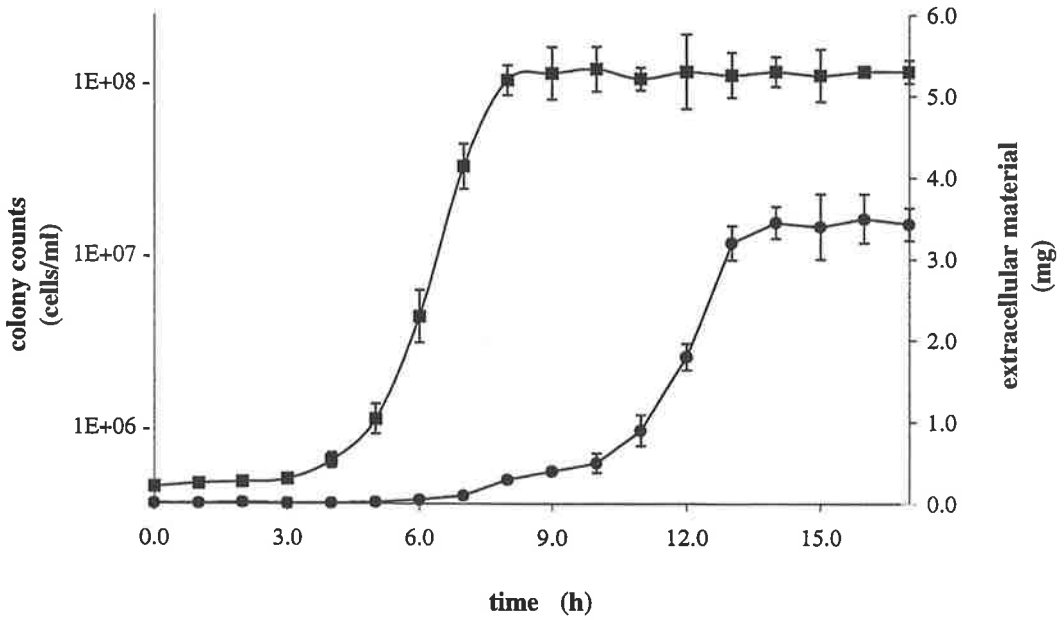
With the addition of concentrations greater than 0.5% w/v tannic acid or condensed tannin to the bacterial growth medium, yields of extracellular material extracted from cultures of *S. caprinus* were found to increase significantly ($P<0.05$, Figure 6.14 and 6.15 respectively). Compared to the control (no tannic acid), a concentration of 5% w/v tannic acid caused a 6-fold increase in the amount of extracellular material produced by *S. caprinus*. A 2% w/v inclusion of condensed tannin in the growth medium resulted in a 2.5-fold increase. At concentrations of tannin greater than 0.5%, growth of *S. bovis* was inhibited and at levels below this, no apparent change in polysaccharide production was detected.

Removal of the extracellular material surrounding *S. caprinus* using sterile sodium acetate extraction before inoculation into mBHI medium containing increasing concentrations of tannic acid resulted in an increase in the lag period of the bacteria (significant compared to the control, ie no removal of the extracellular material, $P<0.1$, Figure 6.16).

The addition of other phenolic acids, such as gallic acid, p-coumaric acid and ferulic acid to the bacterial growth medium appeared to have no significant effect on the amount of extracellular material produced by either *S. caprinus* or *S. bovis*.

Co-culture of *S. caprinus* in the presence of tannic acid and growth-limiting concentrations of other phenolic acids (determined from results displayed in Figure 3.9) did not alter the bacteria's tolerance to these phenolic acids (results not shown).

Figure 6.13 Production of extracellular material by *S. caprinus*

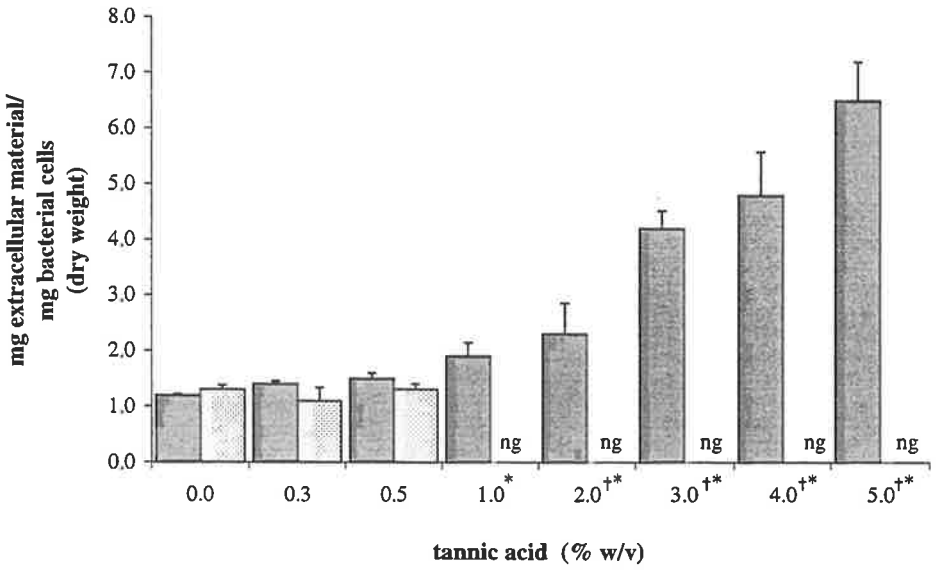


Growth in mBHI medium ■

Corresponding amount of extracellular material produced ●

S. caprinus was incubated in mBHI medium supplemented with 1.0% w/v tannic acid. At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count (left axis). The corresponding amount of extracellular material produced by the bacteria was determined through colorimetric analysis and expressed as glucose equivalents (right axis). Points represent the average of triplicate trials; bars represent the standard error.

Figure 6.14 **Effect of tannic acid on the production of extracellular material by *S. caprinus* and *S. bovis***



S. caprinus ▨ *S. bovis* ⊡

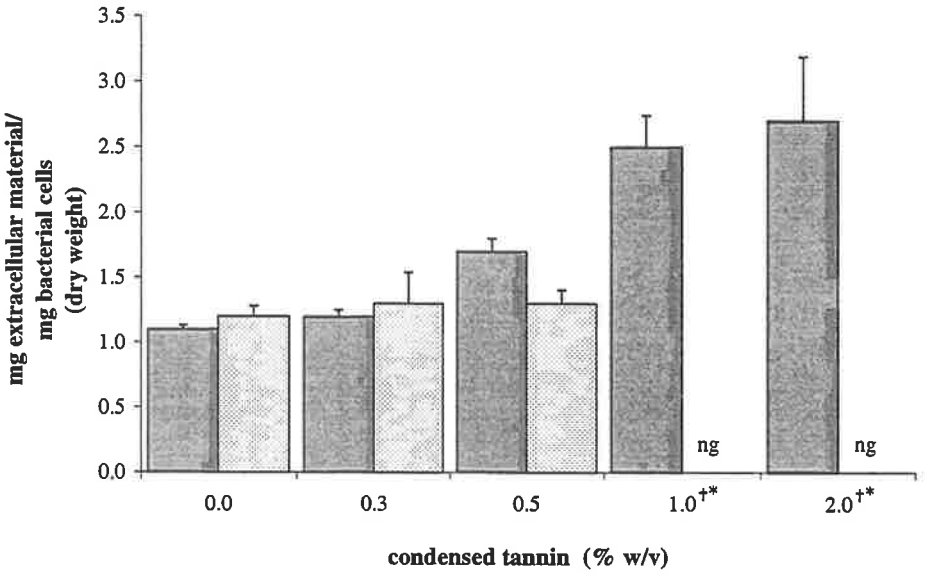
Extracellular material was isolated from *S. caprinus* and *S. bovis* following 48 h growth in mBHI medium supplemented with increasing concentrations of tannic acid. Tannic acid was removed by precipitation with 1% PVP and extensive dialysis against CE buffer. The material was partially purified as described in 2.33 and the amount of extracellular material produced by the bacteria (expressed as glucose equivalents) was determined and correlated to the dry weight of the cells. Data shown represents the average of triplicate trials; bars represent the standard error.

ng: no growth

† Significantly different from control (no tannic acid), $P<0.05$

* Significantly different between bacteria, $P<0.01$

Figure 6.15 **Effect of condensed tannin on the production of extracellular material by *S. caprinus* and *S. bovis***



S. caprinus ▨ *S. bovis* ⊡

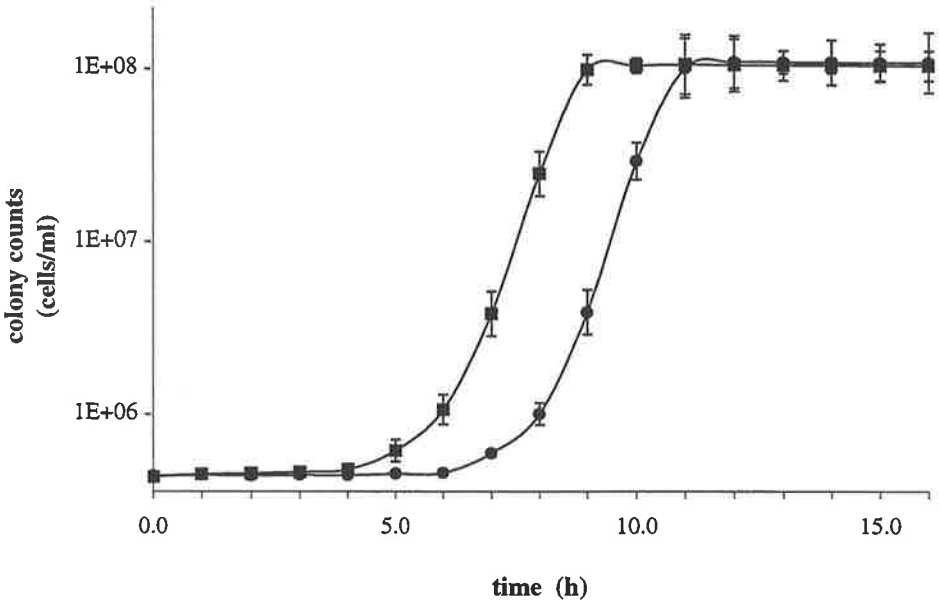
Extracellular material was isolated from *S. caprinus* and *S. bovis* following 48 h growth in mBHI medium supplemented with increasing concentrations of tannic acid. Tannic acid was removed by precipitation with 1% PVP and extensive dialysis against CE buffer. The material was partially purified as described in 2.33 and the amount of extracellular material produced by the bacteria (expressed as glucose equivalents) was determined and correlated to the dry weight of the cells. Data shown represents the average of triplicate trials; bars represent the standard error.

ng: no growth

† Significantly different from control (no tannic acid), $P<0.05$

* Significantly different between bacteria, $P<0.01$

Figure 6.16 **Growth of *S. caprinus* in mBHI medium containing tannic acid following removal of extracellular material**



Growth of *S. caprinus* ■

Growth of *S. caprinus* following removal of extracellular material ●

S. caprinus was incubated in mBHI medium supplemented with tannic acid (1.0% w/v). Following 48 h growth the culture was divided into two. Extracellular material was removed from *S. caprinus* cells from one half before the cells were inoculated into fresh mBHI medium containing 1.0% w/v tannic acid. Cells present in the second half were washed in CE buffer before re-inoculation (control). At regular time intervals, aliquots were removed and growth was determined on mBHI by serial dilution and viable cell count. Points represent the average of triplicate trials; bars represent the standard error.

6.3 Discussion

6.3.1 Microscopic analysis

Previous reports have described changes in the morphology of bacterial cells following growth in the presence of various phenolic acids (Stack and Hungate 1984; Jones *et al* 1994). As shown by results presented in this study, the presence of condensed tannin or tannic acid in the bacterial growth medium also caused morphological changes to the cells. However, *S. bovis* appeared to be more affected by the presence of tannins than *S. caprinus*. Both bacterial species responded to the presence of tannins by a reduction in size as well as a reduction in cell wall thickness compared with the normal 30 to 70 nm Gram positive wall (Tortora *et al* 1992). This thinness may account for the variable Gram staining observed following growth of the bacteria in the presence of tannins as the Gram stain complex would not be retained by the thin wall structure (Beveridge and Graham 1991). The thin cell wall may also explain the increased cell lysis observed. The cells also displayed extensive chain formation and flocculation. However, in *S. bovis*, the chains were composed of elongated and irregularly shaped cells resulting from the initiation of multiple divisional planes and failure of the daughter cells to separate. These results suggested that the bacterial cell surface was not the only target for the action of tannins but that tannins interfered with normal cell division. This proposal is in agreement with Jones *et al* (1994) who reported similar responses for cultures of *B. fibrisolvens* and *S. bovis* following growth in the presence of condensed tannins.

While light microscopic methods failed to indicate the presence of capsular material surrounding the bacteria, electron microscopy revealed a fibrillar amorphous matrix of

condensed extracellular material extending from the surface of *S. caprinus* and apparently interconnecting the bacterial cells. Initially it was assumed that this electron dense material represented condensation of tannin on the surface of the bacterial cell, a suggestion put forward by Bae *et al* (1993) for *F. succinogenes* S85 cells grown in the presence of condensed tannin. However, the extracellular material was still present following washing of the cells in CE buffer containing PVP, a method found in previous experiments to be effective in removing tannin. The addition of ruthenium red, a polycationic dye that has widespread use as a cytological stain because of its specificity for polyanionic polymers (Read and Costerton 1987), to the EM preparation procedures enhanced visualisation of the extracellular material adding further credence to its biological nature.

Based on its appearance under EM and the compositional analysis, it was determined that the extracellular material was characteristic of a bacterial exopolysaccharide (EPS), a general term used to describe all forms of bacterial polysaccharides found outside the cell wall (Akin 1976; Lambe *et al* 1984, 94; Jacques *et al* 1990; Evans *et al* 1998). Distinction between capsular polysaccharides and slime polysaccharides has been defined by the degree of cell association following centrifugation (Whitfield 1988). In this study, the polysaccharides produced by *S. caprinus* were predominantly cell associated which suggested that the material was capsular in nature. However, as previously mentioned, the EPS produced by *S. caprinus* was not morphologically demonstrable at the light microscopy level and could only be visualised by electron microscopy and this type of exopolysaccharide has been termed "pseudocapsule" (Read and Costerton 1987).

Conventional fixation for thin section electron microscopy is insufficient to preserve bacterial capsular material and even non-detection of these structures by electron microscopy is not unusual (Lambe *et al* 1984, 1994). The predominant constituent of bacterial polysaccharides is water and therefore they are particularly susceptible to the dehydration and embedding steps required for thin sectioning electron microscopy (Sutherland 1988). While the addition of ruthenium red has been shown to add chemical stability through salt interactions between the polymers and reduces the degree of collapse during the dehydration process, it does not protect capsular material against total collapse and condensation unless the cells have formed an intercellular matrix or have been stabilised by other means, e.g. specific EPS antiserum (Jacques *et al* 1990; Johne *et al* 1989; Osawa *et al* 1993). The development of an intercellular matrix allows the fibres to maintain their extended configuration and the results presented in this study suggest that *S. caprinus* cells have this matrix forming ability.

In the majority of experiments presented in this study, *S. bovis* was included as a control. Electron microscopic analysis was no exception. Meagre indications of the presence of exopolysaccharide were detected surrounding *S. bovis* although isolation of the material suggested that *S. bovis* produced similar quantities of extracellular material to *S. caprinus*. The spider-like reticulum of extracellular material was not observed interconnecting cells of *S. bovis*, although cell surface blebs were observed by TEM following growth of the bacteria in the presence of non-growth inhibiting concentrations of tannins. These structures have previously been described on the surface of a number of bacteria and are reported to reflect areas of polysaccharide that have condensed during fixation procedures and/or surface polysaccharide secretion

points (Lambe *et al* 1984; Hespell *et al* 1993). The addition of other stabilising compounds such as lysine to the fixation procedure for *S. bovis* also failed to mimic the extended polymer matrix seen around *S. caprinus* cells. This technique has been successful in EPS preservation through its ability to form large complexes with glutaraldehyde. These complexes are positively charged at physiological pH and can therefore cross-link negatively charged sites within the bacterial capsule. The cross bridges of various lengths provide highly effective cross-linking and consequently enhance the stability of capsular constituents during subsequent dehydration (Jacques *et al* 1990). The lack of polysaccharide matrix interconnecting *S. bovis* cells suggests that these bacteria are incapable of forming a bacterial network and may provide an important clue as to how *S. caprinus* can cope with the presence of tannins in its environment.

6.3.2 Compositional analysis of extracellular material

Quantification and compositional characterisation of the polysaccharide produced by *S. caprinus* required its removal from the cell surface and this initially proved difficult. Washing the cells in distilled water (Roberts *et al* 1995), phosphate buffer (pH 7, Sutherland 1989) or NaCl (Hespell *et al* 1993) proved ineffective at striping the cell surface of all extracellular material (determined by TEM). The most effective method was found to be washing the cells in sodium acetate (pH 4.2, Kasper 1976) which, as it was a relatively harsh technique, provided further evidence for the close association of the EPS with the surface of bacterial cells. Identification of the linkage between EPS and the cell surface was not attempted in this study, however it would appear from these results that the linkage moiety was resistant to alkaline and neutral

or salt solutions. In several *E. coli* and some *N. meningitidis* species, a diacyl glycerol moiety has been identified (Troy 1979; Whitfield 1988) although lipids have also been found (Kennedy and Sutherland 1987).

In the experiments conducted in this study, both *S. caprinus* and *S. bovis* were found to produce heteropolysaccharides of different monosaccharide constituents. Classification of the type of exopolysaccharide as defined by Whitfield (1988) and described in Chapter 1, could not be established from the information gathered in these experiments, although Groups I and III can be discarded. Determination of the EPS structure using mass spectrometry would facilitate this classification and may prove interesting, especially if found to be related to the formation of a polysaccharide matrix. However, in relation to tannin tolerating ability, the results presented in this study imply that the copious quantities of polysaccharide produced by the bacteria in response to the presence of tannins may be the more important aspect.

Neither bacterial species was found to produce polysaccharides containing substances with high affinity for tannins, for example high proline containing glycoproteins such as those found within the spore matrix of *Colletotrichum graminicola* (Nicholson and Moraes 1980; Nicholson *et al* 1986). In this fungal species the authors propose that passive binding of these substances may reduce the accessible concentration of toxic phenols to levels that are not inhibitory and may be indicative of a mechanism by which fungi inactivate toxic phenolic compounds in their environment. The increase in the amount of uronic acids present in the EPS following growth of the bacteria in the presence of tannins is interesting however. The presence of these acids has been

shown to increase resistance of *P. aeruginosa* to the antimicrobial compound, povidone-iodine (Whitfield 1988, Brown *et al* 1995). In these papers the authors propose that the uronic acids interact with and neutralise the antimicrobial agent. While the addition of uronic acid in the form of D-mannuronic acid lactone to bacterial culture medium did not increase the resistance of *S. bovis* to tannic acid, increase in uronic acid content in response to tannins may yet be another response by *S. caprinus* to combat tannins.

6.3.3 Growth conditions for exopolysaccharide synthesis

Bacterial EPS's have been reported to be synthesised at different growth phases and under a variety of growth conditions, depending on the organism studied (Chan *et al* 1984; Bae *et al* 1993; Bonet *et al* 1993; Baselga *et al* 1993; Quessy *et al* 1994). Experiments in this study indicated that production of EPS by *S. caprinus* and *S. bovis* occurred primarily at the end of logarithmic and during stationary growth phase. However, using the methods employed in this study, any EPS synthesised during an earlier phase of bacterial growth could be difficult to detect due to the low density of bacteria at this stage. Thus it is possible that a small quantity of EPS may be all that is required by the bacterium to overcome the detrimental effects of tannins in its environment and allow the population to grow in high levels of tannic acid. This possibility however, would be difficult to clarify. Production of EPS during late log and stationary bacterial growth phase has previously been reported in *A. salmonicida* (Bonet *et al* 1993) and it has been proposed that EPS production is inversely related to the growth efficiency of the producing organism (Linton 1991). Bonet *et al* (1993) suggest that bacterial populations go through several periods of growth and non-

growth in response to changes in their microenvironment, with non-growing bacteria using their resources for the production and release of exopolysaccharides. Production of EPS at this late stage of growth has also been found as a common response among bacterial populations to the presence of excess carbohydrate in the growth medium (Bonet *et al* 1993; Russel 1998) as well as during times of metabolic stress (Caputy and Costerton 1982).

Physiological studies have shown that the type and quantity of capsular material is dependent on carbon, nitrogen and phosphorous sources, on oxygen and pH levels, and on growth temperature and growth phase (Chan *et al* 1984; Cerning 1990; Kojic *et al* 1992; Bonet *et al* 1993; Baselga *et al* 1993). Costerton (1981, 1997) has reported that a high carbon to nitrogen ratio generally favours capsule formation. In the experiments presented in this study, an increase in EPS yield was obtained from cultures of *S. caprinus* and *S. bovis* with a corresponding increase in the amount of carbohydrate present in the growth medium. This appeared to be a general phenomenon as both bacteria responded to the increase in approximately equal amounts. The amount of EPS produced by the bacteria did not change with the incorporation of different carbon sources to the growth medium, nor did the composition and the relative ratios for the neutral sugar components of the EPS vary. This continuity of composition under different growth conditions has previously been described (Bayer and Thurow 1977; Bonet *et al* 1993). In strains of *E. coli* and *Enterobacter aerogenes*, polysaccharide production increased by providing excess carbohydrate and under conditions where growth was limited by the nitrogen, phosphorus, sulphur or potassium content of the medium. However, the composition

of the polysaccharide was found to be independent of the nature of the nutrient limitation or of the quantity or identity of the carbon substrate (Bayer and Thurow 1977; Bonet *et al* 1993). In experiments presented here, limitation of carbon and energy sources resulted in minimal EPS production.

Experiments in this study showed that growth of *S. caprinus* in the presence of tannic acid or condensed tannin resulted in a significant increase in the amount of EPS produced by the bacteria whereas EPS production by *S. bovis* did not alter at non-growth inhibitory levels of tannin. Further experiments indicated that production of EPS by *S. caprinus* was proportional to the amount of tannin present in its growth medium. The results were qualitatively described by TEM and FESEM preparations of the cells and then quantified following isolation of EPS from the cells. Thus it appears that tannins act as a signal for EPS synthesis by *S. caprinus*. Similar induction of EPS biosynthesis has been described in a number of bacteria (Stack and Hungate 1984; Whitfield 1988; Singh *et al* 1992; Kidambi *et al* 1995). Stack and Hungate (1984) determined that *R. albus* 8 cells grown in the presence of 3-phenylpropanoic acid (PPA) revealed an extensive, lobed capsule surrounding the cell wall in contrast to PPA-deprived cells devoid of capsule (visualised by TEM). However in that case, the authors suggested that the necessity for PPA may have been due to its inclusion as a component of the capsule. Incorporation of tannic acid, gallic acid or their breakdown products into EPS did not appear to occur in the streptococcal species studied in this thesis. Production of EPS in response to toxic compounds has also been documented and is associated with an increased bacterial tolerance to the offending substance (Kidambi *et al* 1995, Goven and Fyfe 1978). For example,

Kidambi *et al* (1995) reported that plant associated pseudomonads, such as *P. syringae* sp. *syringae* F85 that are commonly exposed to copper bactericides have developed a specific resistance or tolerance to copper salts due to the increased production of EPS. On similar lines, mucoid variants of *P. aeruginosa* showed enhanced resistance to carbenicillin (Goven and Fyfe 1978) and povidone-iodine (Brown *et al* 1995) and induced slime production and capsule synthesis was also found in *Lc. Lactis* ssp. *Cremoris* following growth in the presence of p-fluorophenyl-alanine (Efiuvwevwere *et al* 1999). In the above cases, the authors propose that induction of EPS production was a protective mechanism employed by the bacteria in an attempt to overcome exposure to toxic compounds. Removal of the EPS layer from around *S. caprinus* decreased the tolerance of the bacteria to tannic acid suggesting that production of EPS by *S. caprinus* was also a protective mechanism employed by the bacteria in an attempt to overcome the detrimental effect of tannins. Brown *et al* (1995) suggested that the polyanionic nature of the EPS created a diffusion barrier which acted to retard the antimicrobial agent and in light of the experiments reported in this chapter, this also appears a feasible explanation for *S. caprinus*. Diffusion through the polysaccharide may be affected by charge (ionic) interactions between the EPS and the antimicrobial agent, by an increase in the distance the agent must diffuse, by molecular sieving (size exclusion), and by the viscosity of the EPS barrier (Dudman 1977; Beveridge 1979; Costerton *et al* 1987, 1995).

No other phenolic acid was able to induce EPS synthesis by *S. caprinus* cells suggesting specificity for the tannins and no increased tolerance to other phenolic

acids occurred following EPS induction by tannic acid. The ineffectiveness of the EPS to reduce the bacterial tolerance to other phenolic compounds is likely a reflection of the size difference between low molecular weight simple phenolic monomers that can penetrate the extracellular mass and the high molecular weight tannins that cannot (size exclusion). However, due to the binding nature of tannins, it is also likely that charge interactions between the tannins and the EPS play a significant role in inhibiting the access of tannins to the bacterial cell wall and cytoplasmic membrane.

Chapter 7

General discussion and conclusions

Chapter 7 General discussion and conclusions

The presence of condensed tannins and hydrolysable tannins, found as secondary compounds in a number of important forage plants, can have a large influence on the nutritive value of these plants. In grazing ruminants, high concentrations of tannins adversely affect ruminant nutrition by the production of insoluble tannin-protein complexes which are poorly digested in the rumen and lower digestive tract. They also inhibit microorganisms and microbial enzymes involved in fibre degradation and produce an astringent taste. The combination of these factors results in the inhibition of growth and productivity of animals grazing tannin-rich forages (Clausen *et al* 1990).

It has been observed that some animals, for example wild goats and other feral ruminants, appear to thrive on plants containing high concentrations of tannins and it has been suggested that these animals harbour novel microorganisms that aid in their ability to tolerate tannins. There are a number of possible mechanisms by which this could be achieved. Firstly, the ruminant microorganisms may be unaffected by the presence of tannins and are thus capable of carrying out their normal roles in rumen metabolism. Secondly, the microorganisms may be capable of providing a protective barrier that inhibits the detrimental effects of tannins or alternatively, they may be capable of altering the chemical structure of tannins to produce a less toxic form.

S. caprinus was isolated from the rumen of feral goats known to be grazing tannin-rich *Acacia* by the formation of clearing zones surrounding the bacterial colonies

during growth on nutrient agar medium containing the hydrolysable tannin, tannic acid (0.5% w/v). The bacteria were shown to tolerate high (>7% w/v) levels of hydrolysable and condensed tannins in its growth medium. In comparison, the more common ruminal streptococcal species, *S. bovis* was found to be inhibited by significantly lower concentrations of tannins in its growth medium (<0.75% w/v) and did not form zones of clearing when grown on tannic acid-nutrient agar medium (L.O'Donovan, Honours thesis, Brooker *et al* 1994).

This research project aimed to characterise the ability of *S. caprinus* to tolerate tannins and determine the mechanism(s) the bacteria employ to combat their detrimental effects. However, while the aims of this project have been largely fulfilled, the role of *S. caprinus* in the ability of feral goats to efficiently metabolise plants containing high concentrations of tannins remains equivocal.

The information presented in Chapter 3 of this study described the effect of hydrolysable tannins, condensed tannins and other more simple phenolic monomers on the growth of *S. caprinus* and *S. bovis*. The results indicated that *S. caprinus* was generally more tolerant to the presence of phenolic compounds, particularly the more complex tannins, in the growth medium than *S. bovis*, although neither bacterium was capable of utilising any phenolic compound as a sole energy source. The increased tolerance of *S. caprinus* to a number of phenolic compounds however indicates a possible connection between this activity and the ability of feral goats to consume a diet rich in tannins. Subsequent experiments showed that while *S. caprinus* could tolerate high levels of tannins in its growth medium, it was not unaffected by their

presence. With an increase in the concentrations of tannins in the growth medium, a proportional increase in the lag period was observed. A more substantial increase in lag period was also observed for *S. bovis* at non-growth inhibitory concentrations of tannin. Once turned on, maximum cell density of *S. caprinus* was achieved although *S. bovis* remained susceptible to the higher levels of tannins. Repeated subculturing of the bacteria into tannin-containing nutrient medium resulted in immediate growth of the bacteria although no increase in tannin tolerance was observed in cultures of *S. bovis*. *S. bovis* also appeared more susceptible to the presence of simple phenolic acid monomers in the growth medium than *S. caprinus* and as with the tannins, extensions in lag period were observed for both bacteria.

As lag periods are reported to represent times of intense metabolic activity, it is suggested that upregulation of enzyme activity or gene synthesis was occurring at this time and therefore the increased lag periods may be representative of a number of active responses by the bacteria to the presence of these compounds.

While the results presented in Chapter 5 of this study indicate that the enzymatic basis of tannic acid degradation by *S. caprinus* was questionable, degradation of gallic acid was achieved though decarboxylation of the phenolic acid by gallate decarboxylase. Switching on the gene(s) responsible for this enzyme and/or increased synthesis of the product may therefore account for the extended lag period observed in these bacteria following growth in the presence of tannins. Similarly, the decrease in lag times following subculture of the bacterium may be indicative of an erstwhile functioning enzyme. The similar extension in lag period observed with *S. bovis* following growth

in the presence of low concentrations of tannins may be indicative of transcription of the gene(s) without translation of the product or translation of the gene transcripts resulting in an inactive enzyme product. Similar reasoning may be used to explain the extended lag period observed following growth in the presence of the other phenolic acid monomers tested as neither bacterial strain appeared capable of degrading these compounds.

Characterisation of gallate decarboxylase activity in *S. caprinus* could only be achieved in whole cell suspension preparations. Based on previous studies of this enzyme in other bacterial systems, the lack of activity in cell-free extracts was likely due to the oxygen sensitivity and labile nature reported for gallate decarboxylase. However, other decarboxylase systems, such as those required in pyruvate metabolism require not only the presence of Mg^{2+} (included in experiments in this study) but also a tightly bound co-enzyme, thiamin pyrophosphate. Thus it is also possible the lack of gallate decarboxylase activity by *S. caprinus* was caused by the loss or destruction of a necessary co-factor following the preparation of cell free extracts. In order to further examine this enzyme in *S. caprinus*, the assay procedure must be refined to enable cell free extracts and ultimately purified protein to be used. This may be overcome by conducting the assay procedure under strictly reducing conditions using total cell-free extracts.

Preliminary characterisation of the enzyme responsible for the degradation of gallic acid by *S. caprinus* suggested a specificity for gallic acid and tannic acid, a characteristic displayed by other gallic acid degrading bacteria such as isolated from

E. oxidoreducens (Krumholz *et al* 1987). Further characterisation demonstrated that expression of enzyme activity was increased in *S. caprinus* following growth in the presence of gallic acid and tannic acid compared to cells grown in the absence of phenolics or in the presence of non-gallic acid containing phenolic compounds such as condensed tannin, ferulic and p-coumaric acids. These results provide further evidence that this enzyme may play a role in the biodegradation of tannic acid and/or gallic acid.

Growth experiments however, indicated that the presence of gallic acid did not produce an inhibitory effect on the growth of *S. caprinus* and subsequent experiments indicated that the bacteria did not use gallic acid as a substrate for growth. Thus, the question arises as to what *S. caprinus* gains from the alteration of this phenolic compound? The answer to this question may be explained through the calculation of Gibbs standard free energy change for the degradation of gallic acid and hence the generation (or loss) of energy in the form of ATP brought about by this reaction. The efficiencies of ATP production, transfer and utilisation during growth are important factors influencing the survival of microorganisms in an environment such as the rumen where energy sources are only occasionally abundant. In fermentation, microorganisms derive their energy from substrate level phosphorylation reactions and organic compounds serve as electron donors and acceptors. Most organisms however, can conserve energy either in the form of a transmembrane electrochemical gradient of protons or proton-motive force (Russell and Hespell 1981; Russell and Cook 1995; Russell 1998)

The hypothetical conversion of gallic acid to pyrogallol is as follows:

$$C_7H_5O_5^- + H^+ \rightarrow C_6H_6O_3 + CO_2$$

gallic acid → pyrogallol + carbon dioxide

From this equation it is possible to determine the Gibbs standard free energy change ($\Delta G^{\circ\prime}$ kJ/mol) from the standard free energies of formation ($\Delta G^{\circ\prime}_f$) of the substrates and products. That is, the sum of the standard free energies of formation of the products minus the sum of the standard free energies of formation of the reactants, taking into account the stoichiometry of the reaction. Therefore, using the $\Delta G^{\circ\prime}_f$ published by Thauer *et al* (1977) and the value for pyrogallol estimated by Kaiser and Hanselmann (1982), the standard free energy change of the above reaction can be calculated as follows

	$C_7H_5O_5^-$	+	H^+	→	$C_6H_6O_3$	+	CO_2
$\Delta G^{\circ\prime}_f$	(– 706)	+	(– 39.87)		(– 358)	+	(– 395.2).

Thus the $\Delta G^{\circ\prime}$ of the above reaction is – 7.33 kJ/mol which suggests that the reaction is thermodynamically favourable (ie $\Delta G^{\circ\prime}$ negative) and may provide an explanation of why degradation of gallate may be advantageous to *S. caprinus*.

In this case however, the free energy change of the reaction is small and equivalent to only approximately one tenth of the energy required for ATP synthesis from ADP and phosphate under physiological conditions (Lehninger 1977). Therefore the decarboxylation energy cannot be used in substrate level phosphorylation. It is possible that the decarboxylation energy is either converted by membrane bound primary decarboxylase sodium ion pumps into an electrochemical gradient, or alternatively, an electrochemical proton gradient can be established by the combined

action of a decarboxylase with a gallate/pyrogallol antiporter. The generated electrochemical Na^+ or H^+ gradients can then be exploited for ATP synthesis by Na^+ or H^+ coupled F1F0 ATP synthases which can be used for a variety of functions, including polysaccharide production. This energy conservation has been shown to occur in several anaerobic bacteria following decarboxylation of dicarboxylic acids such as oxalate, malonate, succinate, glutarate, and malate and has been termed decarboxylation phosphorylation (Dimroth and Schink 1998).

The production of a proton-motive force may provide some insight into the formation of the cleared regions surrounding *S. caprinus* during growth on nutrient agar containing tannic acid (0.5% w/v). Previous studies have suggested that acid hydrolysis of the bonds between tannic acid and protein resulted in the formation of the clearing zones (L. O'Donovan, Honours thesis; Brooker *et al* 1994). In this study however, it was determined that lactic acid production by *S. caprinus* and *S. bovis* was similar and did not change following growth in the presence of tannic acid. No other acid was found to be produced by the bacteria in sufficient quantities to form a cleared region. However, when combined with normal cell metabolic products (e.g. lactate), the protons pumped out of the cell as part of a proton-motive force may be sufficient to lower the pH in the immediate bacterial vicinity and cause hydrolysis of the tannin-protein complex resulting in the production of clearing zones. The inability of *S. bovis* to produce cleared zones at this non-growth inhibitory level of tannic acid would therefore relate to its inability to degrade gallic acid.

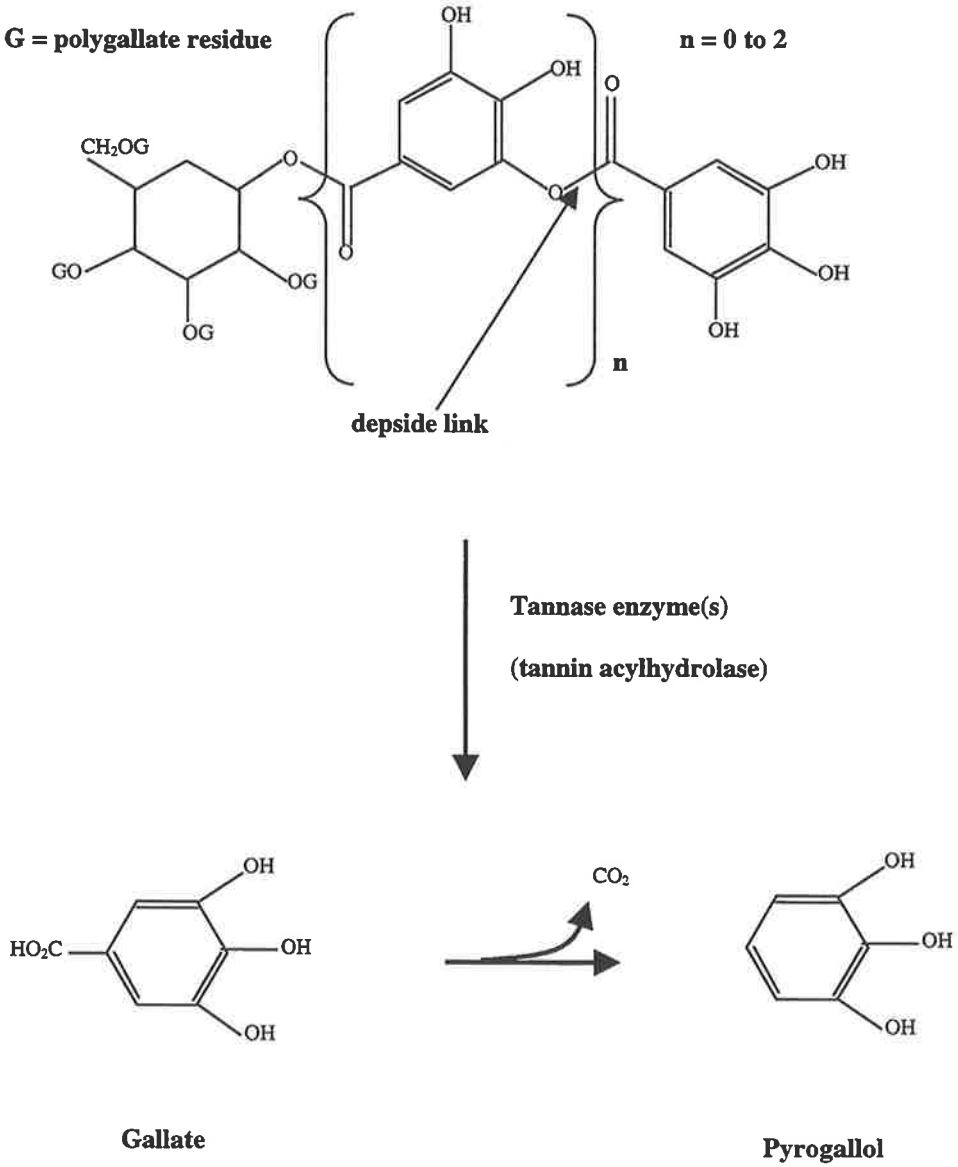
This proposed mechanism for the uptake of gallic acid and secretion of pyrogallol via a co-transport mechanism conflicts with that indicated in *P. acidigallici* and *E. oxidoreducens* (Brune and Schlink 1992; Krumholz *et al* 1987). In these bacteria it was proposed that the equilibrium of the gallate decarboxylase reaction is responsible for creating a substrate sink in the cell in order to enable gallate uptake by facilitated diffusion. Creation of a substrate sink to facilitate substrate uptake has also been postulated for hydroxybenzoate uptake in *Rhodopseudomonas palustris* (Harwood *et al* 1998). In experiments conducted in this study however, gallate uptake via a co-transport mechanism may also provide another explanation for the increased lag periods observed following growth of the bacteria in the presence of gallic acid. These periods may represent synthesis of proteins required for the uptake of gallic acid into the cell during early bacterial growth and/or the secretion of pyrogallol out of the cell at stationary phase.

As mentioned previously, the experiments conducted in this study failed to determine an enzymatic basis for tannic acid degradation by *S. caprinus*. Previous reports however, have ascribed tannin acylhydrolase activity to the degradation of tannic acid in strains of tannin-tolerant Streptococci (Osawa, 1990; Osawa and Walsh 1993b; Nelson *et al* 1995). Osawa (1990) reported that the production of cleared regions surrounding *S. bovis* biotype 1, aka *S. gallolyticus* (Osawa *et al* 1995) following growth on nutrient agar containing tannic acid was indicative of tannin acylhydrolase activity. Similarly, Nelson *et al* (1995) suggested that hydrolysis of tannic acid to pyrogallol displayed by gas chromatography and thin layer chromatography was achieved via tannin acylhydrolase followed by gallate decarboxylase activity. In both

cases however, the evidence was circumstantial as no assays were conducted for either of the two enzymes to support their statement. Figure 7.1 outlines the proposed mechanism for tannic acid degradation by the Nelson isolate. Should this bacterium be capable of hydrolysing the galloyl glucose ester bonds and the intergallate depside bonds by tannin acylhydrolase, glucose as well as gallic acid would be released into the growth medium. Thus the bacterium should therefore be capable of utilising the liberated glucose for growth in a situation analogous to that described for *S. ruminantium* subsp. *ruminantium* (Skene and Brooker 1995). The expression of tannin acylhydrolase activity should therefore allow the bacterium to grow on tannic acid as a sole carbon source. The authors report however that this is not the case for their isolate. While *S. caprinus* and *S. gallolyticus* have recently been reported to be subjective synonyms (Sly *et al* 1997), the relationship of the Nelson isolate to these bacteria is unknown, however evidence suggests they are closely related. Therefore the presence of tannin acylhydrolase activity in the Nelson isolate should be examined using the assay described by Skene and Brooker (1995) and its relationship to *S. caprinus* determined through DNA-DNA hybridisation and ribosomal RNA sequencing.

While the enzymatic degradation of tannic acid was not determined in this study, the presence of an enzyme capable of degrading a portion of the tannic acid molecule cannot be ruled out. It may be possible for this enzyme to act to hydrolyse the depside bonds thereby releasing gallic acid but cannot degrade the galloyl-glucose ester bonds thus leaving a number of gallate groups attached to the central carbohydrate core. This degradation of intergallate depside linkages could destabilise the tannin-protein

Figure 7.1 **Proposed mechanism for tannic acid degradation**



Nelson *et al* 1995

complexes and result in the resolubilisation of protein and peptides which may also describe the cleared regions surrounding the bacteria following growth on tannic acid-nutrient agar. Preliminary experiments in Chapter 5 of this study however, described the unstable nature of tannic acid and an alternative non-enzymatic mechanism for the degradation of tannic acid by *S. caprinus* was proposed. This hypothesis is based on an equilibrium situation existing between tannic acid and gallic acid. In this proposed scenario, uptake and degradation of gallic acid by *S. caprinus* leading to its effective removal from the growth medium, causes an equilibrium shift towards gallic acid. As *S. caprinus* was unable to utilise tannic acid as a sole carbon source, in this proposal degradation of tannic acid is likely to represent hydrolysis of the intergallate depside linkages only rather than total hydrolysis of the tannic acid which would result in the liberation of glucose.

S. caprinus was also shown to be able to tolerate high concentrations of condensed tannins in its growth medium however gas-liquid chromatography analysis did not detect any changes in the condensed tannin profile following incubation with the bacteria. This result may simple reflect the complex nature of the condensed tannin profile and/or may suggest that other mechanisms are in force to enable the bacteria to cope with the presence of this tannin type. Further work however is required to definitively define whether degradation of condensed tannins by *S. caprinus* is achieved.

Results presented in Chapter 6 of this thesis provide an alternative mechanism by which *S. caprinus* copes with the presence of tannins in its environment. In this study

it was revealed that tannic acid and condensed tannin induced the production of exopolysaccharide by *S. caprinus* and that removal of this layer caused an increase in bacterial susceptibility to tannins. This suggested that the formation of a bacterial exopolysaccharide was a protective mechanism employed by the bacteria in an effort to overcome the potentially detrimental effect of tannins. However, experiments in this study also indicated that production of EPS by *S. caprinus* primarily occurred at the end of logarithmic and during stationary growth phase, an unusual time to be produced if its primary role was the protection of bacterial cells. It is possible therefore that the protection afforded by EPS production by *S. caprinus* is simply a bi-product of general metabolism whereby the bacteria utilise their resources to produce and secrete EPS. None the less, production of EPS at this growth stage would provide an effective diffusion barrier to inhibit the action of tannins on the bacterial cell wall. This may be important as it was observed that cell wall thickness was reduced at this stage of bacterial growth possibly rendering the bacteria more susceptible to the presence of tannins in its environment.

Microscopic analysis indicated that the exopolysaccharide extended from the surface of the bacterial cells and formed an intercellular matrix between the bacteria. This structure was not observed around *S. bovis* cells and may therefore provide an important clue as to how *S. caprinus* can cope with the presence of tannins in its environment, both in *vitro* and perhaps more importantly in *vivo*.

This formation has been observed in a number of bacterial species in a number of different environments (McAllister *et al* 1994, Costerton *et al* 1987; 1995 and 1999)

and may represent the ability of *S. caprinus* to interact with and attach to surfaces, such as feed particles or the rumen epithelium. In this study it was shown that the amount of extracellular material produced by *S. caprinus* was greater when the bacteria were grown on solid nutrient agar. In the rumen, this ability may lead to the formation of EPS-enclosed microcolonies and eventually the formation of a bacterial biofilm which has been reported to enhance microbial resistance to environmental stress and antimicrobial agents (Costerton *et al* 1995; Barbeau *et al* 1996; Chen and Stewart 1996).

It would be interesting to conduct experiments to determine whether *S. caprinus* naturally tends towards a biofilm mode of growth and whether tannins have any influence on this ability. Many techniques have been recently described and confocal scanning laser microscopy (CSLM) has proven particularly well suited for the study of microbial biofilms (Costerton *et al* 1995, 1999). CSLM allows the non-destructive, *in situ* analysis of living, fully hydrated biofilms without the need for harsh chemical fixation or embedding techniques that were employed in this study.

In conclusion, the results reported in this study have characterised *S. caprinus'* relationship with tannic acid and condensed tannins and have depicted two ways in which the bacterium was capable of defending itself against the detrimental effects of tannins in its environment. These were:

1. Degradation of tannic acid and its phenolic monomeric constituent, gallic acid to form pyrogallol and

2. Induced production of a protective exopolysaccharide in response to the presence of tannins.

Further work is needed however to determine other mechanisms employed by the bacteria to tolerate the presence of condensed tannins in its environment.

Based on the experiments presented in this study, the following scenarios are proposed to describe the mechanisms employed by *S. caprinus* to overcome the potentially detrimental effects of tannins in its environment.

Characterisation of tannic acid showed that free tannic acid was found in equilibrium with complexed tannic acid-protein in the growth medium. Binding studies indicated that the bound tannic acid was likely to represent the larger molecular weight components of tannic acid while the free tannic acid was representative of the lower molecular weight constituents. A proportion of gallic acid was also found present in equilibrium with tannic acid. The increase observed in bacterial lag times following addition of tannic acid to the growth medium suggested that the presence of this phenolic acid and its constituents results in induced transcription/translation of the enzymes, TAH and/or gallate decarboxylase and/or uptake of tannic acid and/or gallic acid by the bacteria by facilitated diffusion or a co-transport mechanism.

Degradation of tannic acid may then occur either

1. enzymatically. TAH degradation of tannic acid to produce gallic acid, followed by gallate decarboxylase activity resulting in the production of pyrogallol which is then pumped out of the cell or
2. non-enzymatically via a proposed equilibrium reaction with gallic acid. The disappearance of gallic acid into the cell resulting in its effective removal from the growth medium forces the tannic acid-gallic acid equilibrium towards gallic acid. The bacteria degrade gallic acid to pyrogallol intracellularly facilitating continued gallic acid diffusion or active uptake into the cell. Pyrogallol is then pumped out of the cell.

The cleared regions observed around bacterial colonies following growth on tannic acid-nutrient agar may thus be due to the

1. destabilisation of the tannic acid-protein complex following degradation of tannic acid resulting in the resolubilisation of protein and peptides or
2. combination of normal metabolic products (e.g. lactate) combined with the excess expulsion of H^+ ions by the bacterium into the surrounding medium following degradation of gallic acid. Degradation of gallic acid to pyrogallol results in the liberation of energy which can be converted into an electrochemical proton gradient by the combined action of the decarboxylase with a gallate/pyrogallol antiporter. The generated electrochemical H^+ gradients can then be exploited for ATP synthesis by H^+ coupled F1F0 ATP synthases.

Based on stoichiometric considerations, not all of the tannic acid is converted to gallic acid and pyrogallol. Thus it is likely that a proportion of larger molecular weight tannic acid components remain in bound form with protein.

The presence of tannin in the growth medium caused a change in the size and shape of the bacterial cell. Towards the end of bacterial growth, increasing concentrations of tannins resulted in a decrease in cell wall thickness and an increase in the production of exopolysaccharide surrounding the bacteria creating a diffusion barrier to inhibit tannin action.

Appendix

Appendix Media, Buffers and Reagents

Modified BHI medium (mBHI)

/100 ml	
Brain Heart Infusion (Oxoid)	3.7 g
Glucose	0.05 g
Cellobiose	0.05 g
Soluble starch	0.05 g
Cysteine.HCl	0.05 g
Hemin solution	0.05 ml
Resazurin (0.1% stock solution)	0.05 ml

NB media (Nili and Brooker 1995)

/100 ml	
NB Mineral Solution I	6.0 ml
NB Mineral Solution II	6.0 ml
Trace Element Solution	0.5 ml
Hemin/napthoquinone Solution	1.0 ml
VFA Solution	0.33 ml
Na_2S (5% solution)	1.5 ml
Resazurin (0.1% stock solution)	0.05 ml
NH_4Cl	0.37 g
Cysteine.HCl	0.05 g
Na_2CO_3 (8% solution)	5.0 ml
Vitamin Solution	4.0 ml

Note before adding Na_2CO_3 and vitamin solution, the pH is adjusted to 7.7-7.8. Autoclave.

NB Mineral Solution I

K_2HPO_4	11.84 g/l

NB Mineral Solution II

/l	
KH_2PO_4	7.08 g
NaCl	1.78 g
$MgSO_4.7H_2O$	3.75 g
$MnCl_2.4H_2O$	0.20 g

$CoCl_2.6H_2O$	0.02 g
Na_2SO_4	8.3 g
$CaCl_2.2H_2O$	3.20 g

Note $CaCl_2.2H_2O$ is dissolved separately, mixed and the solution made to volume.

Trace Element Solution

/100 ml	
$ZnSO_4.7H_2O$	10.0 mg
H_3BO_3	10.0 mg
$Na_2MO_4.2H_2O$	10.0 mg
$NiCl_2.6H_2O$	5.0 mg
$CuSO_4.5H_2O$	5.0 mg
$FeSO_4$	10.0 mg
$Al(SO_4)_3$	2.0 mg

Hemin/napthoquinone Solution

Dissolve 50 mg hemin in 5.0 ml 1.0 M NaOH, add 95 ml dH_2O and 10 mg 1,4-napthoquinone.

Vitamin Solution

/300 ml	
biotin	2.5 mg
folic acid	2.5 mg
p-amino benzoic acid	2.5 mg
cyanocobalomin	2.5 mg
Ca pantothenate	20.0 mg
Nicotinamide	20.0 mg
Riboflavin	20.0 mg
Thiamine.HCl	20.0 mg
Pyridoxamine	20.0 mg
Lipoic acid	2.0 mg

VFA Solution

glacial acetic acid	17.0 ml
propionic acid	6.0 ml
n-butyric acid	4.0 ml
iso-butyric acid	1.0 ml
n-valeric acid	1.0 ml

iso-valeric acid	1.0	ml
D-L-α-methyl butyric acid	1.0	ml

LB (Luria Bertani) Medium

/100 ml		
Tryptone	1.0	g
Yeast Extract	0.5	g
NaCl	1.0	g

Autoclave.

SOC medum

/100 ml		
Tryptone	2.0	g
Yeast extract	0.50	g
NaCl	0.06	g
KCl	0.02	g
$MgCl_2$	0.20	g
$MgSO_4$	0.25	g
Glucose	0.36	g

Note Autoclave or filter sterilise and divide into 5-10 ml aliqots.

Modified M10 (mM10)

/100 ml		
Tryptone (Oxoid)	0.20	g
Yeast Extract (Oxoid)	0.05	g
Cysteine. HCl	0.05	g
Resazurin (0.1% stock solution)	0.10	ml
Hemin Solution	0.01	ml
VFA Solution	0.31	ml
B & B Mineral Solution I	3.80	ml
B & B Mineral Solution II	3.80	ml
Na_2CO_3 (8% w/v stock solution)	5.00	ml

Note Adjust pH of medium to 6.8 with 10% NaOH and then make up to volume with dH_2O water. Autoclave.

Hemin Solution

Dissolve 2.0 g hemin in 100 ml of a 1:1 solution of 100% ethanol:0.05 M NaOH.

B & B Mineral Solution I (Bryant and Burkey, 1953)

K_2HPO_4	6.0	g/l

B & B Mineral Solution II (Bryant and Burkey, 1953)

/l		
NaCl	12.0	g
$(Na_4)_2SO_4$	12.0	g
KH_2PO_4	6.0	g
$CaCl_2$	1.2	g
$MgSO_4.7H_2O$	2.5	g

Casein-agar preparation

/100ml		
Casein	5.0	g
Mineral solution I	4.0	ml
Mineral solution II	4.0	ml
Agar	1.0	g

Autoclave.

Modified M9 medium

/100ml		
Na_2HPO_4	0.6	g
KH_2PO_4	0.3	g
NaCl	0.05	g
NH_4Cl	0.1	g

Adjust pH to 7.4, autoclave, then add

1 M $MgSO_4$	0.2	ml
20% glucose	1.0	ml
1M $CaCl_2$	0.01	ml

Anaerobic Dilution Solution (Ogimoto and Imai, 1984)

/100 ml		
B&B Mineral Solution I	3.8	ml
B&B Mineral Solution II	3.8	ml
Resazurin (0.1% stock solution)	0.1	ml

Cysteine. HCl	0.05 g
Na_2CO_3 (8% stock solution)	5.0 ml

Note pH is adjusted to 6.8-7.0.

Sodium Phosphate Buffer, 0.2 M

/100 ml

$Na_2HPO_4.2H_2O$	3.61 g
$NaH_2PO_4.2H_2O$	3.21 g

For pH 6.8, combine 24.5 ml solution 1 and 25.5 ml solution 2. Make to 100 ml/

TE buffer (pH 7.4)

10 mM Tris-Cl (pH 7.4)
1 mM EDTA (pH 8.0)

Filter sterilise

Phosphate Buffered Saline (PBS)

/100 ml

NaCl	80.0 mg
KCl	2.0 mg
Na_2HPO_4	14.0 mg
KH_2PO_4	2.5 mg

Note pH is adjusted to pH 7.4. Sterilise

CE Buffer, 0.1M (pH 10)

50 mM Na_2CO_3
25 mM EDTA

Filter sterilise.

Citrate buffer

/100 ml

0.1 M citric acid	33.0 ml
0.1 M sodium citrate	17.0 ml

Karnovsky Fixative

Paraformaldehyde, 8%	25.0 ml
Glutaraldehyde, 50%	5.0 ml
Phosphate buffer, 0.2 M, pH 7.2	50.0 ml

Spurr Resin

ERL 4206	10.0 g
DER 736	6.0 g
NSA (nonenyl succinic anhydride)	26.0 g
S-1 (dimethylaminoethanol)	0.4 g

Lead Citrate

Lead citrate	1.33 g
Sodium citrate	1.76 g
DH_2O	30.0 ml

Shake in 50 ml flask for ~20 min, add 8.0 ml 1 N NaOH (to pH 12) and dilute to 50 ml with dH_2O.

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