Chapter 1
The antimicrobial activity of lipid in the rumen: a review of its mode of action and amelioration.

1.1. Introduction

A lipid is defined by Gurr and Harwood (1991) as any compound that belongs to a chemically heterogeneous group of substances that share the properties of hydrophobicity and solubility in non polar solvents. The lipids considered in this thesis will be restricted to esters of long chain fatty acid (LCFA), primarily triglyceride derived from plant or animal storage depots, non esterified medium and long chain fatty acid (NEFA)s or non esterified LCFA's, cell membrane lipids and other compounds derived from LCFA and useable as a livestock feed.

The total lipid content of forage, as defined by Gurr and Harwood (1991), can comprise 4 - 6% of its dry matter (Van Soest 1982). About half of this is digestible lipid, primarily membrane lipids, with the remainder as cuticular waxes and carotenoid pigments; all of which are retained in an ether extraction (AOAC 1980, Church 1976, Van Soest 1982, Palmquist 1988). The rumen ecosystem has therefore adapted to an intake of dietary lipid in the form of plant cell membrane phospholipids, waxes, pigments and occasionally small amounts of triglyceride from seeds (Van Soest 1982). Increasing the lipid content of a forage based diet by 2 - 3% in situations where the total digestible lipid content of dry matter of the basal diet was 6% has been observed to affect rumen function in a manner that is detrimental to the nutrition of the animal (Palmquist 1988). The ability of the rumen to ferment plant fibre, particularly cellulose, is reduced and this can result in a reduction in the animal's intake of the forage (Kowalzyck *et al.* 1977, Zinn 1989, Palmquist *et al.* 1993). Decreases in intake concurrent with lipid addition may also arise from metabolic regulation as lipid infusion into the duodenum can also depress intake (Gagliostro and Chilliard 1991).

The digestive advantage of ruminant compared to monogastric livestock lies in their capacity for fermentative digestion of feeds high in cellulose. Supplementation of a ruminant's diet with digestible lipid without reducing the digestion of plant fibre in the rumen has therefore been the subject of considerable research. This research has revealed a number of mechanisms by which certain lipids inhibit rumen fermentative processes and a number of strategies that reduce the effects of these lipids in the rumen. They will be reviewed here.

1.2. The properties of a lipid that influence its antimicrobial activity in the rumen

To exert antimicrobial activity a lipid must possess amphiphilic nature, i.e., the capacity to be in aqueous solution or suspension (hydrophilicity) and remain sufficiently soluble in lipid (lipophilicity) so as to permit adsorption onto a microbial cell surface (Blaxter and Czerkawski 1966, Czerkawski *et al.* 1966, Galbraith *et al.* 1971, Galbraith and Miller 1973a and b, Henderson 1973). Triglyceride, waxes and carotenoid pigments are not amphiphilic and do not exhibit antimicrobial activity. Triglyceride is hydrolysed in the rumen with the production of non esterified fatty acid, eg., lauric acid (Figure 1.1a). Non esterified fatty acid (NEFA) is amphiphilic because of the presence of the polar head group (carboxylate) which confers hydrophilicity and the alkyl chain which confers lipophilicity. Replacing the carboxyl group with an alternate head group such as sulphate, eg., sodium laural sulphate (Figure 1.1b), produces a polar detergent with increased solubility in water while maintaining its lipophilic nature. NEFA and polar detergents can cause stasis or death of some rumen and other microorganisms (Nieman 1954, Blaxter and Czerkawski 1966, Galbraith *et al.* 1971, El Hag and Miller 1972, Galbraith and Miller 1973a and b, Galbraith *et al.* 1971, Henderson 1973, Maczulak *et al.* 1981, Wakita and Hoshino 1987, Ushida *et al.* 1990, Ibrahim *et al.* 1993). The minimum concentrations of non esterified fatty acids observed to exert antibacterial activity when measured under *in vitro* conditions is 5 - 50μg/ml (Nieman 1954, Henderson 1973) or 0.1 - 1 mM (Galbraith *et al.* 1971).

a) Lauric acid b) Sodium laural sulphate

Figure 1.1. Chemical formula for a) the non esterified fatty acid, laurate and b) the polar detergent sodium laural sulphate.

With non esterified fatty acids of plant or animal origin, solubility in water and lipophilicity primarily vary according to:

i) the length of the fatty acyl chain;

ii) the number of double bonds within the fatty acyl chain;

iii) the location of the double bonds between carbon atoms; and

iv) geometrical isomerism, i.e., *cis* or *trans* configuration (Gurr and Harwood 1991 pp. 23 - 31).

These four pieces of information about a fatty acid can be written in short hand notation. The location of a double bond is usually given as the number of carbon atoms preceding the double bond counting the carboxyl carbon atom as 1. The shorthand notation for stearic acid with 18 carbon atoms and no double bonds in the fatty acyl chain can be

written as 18:0. A fatty acid with no double bonds is also referred to as a saturated fatty acid. The short hand notation for *trans* vaccenic acid which has 18 carbon atoms in the fatty acyl chain and one double bond with the *trans* configuration located at the 11th carbon atom can be written as *trans*-11-18:1 (Figure 1.2a). A fatty acid with one double bond is referred to as mono-unsaturated while a fatty acid with two or more double bonds, eg., linoleic acid (Figure 1.2c), is often referred to as poly-unsaturated. Both mono- and poly-unsaturated fatty acids can be grouped as unsaturated fatty acid.

$$CH_3(CH_2)_5\overset{11}{HC}{=}\overset{1}{CH}(CH_2)_9COOH$$

a) *trans* Vaccenic acid (*trans*-11-18:1)

$$CH_3(CH_2)_7\overset{9}{HC}{=}\overset{1}{CH}(CH_2)_7COOH$$

b) Oleic acid (*cis*-9-18:1)

$$CH_3(CH_2)_4\overset{12}{HC}{=}\overset{9}{CH}(CH_2)HC{=}\overset{1}{CH}(CH_2)_7COOH$$

c) Linoleic acid (*cis, cis*-9, 12-18:2).

Figure 1.2. The chemical formula with its shorthand notation in brackets for a) *trans* vaccenic acid, b) oleic acid and c) linoleic acid showing the position numbers of both the carboxyl carbon atom and the carbon atom that defines the location of the double bond.

Variations in the properties listed above will alter the concentration at which a given lipid exerts antimicrobial activity (Blaxter and Czerkawski 1966, Czerkawski *et al.* 1966, Galbraith *et al.* 1971). Galbraith *et al.* (1971) and El Hag and Miller (1972) using rumen and non rumen gram positive bacteria demonstrated that C2 to C6 saturated fatty acids, i.e., fatty acids of 2 to 6 carbon atoms in length and no double bonds in the fatty acyl chain, exerted no bactericidal or bacteriostatic action when measured *in vitro*. Higher members of this fatty acyl series do exert bactericidal or bacteriostatic activity (El Hag and Miller 1972, Galbraith *et al.* 1971, Henderson 1973, Maczulak *et al.* 1981). Galbraith *et al.* (1971) determined a relative inhibitory activity of fatty acids as a function fatty acyl chain length for a range of non rumen microorganisms under *in vitro* conditions as:

caprylic (8:0) < capric (10:0) < lauric (12:0) ≥ myristic (14:0) > palmitic (16:0) ≥ stearic (18:0).

Galbraith *et al.* (1971) suggested that lauric acid is the most inhibitory of the saturated fatty acids as it has a high degree of solubility in both water and lipid compared to the other fatty acids tested. More of it may permeate a bacterial cell membrane and through a protonophoric action across the cell membrane reduce the amount of energy available for cell metabolic activity resulting in cell stasis or death (Galbraith and Miller 1973a,bandc). Galbraith *et al.* (1971) also report that, on an equimolar basis, the C18 unsaturated homologous series of fatty acids, i.e., fatty acids of 18 carbon atoms in length

and with one or more double bonds in the fatty acyl chain, exert a greater antibacterial activity than lauric acid. They report differences in the antimicrobial activity of the C18 series in relation to the number of double bonds, their location from the carboxyl carbon atom and geometric isomerism as:

stearic (18:0) < elaidic (*trans*-9-18:1) < oleic (*cis*-9-18:1) < linoleic (*cis*, *cis*-9, 12-18:2) ≤ α linolenic (*cis*, *cis*, *cis*-9, 12, 15-18:3).

A similar pattern is observed with susceptible rumen microorganisms (El Hag and Miller 1972, Blaxter and Czerkwaski 1966, Matsumoto *et al.* 1991, Chalupa *et al.* 1984). The increase in antimicrobial activity with increasing unsaturation and the *cis* configuration is attributed to an increase in amphiphilic nature, i.e., increased water solubility and lipophilicity, increased surface area requirements upon adsorption onto the surface of a microorganism and the increased ability of a *cis* as compared to a *trans* configuration to disrupt membrane function (Galbraith *et al.* 1971, Gurr and Harwood 1991, Luvisetto *et al.* 1987, Stein 1981, Van Deenan *et al.* 1972). Blaxter and Czerkawski (1966) however, observed that lauric acid exerted a greater inhibitory activity against methanogenic bacteria in the rumen of sheep compared to the C18 unsaturated fatty acids. The apparent difference in activity between lauric acid and the C18 unsaturated fatty acids may result from the ability of the latter to desorb more easily from bacteria than lauric acid (Harfoot *et al.* 1974). This may result in the reversal of a bacteriostatic action of the C18 unsaturated fatty acyl series as compared to a bactericidal action by laurate.

Changing the chemical environment can alter the inhibitory activity of a lipid. To illustrate this point, Galbraith *et al.* (1971) cite the example of sodium lauryl sulphate (Figure 1.1b) which, at pH 8.0, has the same bactericidal activity when measured *in vitro* as laurate (Figure 1.1a). These workers observed that by reducing the pH of media to 6.0, the bactericidal activity of laurate, but not sodium laural sulphate increased. This effect was explained as an increase in adsorption of the unionised acid, lauric acid, at the bacterial cell wall/water interface. A reduction in pH of the media had little effect on the degree of ionisation of sodium laural sulphate and did not change its inhibitory activity. The degree of inhibitory activity of a lipid is therefore dependant upon its physicochemical properties under a given set of chemical conditions.

1.3. The inhibitory action of lipids in the rumen.

The physical and chemical actions of lipids that are reported to inhibit the capacity of the rumen to ferment plant biomass have been summarised by Devendra and Lewis (1974) into four categories:

i) lipid coating theory (where the coating of fibre by lipid prevents the attachment and/or digestion of fibre by rumen microorganisms);

ii) specific modification of the rumen fibrolytic bacterial population;

iii) direct inhibition of rumen microorganisms in general; and

iv) an indirect effect on rumen microorganisms via a reduced availability of calcium or magnesium for their growth.

No single mode of action has been attributed to the antimicrobial activity of lipids. Rather, the available data indicates that there are a number of antimicrobial actions of lipids which may operate in concert dependant upon the conditions that prevail in a particular system. A summary of the evidence for each type of activity is presented below.

1.3.1. Surface coating theory

The addition of plant fibre to a solution of bacteria and LCFA will result in the transfer of the lipid from the bacterial cell coat to the fibre surface (Harfoot *et al.* 1974). This indicates a high affinity of both the bacterial and fibre surfaces for NEFA.

Digestion of fibre in the rumen involves attachment of bacteria to the fibre via the cell coat (Costerton *et al.* 1978, Latham *et al.* 1978, Akin and Barton 1983, Gong and Forsberg 1989, Roger *et al.* 1990). Certain lipids including LCFA have surface active properties which alter the degree of wetting (hydrophobicity or hydrophilicity) of surfaces including that of fibre (Durham 1961). Devendra and Lewis (1974) hypothesised that deposition of triglyceride on plant fibre will reduce surface wetting and therefore the ability of microorganisms to attach to it.

There is however further evidence questioning this theory. Devendra and Lewis' use of non ionic detergents to increase the hydrophilicity of the surface of plant fibre did not alter the degree of attachment of rumen microorganisms. Additionally, increasing the hydrophobicity of a surface is observed to promote attachment of both gram negative and gram positive bacteria. This occurs as a result of a greater reduction in free surface energy on attachment of the bacteria to a less hydrophilic surface, i.e., increasing the hydrophobicity of a surface is energetically favourable for the attachment of bacteria (Van Loosdrecht *et al.* 1987, Legay-Carmier and Bauchart 1989, Rijnaarts *et al.* 1993). This effect may, in part, explain a lack of antagonism to the inhibitory action of triglyceride by surfactants observed by Devendra and Lewis (1974). The presence of LCFA's in the rumen is therefore unlikely to prevent the attachment of rumen microorganisms to fibre.

1.3.2. Modification of the cellulolytic population in the rumen

Supplementation of ruminant livestock with a source of NEFA alters the relative biomass of rumen microorganisms, in particular it reduces numbers of viable cellulolytic and methanogenic bacteria and anaerobic fungi (Blaxter and Czerkawski 1966,

Clapperton and Czerkawski 1969, Maczulak *et al.* 1981, Wolin and Miller 1983, Ushida *et al.* 1992). Different microbial groups and species within groups in the rumen demonstrate different levels of susceptibility to NEFA. These differences will be discussed more fully in section 1.4. The range of concentrations at which NEFA will inhibit the activity of susceptible microorganisms is sufficiently wide so that some species will be inhibited or killed at concentrations of NEFA that will allow another species to continue growing (Henderson 1973, Maczulak *et al.* 1981). The methanogenic bacteria and anaerobic fungi appear to be more susceptible to NEFA than the celluloytic and non cellulolytic bacteria (Blaxter and Czerkawski 1966, Ushida *et al.* 1992). A loss of the anaerobic fungi in the rumen may have a small effect on the rate of fermentation of plant fibre in the rumen as cellulolytic bacteria can perform this function. A loss of methanogenic activity in the rumen will reduce the growth rate of some species of cellulolytic bacteria, eg., *Ruminococcus flavefaciens*, but will have no effect on the growth rate of another cellulolytic species, eg., *Butyrivibrio fibrisolvens* (Wolin and Miller 1983). A graded increase in the amount of NEFA in the rumen is then likely to selectively remove or inhibit the growth of different microbial groups and species within groups that are responsible for the fermentation of plant fibre in the rumen. Given that graded increases in the rate of inclusion of lipid in a ruminant diet can produce a similarly graded reduction in the rate of digestion of plant fibre (Kowalckyk *et al.* 1977, Palmquist and Jenkins 1982), it seems likely that an increase in the rate of growth of the less susceptible cellulolytic bacterial species can only partially compensate for the loss of the more susceptible species.

A sufficiently high concentration of NEFA can also remove all celluloytic microbial activity from the rumen. McAllan *et al.* (1983) report complete inhibition of digestion of xylose and cellulose in the rumen of sheep on inclusion of linseed oil at 7% of the animal's feed intake. The digestion of mannose, arabinose and galactose was reduced by 16 to 40% and digestion of the neutral detergent fibre fraction was reduced by around 38% of the unsupplemented animal. These workers suggest either a complete removal of the hemicelluloytic and cellulolytic bacteria or the complete inhibition of their glycan hydrolase enzymes was responsible for this effect.

1.3.3. Inhibition of rumen microorganisms in general

Inhibition of intracellular processes

Lipid can disrupt microbial intracellular processes as a result of its permeation of a cell membrane. Disruption can occur through protonophoric activity by NEFA within the membrane (Livesey 1985, Luvisetto *et al.* 1987), increasing the permeability of the membrane to ionic species (Stein 1981, Melchior 1981), changing the membrane structure near functionally important proteins and the binding of lipid to membrane proteins which

can lead to a disruption of the functions of those proteins (Gurr and Harwood 1991, p. 24). A reduction in a microorganisms ability to regulate transmembrane ion and metabolite exchange and maintain transmembrane potentials is associated with an increase in its energetic cost of maintenance and growth (Czerkawski 1986, Luvisetto *et al.* 1987, Dürre *et al.* 1988, Ketlaars and Tolkamp 1992, Melchior 1981, Newbold *et al.* 1993). This can result in a reduced rate of growth, stasis or death of the microorganism. Where cell death does not occur, it is possible that the rate of growth of the microorganism may be reduced to the point at which its rate of cell division is insufficient to maintain its presence in the rumen and cell washout may occur.

Inhibition of extracellular processes

Surface proteins in the cell membrane possess anionic, cationic and hydrophobic sites that allow interaction and communication between the cell and the external environment (McHelaney 1982). The binding of LCFA to or near charged groups or hydrophobic sites of membrane proteins or enzymes is capable of altering the charge or the nature of those sites of the cell membrane. This can lead to disruption of the function of the protein or enzyme (Gurr and Harwood 1991, p. 24).

Evidence for inhibition of the function of cell surface proteins was provided by Coles and Lichstein (1963). Uranyl ions were observed to compete with LCFA for binding sites on the cell surface of *Lactobacillus arabinosus*. Inclusion of uranyl ions with LCFA *in vitro* prevented inhibition of malic enzyme (an extracellular enzyme) but not glycolysis (an intracellular enzyme). Coles and Lichstein (1963) suggested that due to the binding of lipid to membrane proteins or enzymes the extracellular enzymes was more susceptible than intracellular enzyme to an inhibitory action of LCFA. Cellulolytic enzymes also act extracellularly. In some cellulolytic rumen bacteria cellulolytic enzyme has been observed to be released as part of a discrete membrane bound organelle into the space formed between the bacteria and the substrate (Coughlan 1991). It is possible cellulolytic enzyme activity may be susceptible to disruption by certain lipids.

1.3.4. An indirect effect on rumen microorganisms via reduced availability of calcium and magnesium for growth

The need for calcium and magnesium for the growth of a number of key rumen cellulolytic bacterial species and including *Fibrobacter succinogenes* was reported by Hubbert et al. (1958) and Bryant *et al.* (1959). Calcium and magnesium are essential for a range of functions performed by microorganisms including:

i) activation of enzyme complexes through binding to cell membrane phospholipid and membrane proteins (Avery and Tobin 1993);

ii) transport of metabolites (Wong 1993);

iii) membrane fusion within and between cells (Gurr and Harwood 1991);

iv) adhesion of bacteria to substrate (Marshall 1971); and

v) initiation of cell growth and division (Friis 1994).

The rumen fibrolytic bacteria and probably fungi also have requirements for calcium for fibrolytic enzyme conformation, stability and attachment (Coughlin 1991). A deficiency of ionised calcium or magnesium in the rumen is therefore likely to reduce the capacity of the rumen to ferment plant fibre.

In aqueous solution non esterified LCFA can react with magnesium and calcium to form a precipitate (Merck 1989). This process reduces the concentration of ionised calcium or magnesium in solution. Ferlay and Doreau (1995) observed rumen fluid ionised calcium concentration, but not magnesium concentration, was reduced in cattle fed a mixed ration of hay, grain and rapeseed oil, the latter being a source of non esterified LCFA's. This indicates that the chemical conditions found within the rumen were unfavourable to the formation of magnesium but not calcium soaps of LCFA's. A deficiency of calcium, but possibly not magnesium, for microbial function could occur under these conditions.

A reduction in the availability of calcium or magnesium for microbial function in response to non esterified fatty acid can not fully explain the toxic effect of the non esterified fatty acid. The polar detergents, eg., sodium laural sulphate (Figure 1.1b) and sodium oleyl sulphate have a limited ability to bind calcium or magnesium (Moilliet *et al.* 1961) but are toxic to a range of rumen and non rumen bacteria (Blaxter and Czerkawski 1966). This indicates that fatty acyl compounds possess an antimicrobial action beyond their capacity to reduce the availability of calcium (or magnesium) for microbial cell processes.

1.4. Sensitivity of different rumen microorganism to lipid

1.4.1. Bacteria

Adhesion and penetration of NEFA onto and into the bacterial cell wall occurs with both gram positive and gram negative bacteria (Galbraith and Miller 1973a,bandc, Ibrahim *et al.* 1993). The gram positive bacteria however appear to be more susceptible to NEFA (Nieman 1954, Galbraith *et al.* 1971). Some putative gram negative species including strains of *Butyrivibrio fibrisolvens* are also susceptible to exogenous NEFA (Henderson 1973, Maczulak *et al.* 1981). In the case of *B. fibrisolvins* this anomaly may be explained by the species possessing a gram positive cell wall structure as determined by electron microscopy (Maczulak *et al.* 1981). Two of the three main fibre digesting rumen

bacterial species *Ruminococcus flavefaciens* and *R. albus* as well as the main methanogenic genera in the rumen, *i.e,. Methanobacterium sp.* are classified as gram variable to gram positive and are susceptible to the antimicrobial action of lipid (Hungate 1966, Maczulak *et al.* 1981). The third important fibre digesting species, *Fibrobacter succinogenes,* possesses a gram negative cell wall structure (Hungate 1966) and is less susceptible to the antimicrobial action of lipids compared to the *Ruminococci.* (Maczulak *et al.* 1981).

The structural differences between the two main cell wall types of bacteria suggest reasons for the differences in susceptibility bacteria to certain lipids (Galbraith *et al.* 1971, Henderson 1973). Gram negative species of bacteria have a high concentration of lipid in their cell wall. These endogenous lipids resist the penetration of the cell membrane by exogenous lipids (Russell *et al.* 1990, Gurr and Harfoot 1991, pp. 265 - 281). The gram positive bacteria possess a simpler cell wall structure with a low lipid content (Gurr and Harfoot 1991, pp. 265 - 281). This type of cell wall is more easily penetrated by exogenous NEFA (Galbraith *et al.* 1971). The higher internal osmotic pressures generated by gram positive bacteria compared to gram negative species may also make this group more prone to lysis when loss of the integrity of the cell wall occurs in response to NEFA (Galbraith *et al.* 1971).

1.4.2. Ciliate protozoa

The rumen ciliate protozoal population is sensitive to the amount of lipid entering the rumen environment. Supplementation or infusion of medium and long chain saturated and unsaturated fatty acids (Czerkawski *et al.* 1975, Ikwuegbu and Sutton 1982, Matsumoto *et al.* 1991, Ushida *et al.* 1992) or alkyl detergents, eg., sodium oleyl sulphate (Orpin 1977, Bird 1982, Yang and Varga 1993) at levels that reduce the rate of fermentation of plant fibre may defaunate the rumen (Bird 1982, Yang and Varga 1993). This suggests that the ciliate protozoa are inhibited at lower concentrations of lipid than the susceptible rumen bacterial population (Ushida *et al.* 1992). This may be due to the slower turnover times of the ciliate protozoa resulting in a reduction in their ability to grow through a period of toxicity induced by supplemental lipid (Ferlay and Doreau 1992) and a lower ability to avoid the uptake of lipid due to its ingestion with food particles and bacteria (Harfoot *et al.* 1974).

1.4.3. Anaerobic fungi

Ushida *et al.* (1992) report a reduction in the number of culturable zoosporangia and zoospores from rumen fluid of sheep supplemented with calcium soaps of medium and LCFA. This effect is probably the result of dissociation of calcium soaps leading to increased exposure of the rumen biota to NEFA (Sukhija and Palmquist 1990).

Under the same conditions, counts of total and cellulolytic bacteria were unaffected by the treatment. This suggests that the rumen anaerobic fungi are more sensitive to a source of exogenous lipid than rumen bacteria and as with bacteria, their susceptibility to certain lipids may result from the structure of their cell wall.

The anaerobic fungi have cell walls that are up to 90% polysaccharide with a low lipid content (Avery and Tobin 1993). The fungal cell wall may therefore be an ineffective barrier to penetration of the cell membrane by lipid. This group may also need higher levels of Ca^{2+} for fibrolytic enzyme function and attachment to substrate (Coughlin 1991). It is likely however that lipid does not penetrate the plant tissue that is accessed by fungal rhizoids and this would offer some protection to the fungi from exogenous lipid. If this hypothesis is correct then the zoospore may be the most susceptible stage of the fungi's lifecycle.

1.5. Antagonists to the antimicrobial activity of lipid

1.5.1. Emulsifying agents

Emulsifying agents such as bile salts, bovine serum albumin, lecithin, α tocopherol, calciferol, ergocalciferol act to stabilise the formation of lipid micelles (Durham 1961, Galbraith *et al.* 1971, Galbraith and Miller 1973b). This reduces the concentration of LCFA in solution and, as a result, their toxicity to microorganisms is also reduced (Hutner 1942, Kodicek and Worden 1945, Galbraith *et al.* 1971, Galbraith and Miller 1973b). The ability of emulsifying agents to moderate the toxic effects of LCFA appears to apply to the rumen environment. Zinn (1989) reported a partial restoration of the digestibility of fibre within the rumen of cattle fed a mixed animal vegetable fat included at 4% of intake when lecithin was included in the diet at 2% of intake.

1.5.2. Adsorbent agents

Adsorbent agents such as cellulose, plant fibre, starch, silicic acid and fullers earth act to remove LCFA from solution and as a result reduce the exposure of microorganisms to lipid (Hutner 1942, Foster and Wynne 1948, Hardwick *et al.* 1951, Nieman 1954, Harfoot *et al.* 1974, Maczulak *et al.* 1981, Kemp *et al.* 1984a and b, Firkins *et al.* 1991). The bacterial surface competes with plant fibre for the adsorption of LCFA but not for triglyceride (Harfoot *et al.* 1974). The addition of adsorbent agents are then observed to reduce inhibitory effects of LCFA on bacterial growth (Maczulak *et al.* 1981, Kemp *et al.* 1984a and b, Firkins *et al.* 1991).

1.5.3. Cations

The presence in solution of calcium and magnesium ions has long been observed *in vitro* to reduce or remove the inhibitory effects of LCFA on microorganisms (Kodicek and Worden 1945, Galbraith *et al.* 1971, El Hag and Miller 1972, Galbraith and Miller 1973aandc, Hoover *et al.* 1989, Firkins *et al.* 1991, Offer and Offer 1992) and enhance the fermentation of plant fibre by rumen microorganisms (El Hag and Miller 1972, Jenkins and Palmquist 1982, Drackley *et al.* 1985, Palmquist *et al.* 1986, Offer and Offer 1992). Calcium ions are more effective than magnesium ions in reducing the antimicrobial action of fatty acids (Galbraith and Miller 1973a). Galbraith *et al.* (1971) reported beneficial effects on bacterial growth rates measured *in vitro* on including calcium ions at up to 20 times the concentration of added LCFA. These workers found that magnesium ions produced only a small reversal effect on the antimicrobial action of LCFA, even when included at 40 times the concentration of the LCFA.

The differences in the effects of calcium and magnesium may relate to differences in solubility and interfacial tensions produced by magnesium as compared to calcium soaps in solution (Galbraith and Miller 1973a). Galbraith *et al.* (1971) suggested that the formation of calcium soaps of lineolate both removes the fatty acid from solution and also increases the interfacial tension between the hydrophobic fatty acids and the media. This may result in a large reduction in concentration of fatty acids in solution. Magnesium salts of lineolate form a limited amount of precipitate only. Other cations including ferric iron, barium, beryllium and strontium are reported as being ineffective in reducing the antimicrobial action of LCFA or are in themselves inhibitory to fibre digestion or bacterial growth (El Hag and Miller 1972, Galbraith and Miller 1973a).

1.5.4. Biohydrogenation

The rumen ecosystem has adapted to deal with the quantities and types of lipids normally found in pasture species. The predominant fatty acid ingested by grazing or roughage fed ruminants is α linolenic, linoleic (Figure 1.2c) and palmitate with smaller amounts of oleic (Figure 1.2b) and stearic acids (Hawke and Silcock 1970, Harfoot 1981). These occur primarily as the galactosyl glycerides and phospholipids of plant membranes (Hawke and Silcock 1970, Harfoot 1981). Although LCFA is not digested within the rumen, poly- and mono- unsaturated fatty acids undergo biohydrogenation and fatty acids may be elongated up to 18 carbon atoms (Wu *et al.*, 1991, Wu and Palmquist 1991). The fatty acid flowing from the reticulo rumen therefore is predominantly stearate (18:0) and vaccenate (*trans*-11-18:1) (Figure 1.2a) (Ekeren *et al.* 1992, Fotuhi and Jenkins 1992, Kemp *et al.* 1984).

The process of biohydrogenation therefore results in the production of LCFA of reduced toxicity to rumen microorganisms (Kemp *et al.* 1984). Increasing the amount of lipid and/or starch in the rumen environment is however observed to reduce the numbers of bacteria involved in lipolysis and/or biohydrogenation (Kemp *et al.* 1984, Gerson *et al.* 1985) and to saturate the biohydrogenating capacity of the rumen (Hawke and Silcock 1970). This results in increased levels of unsaturated fatty acids within the rumen environment (Wu and Palmquist 1991) and in the digesta flowing to the lower tract (Wu *et al.* 1991).

A prerequisite for biohydrogenation of fatty acids is the hydrolysis of the ester linkage between the fatty acid and glycerol moiety of the triglyceride. This action is carried out by acyl hydrolases including lipases, galactosidases and phospholipases and results in the release of fatty acid with a free carboxyl group (Hawke and Silcock 1970, Hawke 1971, Fotouhi and Jenkins 1992). From this point a wide range of fatty acid isomers can be generated by a cohort of rumen microorganisms (Kemp *et al.* 1984a and b). At each step in the process of biohydrogenation, LCFA of lower toxicity is produced. A description of the process and direction of biohydrogenation illustrates the range of fatty acid isomers that can be generated within the rumen.

The first step in the process of hydrogenation of linoleic (*cis, cis*-9, 12-18:2) or α linolenic acids (*cis, cis, cis*-9, 12, 15-18:3) involves in both cases the isomerisation of the *cis*-12 bond to form the *cis*-9 *trans*-11 or *cis*-9 *trans*-11 *cis*-15 conjugated fatty acyl di- or tri- enes respectively (Kemp *et al.* 1984a and b, Gurr and Harwood 1991). This is followed by the hydrogenation of the *cis*-9 bond by a reductase and the hydrogenation of the *cis*-15 double bond in the case of α-linolenic acid to produce the penultimate end product, *trans* vaccenic acid (*trans*-11-18:1) (Figure 1.2a) (Kemp *et al.* 1984a and b, Gurr and Harwood 1991). Hydrogenation of the *trans*-11 double bond results in stearate (18:0) as the end product of biohydrogenation however the production of stearate appears to occur only slowly in the rumen. Harfoot (1981), in a review paper, stated that only two species of rumen bacteria have been isolated on their ability to hydrogenate vaccenate to produce stearate. While the relative biomass of these two isolates was not stated, it appears that there is limited capacity among rumen bacteria to reduce vaccenate to stearate. Vaccenate can therefore form a large proportion of total LCFA flow from the rumen on the addition of vegetable oil to an animal's diet.

1.5.5. Uptake and adsorption of lipid by rumen bacteria

Rumen bacteria are reported to incorporate exogenously derived mono- and poly-unsaturated lipid into their cell membranes (Demeyer *et al.* 1978), form lipid droplets within their cytoplasm (Bauchart *et al.* 1990) and adsorb, predominantly saturated, lipid

onto their exterior surface (Harfoot *et al.* 1974). As it is unlikely that the lipid can be oxidised as an energy yielding substrate (Czerkwaski 1986 p. 202) and the process of biohydrogenation does not regenerate significant quantities of oxidised pyridine nucleotides (Blaxter and Czerkawski 1966), it is unlikely that bacteria benefit significantly from either the uptake of fatty acid or the process of biohydrogenation. The uptake and adsorption of lipid by bacteria will remove it from the rumen environment. Lipid will be removed faster from a rumen that exhibits high rates of turnover of bacteria and small particles (Harfoot *et al.* 1974). The ability of the rumen environment to tolerate lipid is then, in part, proportional to the animal's feed intake.

1.5.6. Rumen soluble calcium

Compounds that provide soluble calcium in the rumen, *eg.*, lucerne ash, calcium chloride, calcium lactate and calcium hydroxide have been included at 1 - 3% of ruminant diets that contain added lipid at 5 - 10% of the diet with the intention of precipitating LCFA as calcium soap (Swift *et al.* 1951, Miller *et al.* 1970, El Hag and Miller 1972, Palmquist *et al.* 1986, Offer and Offer 1992). While these workers report rumen function is improved by this treatment this has not been attributed entirely to the formation of calcium soaps. Jenkins and Palmquist (1982) and Palmquist *et al.* (1986) report that calcium soap formation in the rumen was increased by use of soluble calcium but was primarily dependant on LCFA concentration. This lead Palmquist *et al.* (1986) to conclude that "the beneficial effects of added calcium in high fat diets were not caused by increased calcium soap formation".

Ferlay and Doreau (1995) and Palmquist *et al.* (1986) reported that inclusion of a source of LCFA in the diet of cattle significantly reduced the concentration of ionised calcium but not magnesium in rumen fluid. Ferlay and Doreau (1995) suggested that at least part of the inhibitory action of lipid within the rumen may be a result of a reduction in available or free ionised calcium ($Ca^{2+}.12H_2O$). In the light of the innovative work of Ferlay and Doreau (1995) and that of Palmquist *et al.* (1986) it is appropriate to consider supplementation of livestock with sufficient rumen soluble calcium to meet the requirements of the rumen microorganisms as well as the animal's physiological needs.

1.6. Lipid supplements which have a reduced toxicity in the rumen

By understanding the nature of the antimicrobial action of lipids in the rumen a number of modified fat based supplements have been developed which have a reduced toxicity within the rumen. These include hydrogenated fats, blends of animal and vegetable fats, calcium soaps of LCFA, encapsulated fat and fatty acyl amides.

Hydrogenated fats

Fat supplements can be hydrogenated, i.e., the double bonds in the fatty acyl chain are reduced using heat and catalysts (usually finely divided nickel), to increase the content of saturated fatty acids (Whitten and Gailey 1984). This process increases the melting point and decreases rumen solubility of the fat (Chalupa *et al.* 1986, Grummer 1988, Jenkins and Jenny 1989). The effectiveness of these products in maintaining rumen function is primarily dependant upon the degree of saturation achieved in the hydrogenation process (Drackley and Elliot 1993). The inhibitory effects of the fat are reduced but not completely overcome and enable the inclusion of up to 6% additional LCFA in an animals diet without significantly altering rumen function (Grummer 1988, Jenkins and Jenny 1989, Eastridge and Firkins 1991, Drackley and Elliot 1993).

Dietary lipids with a high content of saturated fatty acid however have a reduced digestibility in the small intestine. Jenkins and Jenny (1989) report a 30% reduction in whole tract apparent digestibility of a hydrogenated (99% saturated medium and LCFA) compared to unmodified fat (43% saturated medium and LCFA). The reduction in intestinal digestibility is proportional to the degree of saturation of the LCFA (Palmquist 1988). Some of the benefit gained in protecting the rumen from the effect of the fat is then lost due to a reduction in the digestible energy value of the diet (Jenkins and Jenny 1989, Eastridge and Firkins 1991, Borsting *et al.* 1992, Weisbjerg *et al.* 1992, Drackley and Elliot 1993).

Blends of animal and vegetable fat

Blending fats with different levels of saturation is reported to reduce both the toxicity of the individual fats and to increase the intestinal digestibility of the saturated fatty acids through a synergistic action with the unsaturated fatty acids (Chalupa *et al.* 1986, Ohajuruka *et al.* 1991, Wu *et al.* 1991, Borsting *et al.* 1992). Ohajuruka *et al.* (1991) report that incorporation of a fat blend at up to 5% of the animals intake had no effect on rumen function. The data supporting the views of Ohajuruka *et al.* (1991) as well as the findings of Chalupa *et al.* (1986), Wu *et al.* (1991) and Borsting *et al.* (1992) were obtained in studies using highly productive dairy cows fed diets high in concentrates. Fibrolytic activity in the rumen of these animals would already be low and the model would be insensitive to the effect of the lipid. This practice may therefore be ineffective in production systems where roughage is a significant component of the animal's feed intake.

Calcium soaps of LCFA

Calcium soaps of fatty acids can have a sufficiently low solubility within the rumen to exert no toxic effect on rumen microorganisms. Supplementation of ruminant

diets with calcium soaps of palm oil fatty acids has allowed the inclusion of additional LCFA up to 9% of total intake without altering the digestion plant fibre or metabolism in rumen (Schauff and Clark 1992).

Calcium soaps can undergo acid hydrolysis in the rumen where they dissociate with the release of NEFA and calcium. The extent to which they become dissociated appears to be proportional to the total number of double bonds in the fatty acyl chain and to number of double bonds with the *cis* configuration and is inversely proportional to the number of carbon atoms in the fatty acyl chain and pH (Sukhija and Palmquist 1990, Ushida *et al.* 1992, Ferlay *et al.* 1992, Doreau *et al.* 1993). Sukhija and Palmquist (1990) reported calcium soaps of fatty acids from soya bean oil, predominantly made up of mono- and poly-unsaturated LCFA's with the *cis* configuration, to be 80% dissociated in a buffer solution at pH 5. Calcium soaps of stearate were 25% dissociated at this pH. At pH 6.5 calcium soaps of soya bean oil were 11% dissociated compared to 1% for those of stearate.

As calcium soaps can dissociate to NEFA within the rumen they cannot be considered to be completely inert. Including a calcium soap in a ruminant diet rather than its equivalent NEFA reduces the degree of exposure of rumen microorganisms to NEFA. They therefore exert a low level of toxicity rather than zero toxicity.

Encapsulated fat

Encapsulation of fat within an insoluble or slowly fermentable organic matrix limits its exposure within the rumen and 'protects' the lipid from biohydrogenation. Formation of a slowly rumen fermentable matrix has been achieved by treating casein and seed proteins with formaldehyde (Storry *et al.* 1973, Goering *et al.* 1977, Sutton *et al.* 1983, McAllan *et al.* 1983, Ashes *et al.* 1992), extrusion of oil seeds under increased pressure with steam to produce the Maillard reaction thereby lowering protein solubility (Kim *et al.* 1993) and heating blood serum to precipitate proteins thereby reducing their solubility in the rumen (Ekeren *et al.* 1992). Dependent upon the efficacy of the encapsulation process, inhibition of rumen function by lipid is reduced or prevented (Storry *et al.* 1973, Goering *et al.* 1977). The reduction in the amount of unsaturated LCFA undergoing biohydrogenation provides a method of increasing the flow of unsaturated fatty acids to the duodenum (Goering *et al.* 1977, Ashes *et al.* 1992). The latter effect is reported to improve digestibility of supplementary fat and alter the fatty acid profile of animal products (Scott and Cook 1975, Ashes *et al.* 1992, Borsting *et al.* 1992, Ekeren *et al.* 1992).

The efficacy of any method of encapsulation in maintaining rumen function and preventing biohydrogenation of the lipid varies according to the extent to which the

lipid is encapsulated and in the degree to which the encapsulating matrix can resist fermentation in the rumen. Ekeren *et al.* (1992) report near complete biohydrogenation of oleate encapsulated with calcium alginate within the rumen of cattle due to the presence of hydrolytic enzymes active against the encapsulant. In this work, the degree to which the supplemental lipid had undergone biohydrogenation was used as an indicator of the efficacy of the encapsulating process. Scott *et al.* (1971) using a lipid supplement encapsulated with formaldehyde treated seed protein report nearly complete prevention of biohydrogenation of the lipid and no disruption to rumen function in sheep fed 6.5% of their intake as encapsulated lipid. Feeding the equivalent unprocessed lipid however, reduced the animal's ability to digest fibre and feed intake. McAllan *et al.* (1983) and Sutton *et al.* (1983), using a lipid supplement encapsulated with formaldehyde treated casein, achieved only partial success in preventing biohydrogenation of the lipid and maintaining rumen fibrolytic activity. Possession of the appropriate technology and technique is therefore a prerequisite for the successful application of this process.

Fatty acyl amides

These compounds, including N-stearoyl-D, L-methionine (Langar *et al.* 1978) and N-linoleoyl-methionine (Fotouhi and Jenkins 1992), contain an ester linkage which is slowly or non hydrolysable in the rumen but is hydrolysed after passing through the abomasum. The fatty acyl amides appear not to react with microorganisms and they do not inhibit rumen function. Due to their high cost of production, these compounds have particular application for research purposes (Fotouhi and Jenkins 1992).

1.7. Conclusion

Ruminant animals fed a roughage based diet consume a number of lipid types, some of which possess antimicrobial activity. Antimicrobial activity of a lipid is the result of the presence of a polar head group and an alkyl chain containing 8 or more carbon atoms which confers both solubility in aqueous solution and lipophilicity. Non esterified fatty acids and polar detergents possess these properties and can exhibit an antimicrobial action. Triglyceride, waxes and carotenoid pigments, while meeting the definition of a lipid do not exhibit an antimicrobial action.

There appears to be no single mechanism that can explain the antimicrobial nature of lipids. A number of mechanisms including altered intracellular and extracellular protein or enzyme function, loss of cell membrane integrity and a reduction in the availability of calcium may act to produce stasis or death of microorganisms. Not all species of rumen microorganisms are susceptible to the antimicrobial action of lipids. The gram positive bacteria, which includes key fibrolytic species, the methanogens, ciliate

protozoa and the anaerobic fungi are susceptible. Their susceptibility appears to relate to having a cell wall structure of low lipid content and to the relative importance of the activity of extracellular enzymes for their maintenance and growth. The gram negative bacteria, which includes the majority of the non fibrolytic bacterial species, are in general, less susceptible to these lipids.

A number of natural processes can be antagonistic to the antimicrobial action of LCFA. Absorbent agents such as plant fibre and starch, emulsifying agents such as lecithin and tocopherol and cations including calcium and magnesium can reduce the concentration of LCFA in solution. These agents reduce the exposure of susceptible microorganisms to the LCFA. Similarly, adsorption uptake and biohydrogenation of LCFA by certain rumen bacteria can also reduce its concentration within the rumen environment. Using processed lipids such as hydrogenated fats, calcium soaps of LCFA's and encapsulated fats in a ruminant diet can also be effective in reducing the concentration of LCFA in the rumen of lipid supplemented animals. Up to the level at which the small intestine can digest dietary lipid, the maximum level of inclusion of lipid in a ruminant's diet may be limited by the point at which it reduces the fermentation of cellulose. In comparison, the maximum level of inclusion of supplemental lipid in the diet of a monogastric animal is primarily set by its requirement for a balanced array of nutrients.

Chapter 2
The effect of LCFA on digestion of dietary components and synthesis of microbial biomass in the rumen: a review.

2.1. Introduction

The previous chapter reviewed the effects of a range of non esterified fatty acids on the growth of microorganisms. The current chapter will focus on the effects of LCFA, i.e., fatty acid with 16 or more carbon atoms in the fatty acyl chain, on various processes involved in the fermentative digestion of dietary components by microorganisms in the rumen.

A review of studies into the effect of LCFA on rumen metabolism and fermentative processes indicates that a diverse range of experimental methods have been used. The methodology is determined by the questions to be answered and the interpretation of the results is restricted by the limitations of the methodology. In an attempt to compare the findings of different researchers three primary categories are defined in this chapter which provide a framework for interpreting the range of different results. The first category is based on the proportion of the animal's diet that was made up of roughage and attempts to reflect the relative importance of the fermentation of plant fibre to satisfy the nutrition of the animal. The second category refers to the level of metabolisable energy intake of an animal relative to its metabolic size and is used to broadly infer the level of turnover of feed from the rumen of an animal. Animals will be categorised as being fed at around maintenance metabolisable energy intake, around 1.5 x maintenance or around 2 x maintenance. The third category refers to the species of experimental animal used. Data arising from studies of cattle will be distinguished from data arising from sheep as a reduction in the digestibility of a diet in response to an increase in dietary LCFA content may disadvantage the capacity of sheep to maintain their dry matter intake to a greater extent than cattle. No distinction has been made for the type of productive function of the animal. This is based on an assumption that type of productive function will have no significant feed back effect on rumen function that can not be accounted for by the animal's energy intake.

2.2. Factors affecting the fermentation of plant structural carbohydrate in the rumen in response to LCFA

Level of inclusion of LCFA

Studies using sheep fed medium to high roughage content diets at maintenance or 1.5 times maintenance energy intake indicate there is a good (negative) correlation between dietary content of rumen soluble LCFA and the digestion of plant fibre in the rumen (Kowalzyck *et al.* 1977, Henderson *et al.* 1977, Ikwuegbu and Sutton 1982). Where the feed intake of cattle fed a medium to high roughage content diet is similarly restricted, as with sheep, linear decreases in the digestion of plant fibre in the rumen have been observed in response to incremental increases in the content of fat or oil in the animal's diet (Zinn 1989, Palmquist *et al.* 1993). This relationship is less apparent in studies using cattle fed at a high level of feed intake or fed a diet with a high starch content (Weisbjerg *et al.* 1992, Jenkins and Jenny 1989).

Type of LCFA

The properties of a LCFA that determine the level at which it will exert antimicrobial activity when measured *in vitro* are its chain length and the number, location and type of double bonds (see section 1.2). The LCFA in lipids derived from natural sources are heterogenous with respect to these properties. The heterogeneity of the LCFA's in naturally occurring triglycerides produces problems in attempting to define key criteria that will quantify and predict its antimicrobial activity in the rumen when included in a diet.

Chalupa *et al.* (1984) suggested that as melting point of a LCFA is determined by its chain length and the number, location and configuration of double bonds, melting point of a lipid may provide sufficient information to provide a relative measure of its antimicrobial activity. Chalupa *et al.* (1984) used changes in the ratios of volatile fatty acids produced by cultures of mixed rumen microorganisms as an index of their antimicrobial activity. These workers found that melting point of LCFA as a function of fatty acyl chain length from C12 to C22 and for C18 fatty acyl series with respect to number of double bonds and their configuration to be negatively correlated ($r = -0.93$ to -0.95) with its antimicrobial activity when measured *in vitro*. Chalupa *et al.* (1986) partially confirmed this finding in the rumen using a low melting point LCFA, oleic acid (*cis*-9-18:1) with a melting point of 16°C, and a high melting point LCFA, stearic acid (18:0), with a melting point of 70°C. The fatty acids were included at 10% of dry matter in a diet containing equal portions of concentrate and roughage and fed to cattle at around their

maintenance energy intake. Stearic acid, decreased the ratio of acetate to propionate in rumen fluid by 20% whereas oleic acid, depressed this value by 60%.

Pantoja *et al.* (1994) suggested the number of double bonds in a quantity of lipid would adequately describe its antimicrobial activity in the rumen. The number of double bonds in a lipid is given by its iodine value, i.e., the weight of iodine which will react with a given weight of lipid. Hydrogenated tallow, tallow and tallow/vegetable oil blend, with iodine values of 18, 62 and 84 gI/100g LCFA (16, 50 and 65 molar % unsaturated LCFA content respectively) was included in a medium roughage content diet at 5% of intake of cattle fed around twice their maintenance energy intake. Digestion of plant fibre (neutral detergent fibre) in the rumen was not affected by inclusion of hydrogenated tallow or tallow but was reduced by 10% on inclusion of the animal vegetable fat blend. This observation lead these workers to suggest that a trigger level for the degree of unsaturation of a lipid must be reached before a lipid is active in the rumen.

Level of feed intake

The inclusion of a source of LCFA in the diet of an animal fed at a high ruminally fermentable energy intake is often observed to have a small effect on the rate of digestion of plant fibre in the rumen whereas similar sources and quantities of LCFA included in the diet of animals fed for low to moderate ruminally fermentable energy intake has depressed the digestion of fibre (Kowalckyk 1977, Palmquist and Conrad 1978, Palmquist and Jenkins 1980, Bird and Dicko 1987, Zinn 1989, Weisbjerg *et al.* 1992, Palmquist *et al.* 1993). This difference may, in part, result from differences in the rate of clearance of LCFA from the rumen. A high intake of a rapidly ruminally fermentable feed is observed to increase the passage rate of the fluid and small particle fractions of the digesta from the rumen to the lower digestive tract (Kashe and Engelhardt 1990). More LCFA is associated with small as opposed to large particles (per unit mass of particles) resulting in selective removal of LCFA from the rumen (Harfoot *et al.* 1974). Increasing the rate of turnover of fluid and small particles from the rumen by increasing the level of intake of an animal, particularly by increasing the digestibility of its diet, may reduce the time that freshly ingested LCFA can disrupt the processes of fermentation. The apparent toxicity of the LCFA would then be reduced.

Level of starch in the diet

A higher level of digestibility of a diet is often achieved by increasing the level of inclusion of starch based concentrates. Increasing the content of starch in the diet of a ruminant is generally observed to reduce the digestibility of fibre in the rumen (Hoover 1986). This response is primarily the result of a reduction in the concentration of fibrolytic

bacteria and the activity of their β-glycan hydrolase enzymes which may result from competition for nutrients by the starch digesting microorganisms, changes in rumen fluid chemistry, particularly pH, and increased rates of dilution of rumen digesta (Latham *et al.* 1972, Hoover 1986, Allen and Mertens 1988). The digestion of starch and soluble sugars in the rumen is reported to be unaffected or increased by the inclusion of a source of LCFA in the diet of ruminants at a level sufficient to inhibit the digestion of plant fibre (Johnson and McClure 1972, McAllan *et al.* 1983, Zinn 1988, Elmeddah *et al.* 1991). As reliance on the fermentative digestion of plant fibre to maintain the animal's nutritional status decreases so will the effect that LCFA exerts on the capacity of the rumen to fermentatively digest the basal diet.

Rumen fluid ammonia concentration

Rumen fluid ammonia concentration has been observed to decrease in response to an increase in the content of LCFA in an animals diet (Henderson *et al.* 1977, Kowalczyk *et al.* 1977, Ikwuegbu and Sutton 1982, Doreau *et al.* 1991,Wiesbjerg *et al.* 1992, Doreau *et al.* 1993). This is correlated with a reduction in the concentration of protozoa in rumen fluid (Henderson *et al.* 1977, Ikwuegbu and Sutton 1982). Restoring ammonia concentration in the rumen to control levels appears not restore the rate of digestion of dry matter in the rumen. In the study by Henderson *et al.* (1977), including tallow at 10% of the intake of pasture hay offered *ad libitum* to sheep reduced the digestion of dry matter in the rumen over 24 hours from 69 g/100 to 63 g/100 and reduced rumen fluid ammonia concentration from 18 to 9.7mg/100ml when measured 4 hours after feeding. Inclusion of urea at 1.5% of dry matter increased the concentration of rumen fluid ammonia to 17.6mg/100ml for the tallow + urea diet however this had no significant effect in elevating the rate of digestion of dry matter. Orskov *et al.* (1978) also observed no reversal of a depression in the rate of digestion of a diet of dried grass fed to sheep at *ad libitum* intake and supplemented with tallow up to 13% and urea at 1.5% of dry matter in the diet. In this study including urea with tallow produced a partial reversal of a depression in feed intake in response to tallow. Tallow at 13% inclusion reduced the animals dry matter intake from 83 to 52g dry matter/kg liveweight$^{0.75}$. Animals supplemented with urea and tallow had a dry matter intake of 63g dry matter/kg liveweight$^{0.75}$. In both studies the inclusion of urea had no effect on the rate of digestion of dry matter in the rumen.

2.3. The effect of LCFA on the processes of fermentation in the rumen

2.3.1. Hydration of freshly ingested feed

Mathematical models proposed by Allens and Merten (1988) and Van Milgen *et al.* (1993) which are descriptive of the process of fermentation in the rumen suggest that the rate of hydration of a feed is a factor contributing to fermentation lag time but not to the extent to which fibre is digested. Hydration of a feed increases its specific gravity (Welch 1986) which assists it to mix with the actively fermenting feed and rumen liquor (Wyburn 1980 p. 37, Welch 1986, Czerkawski 1986 p. 32). When a feed is coated with lipid, the hydrophobic action of the lipid could decrease its rate of hydration and mixing with the actively fermenting rumen contents, increasing the time to commencement of digestion (lag time). Allens and Merten (1988) propose that an increase in lag time will increase the total fibre pool within the rumen and reduce the animals feed intake due to increased gut load.

A study by Drackley *et al.* (1994) appears to support this hypothesis. These workers compared the effect of coating either the roughage portion, concentrate portion or total mixed ration fed to cattle with melted tallow at 5% of the animals intake. The diet was made up of equal portions of roughage and protein and starched based concentrate. A 12% reduction in feed intake was observed in animals when tallow coated the total mixed ration but not the other two treatments. Characteristics descriptive of the rumen fermentation process (pH, VFA production, NH_3 concentration) and dry and organic matter digestibilities were not affected by the treatment suggesting no antimicrobial effect of the LCFA. Drackley *et al.* (1994) concluded that the reduction in the animal's intake was the result of physical changes in feed characteristics.

2.3.2. Microbial Colonisation

The effect of LCFA on microbial colonisation of fibre has previously been discussed (see section 1.3.1). There evidence was presented that the presence of triglyceride or LCFA on a surface may be energetically favourable to the attachment of bacteria. Microbial colonisation of forage in the rumen is also observed to increase on inclusion of triglyceride in an animal's diet.

Legay-Carmier and Bauchart (1989) and Firkins *et al.* (1991) observed that the addition of soya-bean oil to a high roughage content diet to provide LCFA at 14 and 9% of the diet respectively, increased microbial colonisation of the surface of plant fibre in the rumen of cattle. The extent of microbial colonisation of plant fibre was measured both by electron microscopy and the amount of nucleic acid associated with plant fibre. In

explanation of this response, Firkins *et al.* (1991) suggested that rations with a high roughage content may have a sufficiently large surface area so as to disperse triglyceride or LCFA to the point that it has no effect on microbial colonisation. Legay-Carmier and Bauchart (1989) proposed that as their respective treatments contained large amounts of calcium the antimicrobial action of the LCFA may have been reduced. They suggested two mechanisms to explain an effect of calcium and LCFA on microbial attachment to fibre in the rumen.

i) An increase in the concentration of Ca^{2+} on the fibre surface which due to preferential adsorption of LCFA stimulates electrostatic attraction of bacteria to fibre; and

ii) Stimulation of capsule production by bacteria, and therefore an increase in 'stickiness', in response to energy spared from *de novo* synthesis of fatty acids by bacteria due to uptake of exogenously supplied fatty acids.

Regardless of the mechanism involved there is substantive evidence to indicate that the inclusion of triglyceride in an animal's diet will have no detrimental effect on microbial colonisation of plant material.

2.3.3. Enzymic Hydrolysis

Addition of a source of free LCFA in the diet of a ruminant can reduce the rate of digestion of the plant cell wall fraction of a feed in the rumen in a dose dependant manner (Kowalckyk *et al.* 1977, Palmquist and Jenkins 1982, Ferlay and Doreau 1992). At high levels of inclusion the digestion of cellulose can be completely inhibited (Sutton *et al.* 1983). The digestion of the constituent sugars of the hemicellulose fraction is not affected equally so that the digestion of mannose, arabinose and galactose is affected less by the presence of free LCFA than xylose (McAllan *et al.* 1983). In particular the amount of xylose and cellulose entering the duodenum increases in response to an increase in the quantity of free LCFA within the rumen (McAllan *et al.* 1983). The rate of fermentation of plant fibre in the rumen is reduced and this increases the quantity of potentially digestible plant fibre entering the duodenum (Orskov *et al.* 1978, McAllan *et al.* 1983). The amount of potentially ruminally digestible material in a diet therefore does not change. McAllan *et al.* (1983) attribute the reduction in the digestion of plant fibre to either the removal of the hemicellulolytic and cellulolytic bacteria (see sect 1.4) or the inhibition of their fibrolytic enzymes, the β-glycan hydrolases.

There appear to be no reports describing the direct interaction of LCFA with the glycan hydrolase enzymes of microorganisms. A reduction in the concentration of ionised calcium in incubation media is however, observed to alter the rate of digestion and mode of action (endoglucanase to an exoglucanase) of a β-glycan hydrolase isolated from the rumen bacteria *Ruminococcus albus* AR67 (Gregg 1994 pers. comm.). Calcium

specific metallo amylases and glycan hydrolases are common within the rumen (Rumbak *et al.* 1991, Chauvaux *et al.* 1990). This observation has lead Ferlay and Doreau (1995) to speculate that a reduction in the concentration of free ionised calcium in the rumen in response to removal of calcium ion from solution by precipitation with LCFA will reduce fibrolytic enzyme activity.

Amylase activity, as measured by the rate digestion of starch in the rumen, is reported to remain unchanged or has increased on increasing the amount of LCFA in an animal's diet (Johnson and McClure 1972, McAllan *et al.* 1983, Zinn 1988, Elmeddah *et al.* 1991). The apparent increase in amylase activity may however relate to more of the enzyme being present in the rumen of these animals. Concentrations of LCFA's that are inhibitory to fibrolytic bacteria and protozoa have little effect on those microorganisms that ferment starch and soluble sugars (Hungate 1966, Henderson 1973, Maczulak *et al.* 1981). A reduction in the concentration of protozoa in rumen fluid in response to an increase in dietary LCFA content appears to favour the growth of the non fibrolytic/starch fermenting bacteria and this may promote the digestion of starch in the rumen (Ikwuegbu and Sutton 1982, Sutton *et al.* 1983, Weisbjerg *et al.* 1992).

2.3.4. Rumen liquid and solid digesta kinetics

There appears to be limited data describing the response of rumen liquid and solid digesta kinetics to intake by an animal of LCFA of high microbial toxicity. Czerkawski *et al.* (1975) report a 26% increase in rumen fluid volume and a 18% increase in rumen fluid dilution rate (non significant) on inclusion of linseed oil in the diet of wethers at 9% of their dry matter intake as LCFA. Ikwuegbu and Sutton (1982) also report a 26% increase in rumen fluid volume but observed a 15% (non significant) decrease in rumen fluid dilution rate on inclusion of linseed oil in the diet of wethers at 6.3% of dry matter intake as LCFA. In the study by Ikwuegbu and Sutton (1982) the animals that were supplemented with linseed oil increased their feed intake by 9%. In the study of Czerkawski *et al.* (1975) the animals feed intake did not vary with treatment. Given the level of inclusion of linseed oil in the diets used in both studies the digestion of plant fibre is likely to have been substantially if not completely inhibited (Sutton *et al.* 1983). It is likely that in both cases feeding linseed oil has slowed the dilution rate of solids in the rumen. This may have increased the volume of solids in the rumen and as a consequence increased rumen liquid volume.

In studies where inclusion of a source of LCFA in the diet of cattle has little or no effect on digestion of plant fibre in the rumen, rumen liquid and solid digesta volume appears consistently to be reduced in response to an increase in liquid and particle dilution rates (Chalupa *et al.* 1986, Boggs *et al.* 1987, Ohajuruka *et al.* 1991, Weisbjerg *et al.*

1992). The increase in the dilution rate of both liquid and particles may, in part, relate to the nature of a lipid supplement to contribute to the animal's solid matter intake but to associate with the fluid, bacteria and fine particle fractions in the rumen (Harfoot *et al.* 1974). By associating with these fractions a lipid may turnover more quickly from the rumen and increase the average turnover rate for solids compared to the unsupplemented diet.

2.4. The effect of LCFA on nitrogen kinetics in the rumen

2.4.1. Proteolysis - dietary and endogenous protein

Digestion of true protein in the rumen appears to be unaffected by inclusion of a source of LCFA at a level sufficient to reduce the rate of digestion of fibre in feed rations of low, medium or high roughage content (Sutton *et al.* 1983, Boggs *et al.* 1987, Zinn 1989, Ohajuruka *et al.* 1991, Weisbjerg *et al.* 1992, Palmquist *et al.* 1993 and Yang and Varga 1993). This may be the result of the presence of proteolytic bacteria in the rumen in roughage and grain fed animals that are tolerant to LCFA (Hungate 1966, Henderson 1973, Maczulak *et al.* 1981).

2.4.2. Non protein nitrogen

Urease activity and the enzymes required to convert other sources of non protein nitrogen, *eg.*, nucleic acids and their derivatives, amides and nitrate, to ammonia are found in bacteria tolerant to LCFA (Hungate 1966, Henderson 1973, Maczulak *et al.* 1981). It is therefore unlikely that the availability of N from urea or other source of non protein nitrogen in the rumen will be affected by the presence of quantities of LCFA that will disrupt the digestion of plant fibre.

2.4.3. Microbial protein synthesis and turnover

Increasing the concentration of LCFA in the rumen is observed to alter both the efficiency of synthesis bacterial protein per g of organic matter fermented and the amount of bacterial protein flowing to the duodenum. Differences in the solubility and degree of unsaturation of the LCFA, which affects its relative antimicrobial activity, will also alter the extent to which these parameters are affected by it. Increasing the concentration of LCFA of high microbial toxicity, such as unsaturated LCFA with the *cis* configuration, in the rumen environment appears to reduce amino acid and probably peptide uptake by bacteria and reduces predation of bacteria and ingestion of feed particles by protozoa (Galbraith and Miller 1973c, Wallace and MacPherson 1987, Ikwuegbu and Sutton 1982).

Galbraith and Miller (1973c) observed that the rate of amino acid uptake by bacteria was reduced at sub bacteriacidal concentrations of LCFA when measured *in vitro*. These workers propose that this effect is the result of uncoupling of energy dependant processes responsible for amino acid uptake by bacteria, processes which are more sensitive to inhibition by LCFA than that of oxidative phosphorylation and ATP production. In the rumen, this response would be expected to decrease the efficiency of synthesis of microbial 'crude' protein per g organic matter fermented in the rumen (EMCP). In the rumen however, other factors are also involved in determining the EMCP and the inclusion of LCFA of high microbial toxicity has been observed to increase both the flow of microbial cells to the duodenum and EMCP (Ikwuegbu and Sutton 1982, Sutton *et al.* 1983).

Predation of bacteria and probably fungal zoospores by small protozoa is suggested by Wallace and MacPherson (1987) as the most important cause of microbial protein turnover and therefore reduced efficiency of microbial crude protein synthesis in the rumen. Ikwuegbu and Sutton (1982) and Sutton *et al.* (1983) have observed an increase in the flow microbial organic matter in the rumen in response to an increase in LCFA with a high microbial toxicity in an animal's diet. This was correlated with a reduction in the concentration of protozoa in the rumen. In the study by Sutton *et al.* (1983), inclusion of linseed oil in the diet of sheep to provide 6.3% of the animal's intake as additional LCFA resulted in an 80% reduction in the concentration of protozoa in rumen fluid. Organic matter digestibility in the rumen was reduced by 35%, the flow of diaminopimelic acid (DAPA) to the duodenum increased by 80%, RNA by 46% and the EMCP increased two to three fold dependant on the method of estimation (DAPA or RNA concentration) of microbial protein content of digesta. The EMCP in the rumen however appears to respond quadratically to linear increases in the dietary content of LCFA. Ikwuegbu and Sutton (1982) observed efficiency of microbial crude protein synthesis to increase by 50% on inclusion of linseed oil in a medium roughage content diet fed to sheep to provide 3.6% of intake as additional LCFA. Inclusion of linseed oil to provide 1.8 or 5.4% additional LCFA however had no effect on the flow of microbial organic matter from the rumen or EMCP. These workers proposed that inclusion of the LCFA at the higher level reduced both the activity of the small protozoa and the activity of the microorganisms primarily involved in fermenting biomass in the rumen. This offset any gain in microbial flow to the duodenum or EMCP arising from a reduction in predation of microorganisms.

Additional dietary LCFA therefore appears to act at two levels to alter the net synthesis of microbial protein in the rumen. The first level is a reduction in the population of the most sensitive microorganisms in the rumen, including the protozoa, anaerobic fungi and methanogenic bacteria. A reduction in the numbers of small entodiniomorphid

protozoa will reduce the predation of bacteria and therefore increase both the flow microbial cells from the rumen and EMCP (Harwood and Geyer 1964, Demeyer and Van Nevel 1975, Chalupa *et al.* 1984, Ushida *et al.* 1992). A second level of action of LCFA occurs where the growth of both the protozoal population and the microorganisms primarily involved in fermenting biomass in the rumen is inhibited. At this level of inclusion a reduction in the synthesis of microbial organic matter in response to LCFA is not offset by a reduction in the predation of microorganisms by protozoa and therefore microbial protein turnover. Both the flow of microbial cells from the rumen and EMCP is then reduced (Ikwuegbu and Sutton 1982, Sutton *et al.* 1983).

LCFA of moderate solubility and/or toxicity is also observed to alter microbial protein synthesis in the rumen in a quadratic fashion. This may relate to changes in digestion kinetics in response to LCFA. Weijsberg *et al.* (1992) report that the inclusion of tallow to provide 1.8% of intake as LCFA of cattle fed a medium roughage content ration at around 1.5 x their maintenance energy requirement increased the turnover rate of rumen digesta by 14% and increased the microbial protein flowing to the duodenum by 16%. At this level of feed intake, microbial protein flow to the duodenum and rate of turnover of rumen digesta fell to control levels at 5.4% of additional dietary LCFA. When cattle were fed the same diets at around maintenance energy intake the flow of microbial cells to the duodenum, rumen fluid protozoal concentration and organic matter digestion in the rumen were not affected at any level of LCFA supplementation. They concluded that an increase in the turnover rate of rumen digesta was primarily responsible for the increase in microbial protein flow to the duodenum in response to LCFA.

2.5. The production of methane and the volatile fatty acids acetate, propionate and butyrate in the rumen in the presence of LCFA's

The inhibitory effect of lipid on microbial metabolism is specific to certain groups of microorganisms particularly species of cellulolytic bacteria, the ciliate protozoa, anaerobic fungi and methanogenic bacteria (Clapperton and Czerkawski 1969, Maczulak *et al.* 1981, Sutton *et al.* 1983, Zinn 1989, Ushida *et al.* 1992). The effect of this specificity is to change the molar ratios of the VFA produced in the rumen resulting in higher production of propionate and reduced production of acetate and butyrate (Latham *et al.* 1972, Sutton *et al.* 1983, Chalupa *et al.* 1984). This is the result of the following processes:

i) inhibition of the acetate and/or butyrate producing microorganisms;

ii) inhibition of methanogenic bacteria leading to accumulation of hydrogen in the rumen; and

iii) an increase in the partial pressure of hydrogen which favours the production of propionate at the expense of acetate.

The effect of LCFA on the production of VFA in the rumen will be discussed in this section.

2.5.1. Inhibition of acetate and/or butyrate producing microorganisms

Where a ruminant consumes a diet with a medium to high roughage content, the fibrolytic bacteria *Fibrobacter succinogenes, Ruminococcus flavefaciens, R. albus* and *Butyrivibrio fibrisolvens*, cellulolytic ciliate protozoa eg. *Diplodinium*, non cellulolytic ciliate protozoa, i.e., both Holotrichs and Entodiniomorphs and the anaerobic fungi contribute significantly to the rumen fermentative processes (Hungate 1966, Wolin and Miller 1983, Bauchop 1989, Akin and Borneman 1990). Acetate and/or butyrate are the only VFA produced by these species although *F. succinogenes* and *R. flavefaciens* will also produce succinate and this will contribute to the rumen propionate pool (Hungate 1966). All these species appear to be susceptible to inhibition by LCFA (see section 1.4.1). The significant rumen bacterial species that have limited susceptibility to LCFA, eg., *Selenomonas ruminantium, Prevotella ruminicola*, produce relatively more succinate, and ultimately propionate, than these susceptible species (Wolin and Miller 1983). Increasing the LCFA content of a diet with a high roughage content will therefore reduce the capacity of rumen to produce acetate and butyrate but not propionate per unit of organic matter fermented (Deymeyer and Van Nevel 1975, Wolin and Miller 1983). The total VFA concentration is also observed to decrease (Chalupa *et al.* 1984) and this suggests that the rate of fermentation of organic matter has been reduced (Leng 1970).

2.5.2. Inhibition of the methanogenic bacteria

The fermentation of carbohydrate and the production of ATP for microbial growth and maintenance in the rumen is predominantly via the Empden Meyerhoff Parnass pathway and the pyruvate lyase reactions (Deymeyer and Van Nevel 1975). Electron transfer linked phosphorylation also exists in the rumen at least in some bacterial species and probably in the anaerobic fungi (Erfle *et al.* 1986). Substrate based phosphorylation however involves two key reactions and is associated with electron transfer:

i) the NAD^+ linked dehydrogenation of glyceraldehyde-3-phosphate; and

ii) the pyruvate lyase reactions with the production of acetyl Co-A and ultimately acetate.

Reduced pyridine nucleotide cofactors are generated in both these reactions. These cofactors need to be reoxidised to allow the fermentation of carbohydrate to continue. Succinate, lactate, ethanol, formate and hydrogen gas are formed in the process of reoxidation of these cofactors (Hungate 1966).

The methanogenic bacteria utilise the hydrogen formed in this process for the reduction of carbon dioxide to form methane and ATP (Hungate 1966). This reaction is capable of maintaining a low partial pressure of hydrogen in the rumen (below 1 Pa) (Blaxter and Czerkawski 1966, Hungate 1966, Bauchop and Mountford 1981). LCFA's and in particular lauric acid and the unsaturated LCFA's with the *cis* configuration are observed to inhibit the methanogenic bacteria (Blaxter and Czerkawski 1966, Czerkawski *et al.* 1966). Inclusion of LCFA with antimicrobial activity in ruminant dietary rations is therefore associated with a reduction in methanogenic activity and as a result an increase in the partial pressure of hydrogen in the rumen (Clapperton and Czerkawski 1969).

Increased production of propionate at the expense of acetate

The extent to which electrons are directed to the production of acetate or propionate by some microbial species is dependant on the partial pressure of hydrogen in the rumen and therefore the activity of the methanogenic bacteria (Chen and Wolin 1977). Bacterial species including *Ruminococcus flavefaciens, R. albus,* some strains of *Selenomonas ruminantium*, the ciliate protozoa and the anaerobic fungi are able to regenerate oxidised cofactors by the reduction of protons to form hydrogen (Hungate 1966, Chen and Wolin 1977, Latham and Wolin 1977, Wolin and Miller 1983). The ciliate protozoa and anaerobic fungi are able to regenerate oxidised cofactors in a similar manner (Hungate 1966, Wolin and Miller 1983). In protozoa and anaerobic fungi the enzymes required for this process are located in a subcellular organelle called a hydrogenosome (Paul *et al.* 1990, Müller 1993).

In *R. flavefaciens* and *R.albus,* a low partial pressure of hydrogen favours the formation of acetate from pyruvate with hydrogen acting as the electron sink product for the regeneration of NAD^+ (Latham and Wolin 1977). A high partial pressure of hydrogen, *i.e.*, above 1 Pa, produces conditions thermodynamically unfavourable for the regeneration of NAD^+ using this pathway. Regeneration of NAD^+ in *R. albus* is then achieved by the reduction of acetyl-CoA to ethanol. In *R. flavefaciens* and *S. ruminantium* the regeneration of NAD^+ can also be achieved by the reduction of oxaloacetate to malate; a process that is not inhibited by hydrogen (Wolin 1974, Wolin and Miller 1983). Carbon flow is then directed to propionate via succinate. The alternate paths utilised *by S. ruminantium* and *R. flavefaciens* at high and low partial pressures of hydrogen are outlined in Figure 2.1

The hydrogenosomes of those rumen anaerobic fungi and ciliate protozoa studied to date are reported by Yarlett *et al.* (1981), Yarlett *et al.* (1986) and Müller (1993) to ferment pyruvate to acetate with the formation of hydrogen (Figure 2.2). The fermentative pathways outlined in Figure 2.2 appear to be typical of the anaerobic fungal genus *Neocallimastix sp.* with the possible exception of *N. frontalis* (Müller 1993). The

pathways of fermentation of malate/pyruvate for this genus appears to be similar that of the hydrogenosomes of the ciliate protozoa *Dasytricha ruminantium* except that *D. ruminantium* regenerates butyrate $NAD(P)^+$ by the condensation of two Acetyl-CoA molecules (Yarlett *et al.* 1985) and this pathway is not inhibited by high partial pressures of H_2 gas in the rumen.

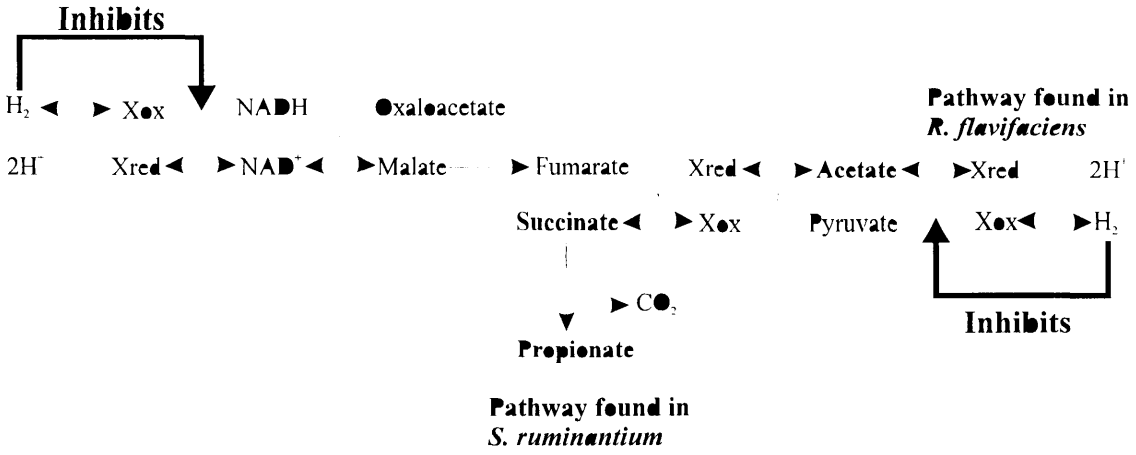

Figure 2.1. Acetate and succinate/propionate formation in *R.. flavefaciens* and *S. ruminantium*. X represents 'intermediate' compounds involved in electron transfer (Wolin and Miller 1983). Inhibition of the transfer of electrons from NADH to an oxidised intermediate occurs at a partial pressure of H_2 exceeding 1 Pa and favours the formation of propionate. An active methanogenic bacterial population will prevent inhibition of these processes and favour the formation of acetate at the expense of succinate/propionate by maintaining H_2 partial pressure below 1 Pa.

A reduction in methanogenesis in the rumen resulting in an increase in the partial pressure of hydrogen inhibits the transfer of electrons from NADPH via ferridoxin to protons. This results in a reduction in the production of acetate and hydrogen (Figure 2.2). The anaerobic fungi, as with the bacterial species described above, then use alternate pathways to regenerate NAD^+ with the production of formate, lactate and ethanol. These alternate pathways however reduce the yield of ATP per unit of carbohydrate fermented and as a result the yield of microbial biomass per unit organic mater (Bauchop and Mountford 1981). The regeneration of $NAD(P)^+$ through the production of butyrate by the ciliate protozoa is not affected by a loss of methanogenesis, however as with the anaerobic fungi, the yield of ATP per unit of organic matter fermented is reduced (Leng 1970, Miller and Jenesel 1979, Wolin and Miller 1983, Yarlett *et al.* 1985).

The antimicrobial action of certain LCFA and other lipids in the rumen can therefore result in a reduction in the contribution to fermentation end product by groups of microorganisms and change the fermentation pattern of key microbial species. Under these conditions the production of propionate is increased and that of acetate and butyrate

decreased. The relative shift in the ratios of production of the volatile fatty acids can therefore be predictive of the degree of toxicity of lipid in the rumen (Czerkawski 1973, Czerkawski *et al.* 1966) when it is considered in association with other factors that influence rumen VFA pattern.

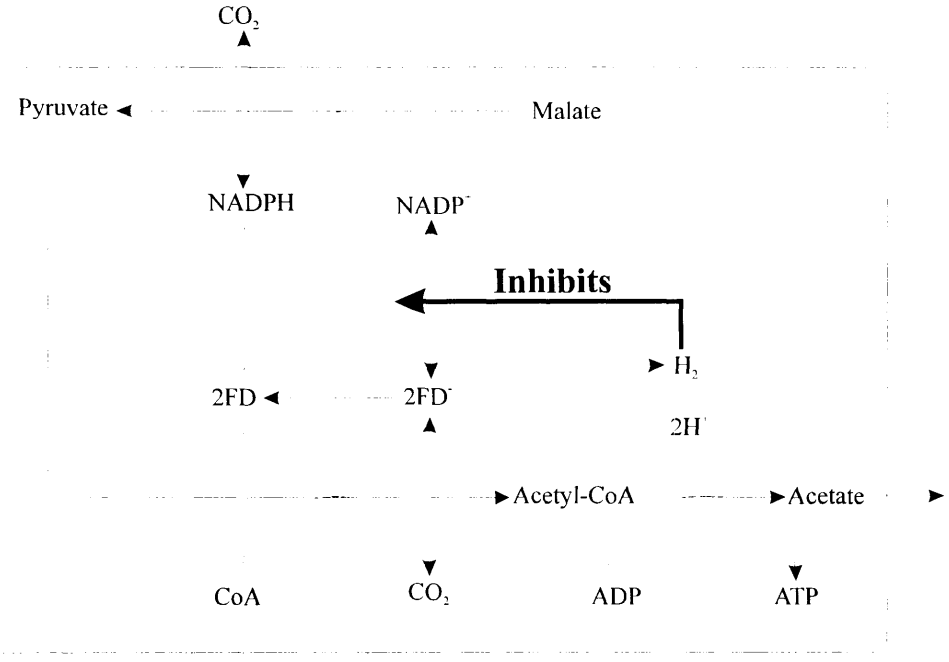

Figure 2.2. The fermentation of malate/pyruvate to acetate and hydrogen within a hydrogenosome typical of the rumen anaerobic fungal genus *Neocallimastix* and the ciliate protozoa (adapted from Yarlett *et al.* 1981 and Muller 1993). The hydrogenosome (delineated by the single line) of the ciliate protozoa however can also regenerate butyrate by condensation of two acetyl-CoA molecules. This pathway is not inhibited by high partial pressures of H_2 gas. As with the rumen bacteria, the pressure of H_2 gas produced in the rumen in the absence of methanogenic activity is sufficient to inhibit the transfer of electrons from NADPH to an oxidised intermediate, in this case ferridoxin. This in turn inhibits the production of acetate.

2.6. Conclusion

Inclusion of a source of LCFA in feed, at a level sufficient to reduce the digestibility of plant fibre in the rumen, appears to have small but variable effects on rumen digesta kinetics and ammonia concentration. The concentration of acetate and butyrate compared to that of propionate in rumen fluid is reduced. The level of feed intake of the animal and the content of roughage in the diet appear to be important determinants of the extent to which LCFA will affect fermentative digestion in the rumen. Animals fed around twice their maintenance energy and/or with 50% or more of the animals dietary energy intake as starch based concentrate appear to be less sensitive to the antimicrobial effects of LCFA compared to animals fed at maintenance energy intake or consuming a diet that provides most of the animals nutrition from the fermentation of roughage in the rumen.

Not all the components of the plant fibre fraction are inhibited by the presence of LCFA's to the same extent. Digestion of xylose and cellulose is depressed to a greater extent than that of arabinose, mannose and galactose. The digestion of plant non structural carbohydrate and dietary crude protein does not appear to be inhibited by LCFA.

The flow of microbial cells to the small intestine and the efficiency of microbial protein production (g MCP/g organic matter fermented in the rumen) appears to increase at levels of inclusion of LCFA in a diet sufficient to suppress the ciliate protozoal population without markedly suppressing the fibrolytic bacterial population. A reduction in the rate of predation of bacteria by the small ciliate protozoa in the rumen appears to increase the number of non fibrolytic bacteria flowing from the rumen. At some point of inclusion LCFA will decrease the concentration of fibrolytic bacteria sufficiently so that a reduction in the rate of fermentation of the basal diet and therefore production of bacterial cells may offset the gains made by the reduction in predation of the non fibrolytic bacteria. Consequently the effect of lipid on the total flow of microbial cells from the rumen appears primarily to be dependant upon a balance between the suppression of the growth of the ciliate protozoa while minimising the fibrolytic bacteria.

Chapter 3
The role of calcium in rumen function: a review.

3.1. Introduction

The concentration of calcium ion in the rumen of sheep and cattle has been observed to decrease in response to increases in dietary content of LCFA as triglyceride (Palmquist *et al.* 1986, Ferlay and Doreau 1995). It is possible that inclusion of LCFA in an animals diet will affect rumen function by causing a deficiency of ionised calcium for microbial growth. This chapter reviews the involvement of calcium in rumen function.

3.2 The recycling of endogenous calcium and its availability from dietary sources

Durand and Kawashima (1979) report that the calcium concentration in the rumen fluid of sheep grazing temperate pasture may vary from 2 - 11mM. Goetsch and Owens (1985) and Yano and Kawashima (1979) report the calcium concentration in the rumen fluid of cattle and sheep fed diets with a cereal grain content of 80% of dry matter and calcium content of 0.25 to 1.1% of dry matter to vary from 0.5 to 6mM.

Sources of calcium entering the rumen include the saliva, net absorption or efflux across the rumen wall and diet. The concentration of calcium in mixed saliva produced by sheep varies from 0.4 to 0.75mM or 16 to 30mg calcium/l saliva (McDougal 1948). Bailey (1958) measured the daily saliva production of 3 adult shorthorn cows eating either grass or hay offered *ad libitum* to be 74 to 110 l/day. The salivary input due to rumination was not measured and would presumably increase these values. If the calcium concentration of mixed saliva from sheep and cattle is approximately equivalent, in animals consuming roughage, saliva may add in the order of 1 to 3g/d of calcium to the rumen of mature cattle and less than 1g/d to the rumen of mature sheep.

Beardsworth *et al.* (1989a and b) report the rate of absorption of calcium from the rumen may be proportional to the concentration of phosphate and possibly the electrical potential difference across the rumen wall. The absorption rate of calcium across the rumen wall was determined from its loss from 2 l of buffered solutions placed in the evacuated and washed reticulo rumen of sheep. At an initial concentration of phosphate of 2.5mM and calcium concentration of 2mM the net absorption of calcium across the rumen wall when measured in 7 animals ranged from -2.5 to 5μmol/l buffer solution/min (Beardsworth *et al.* 1989b). Increasing the concentration of phosphate in the buffer solution appeared to linearly increase the absorption of calcium so that at a phosphate

concentration of 17.5mM the net absorption of calcium ranged from 8 to 22μmol/l buffer solution/min. At concentrations of potassium of 30 and 90mM and no phosphate in a buffer solution the net absorption of calcium varied from 3.2 to 3.7μmol/l buffer solution/min (Beardsworth *et al.* 1989a). Beardsworth *et al.* (1989a) suggested that the concentration of potassium will affect the rate of passive diffusion of calcium ion by changing the potential difference across the rumen wall.

The concentrations of phosphate in rumen fluid from sheep grazing temperate pasture may be 5 to 30mM and up to 45mM in sheep fed a grain based diet (Emmanuel *et al.* 1969, Durand and Kawashima 1979, Yano and Kawashima 1979). Assuming that the values for calcium absorption of Beardsworth *et al.* (1989a and b) will reflect the normally functioning rumen of either cattle or sheep, cattle and sheep with a rumen liquid volume of 50 l and 5 l respectively may have a net absorption of calcium across the reticulo rumen wall in the order of 0 to 0.2 g/d and 0 to 2g/d respectively over a range of ruminal phosphate concentrations from 2 to 20mM. Estimates for the net absorption of calcium from the rumen is then of the same order of magnitude as estimates of inputs of calcium to the rumen from saliva. Ruminal absorption of calcium may then provide a mechanism for the recycling of salvary calcium.

Durand and Kawashima (1979) suggest that dietary sources may supply 95% or more of the calcium entering the reticulo rumen of sheep grazing improved temperate pasture. When ruminants are fed a diet high in concentrates or a diet which is finely ground or pelleted, the importance of diet in supplying calcium to the rumen may be even higher as endogenous supply of calcium may decrease in response to reduced salivary flow while net absorption across the rumen wall may increase where cereal grains increase the concentration of phosphate in rumen fluid. Variation in the amount of calcium in a diet, its rumen solubility and rumen fluid dilution rate may then be the primary factors that determine the concentration of calcium in rumen fluid.

The solubility in rumen fluid of calcium from inorganic sources varies widely. Palmquist *et al.* (1986) measured the dissociation of calcium orthophosphate and calcium carbonate in aqueous solution to be 10% and 0.02% respectively and calcium chloride to be completely dissociated. These workers found aqueous solubility of these compounds to closely reflect their solubility in rumen fluid. Inclusion of calcium chloride in a mixed diet (1:1 cereal grain + roughage) plus tallow at 10% of dry matter, increased the calcium concentration in the diet from 0.60% to 0.96 but increased rumen fluid ionised calcium concentration from 0.60mM to 1.82mM. In the same study inclusion of a mixture (1:2) of calcium orthophosphate and calcium carbonate in the diet increased dietary calcium concentration to 1.10% of dry matter but had no effect on rumen fluid ionised calcium

concentration (0.60 vs 0.62mM). Clark *et al.* (1989) also report the solubility in rumen fluid of calcium carbonate was negligible when it was included up to 2.1% of feed dry matter in a 60% concentrate 40% corn silage diet offered *ad libitum* to cattle. Clark *et al.* (1989) however did not measure rumen fluid calcium concentration but inferred the low solubility of calcium carbonate from the absence of a response of rumen fluid pH and VFA production to inclusion of calcium carbonate in the diet.

Goetsch and Owens (1985) in contrast to Palmquist *et al.* (1986) and Clark *et al.* (1989) report an increase in rumen fluid calcium concentration in response to feeding cattle calcium carbonate. Goetsch and Owens (1985) report that the concentration of calcium ion in rumen fluid of cattle fed at maintenance energy intake increased from 1.4mM to 4.1mM on the inclusion of calcium chloride at 1% of diet dry matter and to 6mM on inclusion of calcium carbonate at 2.5% of diet dry matter. Rumen fluid pH was 5.99 for the unsupplemented diet, 5.93 on inclusion of calcium chloride and 6.05 on inclusion of calcium carbonate which suggests that acid hydrolysis of the calcium carbonate was not responsible for the increase in calcium concentration in rumen fluid (Clarke *et al.* 1989). The animals intake of calcium in this study was reported to increase from 14.4g/d to 63.6g/d, the flow of calcium to the duodenum from 16.9 to 110g/d and net retention of calcium from 11.3 to 55.3g/d on inclusion of calcium carbonate in the diet. The latter set of figures is unlikely (SCA 1990) and Goetsch and Owens (1985) suggested that either recycling of calcium from endogenous sources to the reticulo rumen may have taken place or sampling errors may have occurred.

If recycling has occurred a significant increase in the input of calcium from saliva is unlikely. The concentration of calcium in saliva is near saturation and is fixed by the need to maintain phosphate ion in solution (McDougal 1948). There is also no reason to believe salivary flow to the rumen increased with the addition of calcium carbonate to the diet (Bailey 1958). A net flow of calcium into the reticulo rumen across the reticulo rumen wall would explain these data but would be in disagreement with the findings of Beardsworth *et al.* (1989b) and suggests that the animal has absorbed considerably more calcium from the lower digestive tract than it required for the maintenance of physiological calcium homeostasis (SCA 1990). Physiological calcium homeostasis in ruminants is believed to be primarily controlled by the net absorption of calcium from the lower digestive tract and urinary calcium excretion (Braithwaite 1974, Braithwaite 1978, Care *et al.* 1980). The study by Goetsch and Owens (1985) suggests that excretion of calcium into the reticulo rumen may also have a role in an animal's maintenance of its physiological calcium homeostasis and could modify the concentration of calcium in the rumen. A study by Yano and Kawashima (1979) appears to support this hypothesis. These workers report that rumen fluid calcium concentration increased from 0.67mM to 2.1mM on inclusion of

calcium carbonate at 1.6% of feed dry matter and which increased calcium content of feed dry matter from 0.44 to 1.88% in the diet of sheep fed a 90% concentrate 10% rice straw diet twice daily at maintenance energy intake. This response was observed 6 hours but not 3 hours after feeding. The apparent 6 hour delay in this response may reflect the time course for the passage, absorption from the lower tract and secretion of calcium back into the rumen.

3.3 The role of calcium in attachment of bacteria to plant fibre and the activity of their fibrolytic enzymes

Attachment of bacteria to the surface of plant fibre is recognised as a prerequisite to the digestion of plant fibre in the rumen (Mackie and White 1990). Attachment and adhesion of bacteria to a surface occurs in two phases and involves the extracellular polysaccharide coat of the microorganism (capsule) (Marshall *et al.* 1971, Costerton *et al.* 1978, Latham *et al.* 1978, Vandevivere and Kirchman 1993).

i) Phase one is reversible and involves electrostatic and hydrophobic interactions between the surface of the substrate and the bacterial capsule which hold the bacterium in close association with the substrate.

ii) Phase two is non reversible and involves the formation of covalently bonded linkages between carbohydrate based polymers of the capsule or cell wall of the bacteria and the surface of the substrate.

The extent to which bacteria bind through initial phase processes was interpreted by Marshall *et al.* (1971) as being dependant on a balance between the Vanderwaals attractive forces that exist between the bacteria and the substrate and the repulsive energies associated with an electrically diffuse double layer that exists around the bacteria and the surface of the substrate. Marshall *et al.* (1971) observed that initial phase binding was promoted by an increase in the molar concentration of electrolytes and particularly by the concentration of the divalent cations calcium and magnesium in media. This effect was attributed to a reduction in the width of the electrically diffuse double layer that surrounds both the bacteria and the substrate. Increasing the concentration of these cations allowed the bacteria to associate more closely with the substrate. This in turn increased their opportunities to form covalently bonded linkages (second phase attachment) to the substrate.

The process of formation of covalently bonded linkages between the bacteria and the substrate may also be dependant on the presence of divalent cations. The cations act as ligands between bacterial cell wall or capsule polymers and the substrate by forming a stable structure that promotes the formation of covalent bonds (Costerton *et al.* 1978, Roger *et al.* 1990). Rumen fibrolytic bacteria, with the possible exception of *Fibrobacter*

succinogenes, are observed to bind in this way (Akin and Barton 1983, Latham *et al.* 1978).

Evidence to support the requirement for divalent cations in binding processes may come from the effects of changes in pH in media on attachment of bacteria to surfaces. Roger *et al.* (1990) observed bacterial adhesion to plant fibre *in vitro* was reduced in response to a reduction in the pH of incubation media from 6.0 to 4.5. There was no effect of pH in the range 6.0 to 7.0. Calcium ions are displaced from the surfaces of bacteria and plant fibre by protons as pH decreases (Torre *et al.* 1992) and this would be expected to interfere with the binding processes described above.

Calcium ion is also recognised as having a role in the function, i.e., activity and/or stability, of most extracellular fibrolytic (Petipher and Latham 1979, Gardner *et al.* 1987), amylolytic (Rumbak *et al.* 1991) and proteolytic enzymes (Hazlewood and Edwards 1981) produced by rumen bacteria. In particular, the rate of hydrolysis of cellulose by enzymes produced by anaerobic bacteria appears to be dependant upon the concentration of calcium ion (Coughlin 1991, Beguin and Aubert 1994). Mg^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+} have not been observed to have a role in the activity of cellulases isolated from rumen bacteria (Gardner *et al.* 1987).

3.4 The effect of calcium ion concentration on fibrolytic enzyme activity by rumen microorganisms

The minium concentration of calcium ion sufficient to maximise the activity of cellulases isolated from the rumen has not been determined. The activity of one cellulase isolated from a non rumen bacteria, *Clostridium thermocellum*, was observed to be 0% at a concentration of calcium ion of 0.1mM increasing to 100% activity at 1mM (Morag *et al.* 1991). Durand and Kawashima (1979) report a calcium concentration of 0.25 to 1mM is required to reliably grow pure and mixed cultures of rumen microorganisms. Bales *et al.* (1978) report a concentration of calcium of 0.25mM was sufficient to maximise the digestion of sorghum stalk by rumen microorganisms when measured *in vitro*.

At sufficient concentration calcium ion may also depress the rate of digestion of plant fibre by rumen microorganisms. In the study by Bales *et al.* (1978) digestibility of sorghum stalk was reduced at a calcium concentration of 4.75mM. Goetsch and Owens (1985) report the rate of digestion of dietary acid detergent fibre fraction was reduced from 61.4% to 52.7% in the rumen of cattle fed a diet containing 80% corn grain at maintenance energy intake on increasing the calcium concentration of rumen fluid from 1.4mM to 4.1mM. The increase in calcium concentration was achieved by adding calcium chloride to the diet and had no effect on rumen fluid pH. In the study by Yano and Kawashima (1979)

(see section 3.2), the inclusion of calcium carbonate at 1.6% of feed dry matter also decreased rumen fluid phosphate concentration from 36.8mM to 27.8mM at 6 hours but not 3 hours after feeding but had no effect on rumen fluid pH, magnesium, potassium or sodium concentration. Yano and Kawashima (1979) suggest that the formation of complexes between calcium and phosphate reduced the concentration of phosphate in rumen fluid. The diets used by Yano and Kawashima (1979) contained sufficient P (0.34%) in a rumen available form (cereal grain) so that the reduction in rumen fluid phosphate concentration in response to inclusion of calcium carbonate would not have reduced the growth of rumen microorganisms (Durand and Kawashima 1979). It seems possible that a reduction in the concentration of phosphate in rumen fluid in response to increased dietary concentration of calcium could reduce microbial growth where the diet was low in rumen soluble P. Martinez (1972) cited by Bales *et al.* (1978) reported that a depression of cellulose digestion by rumen microorganisms *in vitro*, which was caused by a high concentration of calcium, was partially reversed by increasing the concentration of magnesium. Bales *et al.* (1978) suggested that calcium ion may cause a reduction in digestion of plant fibre by competitive inhibition of the uptake of other divalent cations by microorganisms or their enzymes. The effect of high levels of calcium ion in rumen fluid on the rate of digestion of plant fibre in the rumen may therefore be dependant on the concentration of other minerals necessary for microbial growth rather than its concentration *per se*. This suggests that a diet may need to be balanced both for its mineral content and for the rumen availability or solubility of those minerals to avoid causing a reduction in rumen microbial activity.

3.5 Cation exchange in the rumen

The fibre fractions of plant material possess cation exchange capacity over the physiological range of rumen fluid pH exchanging metal cations, particularly calcium, magnesium, sodium, potassium and protons from rumen fluid (Van Soest 1982, p. 184, Ha *et al.* 1983, McBurney *et al.* 1986, Torre *et al.* 1992, Salimei *et al.* 1994). Plant fibre can therefore provide a reserve of calcium and other cations and by a process of exchange buffer the rumen against changes in the concentration of these cations and pH. McBurney *et al.* (1986) have measured the cation exchange capacities of rye grass, lucerne, grain oats and cottonseed hulls to be 350, 450, 300 and 1450meq./kg dry matter respectively. In contrast the endosperm of seed grains may have a cation exchange capacity of 1 to 3meq./kg so that it is the hull material that contributes most of the cation exchange capacity. Cation exchange capacity of plant material will increase as digestive fermentation proceeds so that rumen solids may have at least the cation exchange capacity of their parent material (Salimei *et al.* 1994). Cation exchange may then provide greater buffering capacity in the rumen of a replete animal grazing pasture than that provided by

bicarbonate at 50meq./l and orthophosphate at 40meq./l in rumen fluid and of a similar order of magnitude in the fasted animal (Turner and Hodgetts 1955a and b, Emmanuel *et al.* 1969).

A ruminant fed a diet with a high roughage content will then have an increased capacity to buffer against changes in the concentration of cations and pH in rumen fluid compared to one fed a concentrate based diet. A concentration of calcium in rumen fluid that is either too high (see section 3.2) or too low (see section 1.5.6) is observed to reduce microbial growth and the digestion of plant fibre. Maintaining a high cation exchange capacity in the rumen may then be of particular benefit for rumen digestive function where the animal's diet contains either a high concentration of rumen soluble calcium, eg., calcium chloride, or a high concentration of a compound that binds calcium, eg., LCFA, as large and rapid changes in calcium concentration may be avoided.

3.6. Conclusion

Calcium ion performs a number of roles central to the fermentation of fibre in the rumen. Calcium is involved in attachment processes for certain groups of rumen microorganisms, fibrolytic enzyme function and as part of the cation exchange complex on plant fibre provides a ionic and pH buffering mechanism in the rumen. Rumen calcium is primarily derived from dietary sources although saliva may provide significant amounts of calcium ion to the rumen in diets with a very low rumen soluble calcium content. Legumes tend to have higher calcium content than grasses however the solubility of calcium may be higher in grasses than legumes. The availability of calcium to the rumen from inorganic sources varies according to the nature of the source and rumen fluid pH. Calcium chloride appears to be completely soluble in rumen fluid while calcium orthophosphate and calcium carbonate may effectively be insoluble in the rumen of roughage fed animals. Animals fed diets with a high roughage content may have a higher cation exchange capacity of rumen contents compared to animals fed diets high in concentrates. A high cation exchange capacity in the rumen may be of benefit to rumen digestive function where a diet has a high concentration of calcium or a high concentration of LCFA as large or rapid changes in the concentration of calcium in rumen fluid may be avoided.

3.7. Basis for further research

Previous research has demonstrated that the presence of free LCFA in the rumen can alter the contribution of key microbial species to the fermentation of plant fibre. The rate of digestion of plant fibre in the rumen as well as the molar ratio of (acetate + 2×butyrate):propionate and the concentration of ionised calcium in rumen fluid is observed to decrease with an increase in the concentration of LCFA in a diet. The microbiology and

biochemistry relating to the change in the ratio of the major VFA's on exposure of rumen microorganisms to LCFA is well understood. It therefore seems reasonable to use both the rate of digestion of fibre and ratios of the VFA's to indicate the extent to which rumen fermentation has been affected by LCFA.

Palmquist *et al.* (1986) report that inclusion of rumen soluble calcium in a diet can reduce the effect of LCFA on the digestion of plant fibre in the rumen. The extent to which calcium soap formation was responsible for the restoration of the normal digestion of plant fibre in the rumen was equivocal. Studies conducted i*n vitro* have demonstrated that rumen microorganism and at least some of their fibrolytic enzymes require calcium ion to maintain growth and activity. It is therefore likely that rumen fermentative capacity, particularly with respect to the fermentative digestion of plant fibre, is sensitive to calcium ion concentration.

These findings suggest the hypothesis that providing soluble calcium to the rumen at an appropriate rate will improve the ability of rumen microorganisms to digest plant fibre in the presence of supplemental LCFA's. This will occur by neutralising the antimicrobial activity of LCFA's through the formation of associations between calcium and LCFA and by preventing a deficiency of calcium for rumen microbial function. A successful methodology for utilising additional dietary LCFA would maintain rumen function at normal levels as measured by the production of VFA, microbial cells and rate of digestion of plant fibre and would enable the animal to increase its intake of energy by increasing its intake of LCFA while maintaining intake of the basal diet. The benefits of feeding cottonseed oil, as part of the whole seed or refined product, to improve the nutrition of the animal would be observed as an increase in the rate of accretion of energy in the animal's carcass with no reduction in the rate accretion of lean tissue.

Chapter 4
General materials and methods.

4.1. Management of experimental animals

Animals in metabolism or growth experiments were housed individually indoors on wire mesh or wooden slatted floors. Housing was continuously lit during experimental periods. Digestibility and metabolism studies were conducted in temperature controlled rooms set to 22°C ± 2°. All animals received a single drench of a broad spectrum anthelmintic (Ivermectin®) on entering indoor housing facilities and were subsequently drenched every 6 months. Animals with rumen cannula not being used for experimental purposes were kept in individual pens and fed a mixture of oaten and lucerne chaff (3:1) at the estimated maintenance level of feed intake. The area of skin under and surrounding the cannula was shorn of wool to a length of 2mm and the cannula cleaned at 4 weekly intervals or sooner as necessary.

4.2. Diets

The basal diet consisted of a mixture of oaten and lucerne chaff (9:1) with 3% added minerals and 1% added urea (basal diet). The mineral mix consisted of 400g/kg $CaHPO_4$, 200g/kg NaCl, 200g/kg Na_2SO_4 and 200g/kg Pfizer® Vitamin and Trace Mineral Premix. The level of inclusion of treatment feed materials as a proportion of dry matter intake is outlined in Table 4.1. Just prior to feeding out, the supplements and the mineral additives were mixed with 300g of oaten chaff and cottonseed oil or water (100ml) as appropriate was added to bind the mineral mix to the chaff. The supplements were then mixed by hand into the basal ration.

Table 4.1. Level of inclusion of supplements for treatment diets on a dry matter basis.

Diet	Supplement	Level of Inclusion (g/kg)
Basal	Nil	Nil
+CSO	Cottonseed Oil	5.1
+CSM	Cottonseed Meal	12%
+WCS	Whole Cottonseed	25%
+CSM+CSO	Cottonseed Meal + Cottonseed Oil	12% and 5.1%

The basal and +CSO supplemented diets and the +CSM and +WCS diets were formulated to be isonitrogenous. Those diets containing cottonseed oil were formulated to be iso energetic. Calcium hydroxide was added to each diet at 0 or 2% of intake as appropriate. Cottonseed oil was supplied by Cargill Pty. Ltd. Brisbane, Australia. Cottonseed meal was supplied by Cargill Pty. Ltd. Narrabri, Australia. Whole cottonseed

was supplied by Cottonseed Distributors, Narrabri, Australia. Calcium hydroxide was in the form of 'Building Lime' produced by Blue Circle, Tamworth, Australia with 98% of calcium as aqueous soluble calcium.

Whole cottonseed meal was prepared from black (acid delintered) cottonseed in an 18" hammer mill with bagging cyclone (JAS Smith Pty. Ltd. of Ballarat, Australia). The meal was produced by supplying seed into the grain chute at a rate of 1.6 tonnes/hour with the dust extraction vent adjusted so that 95kg/100kg of material fed into the grain chute was recovered. The meal consisted of a kernel fraction with 80g/100g of kernel particles being less that 2mm in diameter and a hull fraction with 50g/100g of hull particles greater than 2mm in diameter.

4.3. Sample collection and analysis

4.3.1. Feed and faeces

Feed refusals from animals housed in metabolism crates were collected just prior to commencement of feeding for the next 24 hour period. Faecal production for each 24 hour period was collected into a plastic bag harnessed to the hindquarters of the animal. The harness consisted of a PVC ring 110mm in diameter and 20mm in width placed to surround the animal's anus. The ring was attached to the animal's upper hind legs, back and belly using contact adhesive and cotton cloth strips. A 10% subsample of faeces representative of the total 24 hour collection was bulked with the previous days sample and stored at -20°C.

4.3.2. Wool growth

A strip of wool 100mm long was dyed at the skin surface (dye banding) using Durafer Black R solution (I.I.A.N.Z. Pty. Ltd.) initially, one week after commencement and finally, two weeks prior to completion of the experiment period (Langlands and Wheeler 1968). Dye bands were removed on the final day of the experiment. Animals were shorn and the fleece weight recorded within three days of completion of the experiment period. Wool growth over the experiment period was determined as a proportion of total fleece weight which was represented by the amount of wool between the dyebands. Preexperiment rate of wool growth was used as a covariate to correct for between animal variation.

4.3.3. Rumen fluid

Sampling via cannula

Rumen fluid was withdrawn through a metal probe into a 25ml syringe from the ventral sac from sheep fitted with a Jarret type cannulae. The probe consisted of 300mm of 4mm internal diameter metal tubing fitted with a metal cage 40mm in length by 15mm in diameter and covered with a layer of nylon gauze with a pore size of 40μm. The proximal end of the probe was fitted with a length of plastic tubing to facilitate attachment of a disposable plastic syringe. Sampling consisted of withdrawing and discarding an initial 20ml of rumen fluid. A second 20ml of rumen fluid was withdrawn for analysis.

Sampling via oesophageal tube

Rumen fluid from animal's not fitted with a rumen cannula was obtained by inserting a 1.2m length of 15mm internal diameter plastic hose into the reticulo rumen via the oesophagus and allowing rumen fluid to flow out of the reticulo rumen assisted by gravity and under positive pressure created by ruminal contractions. The tube was flushed with a volume of rumen fluid (around 100ml), to avoid contamination of the sample with saliva, and discarded. A second volume of rumen fluid (100 to 150ml) was collected into plastic beaker for use.

Volatile fatty acid concentration

20ml of rumen fluid to be used for determination of volatile fatty acid content was dispensed into a 30ml screw cap glass bottle containing 0.15g (4.5mmol) of conc. H_2SO_4. The sample + acid was stored at -20°C until required. Just prior to analysis the frozen sample thawed at 4°C then centrifuged at 1600g for 15minutes. The supernatant which was decanted into a fresh 30ml bottle to produce 'clarified' rumen fluid. Concentration and molar proportions of volatile fatty acids were determined using a gas chromatograph (Packard Model 427) according to the method of (Geissler *et al.* 1976) with isocaproate as the internal standard.

Ammonia concentration

Rumen fluid was prepared as for determination of volatile fatty acids. NH4 + - N concentration was determined colourimetrically using an auto analyser (Technicon Instruments Corporation, USA) according to the method of Bietz (1974).

pH and free ionised calcium concentration

pH and free ionised calcium were determined using a glass calomel pH electrode and a calcium ion specific electrode with reference electrode connected to a programmable pH/millivolt meter (Activon Scientific Products Company Pty Ltd, Sydney, Australia). The instrument was calibrated to a 2 point standard curve for determination of pH (5 and 7) and a 4 point standard curve (10^{-5}mM, 10^{-4}mM, 10^{-3}mM and 10^{-2}mM [Ca^{2+}]) for determination of free ionised calcium. Calibration of the pH and calcium ion specific electrodes was checked between samples using pH 7 buffer and calcium chloride solution at 10^{-3}mM. The standard curves for both electrodes were recalibrated hourly.

Ciliate protozoa

4 ml of freshly sample rumen fluid was added to 16ml of formal saline solution (0.9% NaCl, 4% formaldehyde) and stored at room temperature. The concentration of holotrich and small entodiniomorphid protozoa in this prepared sample was determined using a Hawkesley Cristalite counting chamber (0.2mm deep with 1/16mm^2 graduations).

Kinetics

Rumen fluid volume and outflow rate were estimated from the dilution of a single dose of CrEDTA injected into the ventral sac of the rumen. Twelve samples of rumen fluid were withdrawn at regular intervals over a 24 hour period commencing 3½ hours following injection of the CrEDTA dose. The concentration of chromium was determined on prepared rumen fluid (see section 4.2.1) by atomic absorption spectrophotometry (Perkin-Elder, Model 360, USA) using an acetylene and nitrous oxide flame.

4.3.4. Blood plasma urea

10ml of blood was withdrawn from the jugular vein by venipuncture 4 - 4.5 hours following feeding, dispensed into centrifuge tubes containing lithium heparin and stored on ice. Within three hours of sampling these samples were centrifuged at 1800g for 15 minutes. The plasma was decanted into 15ml glass screw cap bottles and stored at -20° C. Blood plasma urea concentration was determined colourimetrically using an autoanalyser (Technicon Instruments Corporation, USA) according to the method of Marsh *et al.* (1957).

4.3.5. Dry and organic matter content

Dry matter content of feed, feed refusals and faeces was determined on samples dried to constant weight at 85°C. Following drying, all feed and faecal samples were

ground using a bench mounted hammer mill fitted with a 1mm sieve. Organic matter content of these samples was determined as the loss of mass upon combustion in a muffle furnace heated to 600°C for 4 hours.

4.3.6. Organic nitrogen and macrominerals

Samples of feed faeces and blood plasma to be tested for mineral content (calcium, phosphorus, magnesium and sulphur) were oxidised (wet ashed) using perchloric acid solution sealed flasks incubated at 80°C (Anderson and Henderson 1986). Mineral content was determined using an Inductively Coupled Plasma Spectrophotometer (3520 ARL, Bausch and Lomb, USA). Total organic nitrogen content of fluid or dry matter samples with 0.5% or greater organic N content was determined using an Organic Nitrogen Determinator (FP-228, Leco Corporation, USA).

Total organic nitrogen in samples with less than 0.5% N content and including urine and rumen fluid was determined following conversion of organic N to NH_4^+-N by micro Kjeldahl oxidation (AOAC 1980). The oxidised samples were made alkaline by addition of NaOH solution (50%w/v) followed by steam distillation and collection of NH_3 into boric acid solution (2%w/v) buffered at pH 5. These samples were then back titrated using 0.01N H_2SO_4.

4.3.7. LCFA analysis

LCFA content of feed or faeces was determined by direct extraction and methylation of fatty acids. Between 150mg to 300mg of sample was weighed into 150mm x 15mm screw top culture tubes and 100 to 200µg of C17:0 fatty acid methyl ester in hexane was then accurately pipetted into each tube as an internal standard. Methanol: benzene (4:1v/v) solvent (5ml) followed by acetyl chloride (0.3ml) was added to each tube with constant mixing (Vortex mixer). The tube was sealed using two layers of teflon tape and the screw cap and placed in a water bath for one hour at 100°C. Separation of the non polar (organic) and aqueous phases was achieved by addition of 5ml K_2CO_3 (12%W/V) followed by centrifugation at 200g for 5 min. The organic layer was removed using a Pasteur pipette and the benzene allowed to evaporate under an N_2 gas flow. The remaining material was taken up in 0.2ml of hexane. C12 - C22 fatty acid methyl ester content of samples was determined on a Hewlett Packard 5890 series II gas chromatograph with Restec 'FFAP' 30m by 0.53mm internal diameter column. External standard contained C16:0, C18:0, C18:1, C18:2, C18:3 fatty acid methyl esters.

4.3.8. In sacco dry matter digestibility

Roughage's were ground using a bench mounted hammer mill fitted with a 2mm sieve. Cottonseeds and CSM were reduced to finely divided particles in a coffee mill that utilised blades spinning at high speed. About 400mg of sample was accurately weighed into dacron bags, 120mm x 60mm with a mesh size of 40μm. A glass marble was also added to increase bag weight and aid in maintaining the bag in position below the surface of the rumen digesta raft (Perdok 1987). Each determination consisted of three replicate bags containing the test feed and marble placed into the ventral sac of the rumen for the specified period. This procedure was repeated in the same animal 24 hours later. Bags observed to be 'floating' on top of the digesta raft were excluded from further analysis. Following incubation in the rumen up to 36 sealed bags with samples were rinsed with agitation 3 times in 10 l of warm water containing 10 ml of Teepol® for a total of 15min. Samples were dried at 60°C to constant weight prior to gravimetric determination of dry matter loss. Dry matter content of three replicate samples of the test feeds was determined concurrent with the rumen-incubated samples.

4.3.9. Cross sectional area of the Longissimus dorsi muscle and carcass fat

A relative measure of changes in the body composition of an animal was determined using computer aided tomograph images (CAT scan). Images were 5mm wide and in transverse section at a point that bisected the twelfth rib 110mm from the dorsal spinal process of the associated vertebra. The scan also bisected an area close to the 2nd lumbar vertebra although the precise point of bisection varied within and between animals. Images were analysed for changes in cross sectional area of carcass fat and eye muscle (Longissimus dorsi).

4.4. Animals, allocation to treatment and feeding regime used in growth experiments - Chapters 7 and 8.

Seven month old 1st cross wether lambs (Border Leicester x Merino) were housed indoors in individual pens on slatted floors. Ten days prior to commencement of the experiment period animals were grouped into one of eight strata based on liveweight. One animal was then selected randomly from each of the stratified groups and allocated to each of ten dietary treatments (80 animals in total) that form the basis of the studies described in Chapters 7 and 8. Animals were allocated randomly to pens. The mean liveweight of treatment groups was 21.7kg with standard error of liveweight within groups of 1.6kg. Animals were initially offered half their estimated *ad libitum* feed intake and feed on offer was increased to *ad libitum* plus 10-15% over ten days at which time the experiment commenced. Feed was offered at 10-15% above an average of the previous

three days intake throughout the experiment period, adjusted twice weekly. Feed refusals were collected, their weight was recorded and animals were fed once daily between 8am and 11am.

Chapter 5
The use of calcium salts to reduce the antimicrobial action of cottonseed oil: Fermentation of oaten chaff by mixed rumen microorganisms *in vitro*.

5.1. Introduction

Rumen microorganisms digest a variety of feed materials including structural and non structural carbohydrate and protein. In the rumen, carbohydrate is primarily fermented to acetate, propionate and butyrate with the production of carbon dioxide and hydrogen. Hydrogen pressure is kept low (1 Pa) through methanogeneis, i.e., the reduction of carbon dioxide to methane (Blaxter and Czerkawski 1966, Hungate 1966, Wolin and Miller 1983).

While rumen microbes can hydrolyse a variety of substrates the ability to cleave the β 1-4 linkage of the hexose and pentose sugars comprising structural carbohydrate (cellulose and hemicellulose) is restricted to certain species of fibrolytic microorganisms including bacteria, ciliate protozoa and anaerobic fungi (Hungate 1966, Wolin and Miller 1983, Bauchop 1988). The primary end product of the fermentation of cellulose is acetate (Hungate 1966, Wolin and Miller 1983). Some succinate is also produced as an intermediate but is rapidly decarboxylated to propionate by non cellulolytic species. A reduction in the rate of fermentation of cellulose will therefore reduce the production of acetate (Wolin and Miller 1983).

The use of chemical agents that reduce the activity of the methanogenic bacteria will increase the partial pressure of hydrogen in the rumen (Blaxter and Czerkawski 1966). This inhibits the production of acetate by cellulolytic microorganisms and increases the production of propionate by non cellulolytic bacteria (Blaxter and Czerkawski 1966, Czerkawski 1966, Wolin and Miller 1983). The rate of digestion of substrate high in structural carbohydrate and the molar ratios of the major VFA, under specified conditions, can therefore act as an indicator of the relative activity of the fibrolytic microorganisms both *in vivo* and *in vitro*.

LCFA's of 8 to 18 carbon atoms in length act as antimicrobial agents at low concentrations *in vitro* and *in vivo* (Galbraith *et al.* 1971, Galbraith and Miller 1973c, Henderson 1973). This action is observed with rumen microorganisms with the gram positive cellulolytic and methanogenic bacteria, the ciliate protozoa and the anaerobic fungi particularly susceptible (Nieman 1954, Galbraith *et al.* 1971, Galbraith and Miller 1973c, Henderson 1973). Cottonseed oil or other triglyceride is rapidly hydrolysed to

glycerol and long chain fatty acids (LCFA's) by mixed fermentations of rumen microorganisms and is therefore a source of LCFA's (Harfoot *et al.* 1974). The addition of cottonseed oil to a fermentation of oaten chaff by mixed rumen microorganisms should therefore result in a decrease in both the rate of digestion of oaten chaff (dry matter loss) and the production of acetate. Addition of a compound that removed the antimicrobial action of LCFA's should return digestion and the pattern of VFA production to that of the fermentation receiving no cottonseed oil.

LCFA's can react with calcium ion to form highly insoluble precipitates possessing no antimicrobial activity (Palmquist *et al.* 1986). The addition of calcium ions to a fermentation system in stoichiometric quantities should precipitate any added LCFA's and remove their antimicrobial action (Miller *et al.* 1970, Jenkins and Palmquist 1986, Palmquist *et al.* 1986). As the ratio of calcium soap to free LCFA is increased with increasing pH (Sujhika and Palmquist 1990), calcium hydroxide, an alkalinising salt, may be more effective than calcium chloride in promoting calcium soap formation. The addition of preformed calcium soaps should exert no antimicrobial action (Palmquist *et al.* 1986). A series of *in vitro* fermentations were conducted to test these hypotheses.

5.2. Materials and Methods

5.2.1. Experimental design

There were 7 treatment regimes in which rumen fluid was incubated with cottonseed oil (CSO), calcium salts or calcium LCFA soaps (CaLCFA). These treatments are summarised in Table 5.1.

Table 5.1. The level of inclusion of cottonseed oil, calcium salts or calcium soaps in each flask.

Treatment	[1]CSO LCFA	$CaCl_2$	$Ca(OH)_2$	[1]CaLCFA
	µmol	µmol	µmol	µmol
Control	0	0	0	0
Calcium hydroxide	0	0	200	0
Calcium chloride	0	200	0	0
Cottonseed oil	375	0	0	0
Cottonseed oil + calcium hydroxide	375	0	200	0
Cottonseed oil + calcium chloride	375	200	0	0
Calcium LCFA	0	0	0	200

1. CSO refers to cottonseed oil, CaLCFA to calcium soaps of LCFA's.

LCFA's derived from cottonseed oil were included at 20% of the dry weight of the oaten chaff. Calcium was added in excess (10%) of stoichiometric amounts to produce calcium soap Ca $(LCFA)_2$ according to the equation

$$Ca^{2+} + 2.LCFA^{-} \Rightarrow Ca(LCFA)_2.$$

The level of inclusion of preformed CaLCFA provided the same additional calcium ion as with the other calcium supplemented treatments and 400μmol of LCFA's.

5.2.2. Experimental procedure

Rumen fluid was collected from sheep (see section 4.3.3) and placed into sealed flasks in a 39°C water bath. A buffer solution (pH 6.7) was prepared according to the composition for synthetic saliva suggested by McDougall (1948). Incubations took place in sealed 100ml serum bottles. Each bottle contained 40 ml buffer solution, 500mg of ground oaten chaff (see section 4.3.8.), 10ml of rumen fluid and the respective treatment compounds (see Table 5.1). The gas space in the flask was flushed with carbon dioxide and nitrogen (1:1v/v), sealed with a butyl rubber septum and a 20 gauge hypodermic needle attached to a 10ml syringe barrel was inserted through the septum. 2ml of distilled water was added to each syringe. All bottles were incubated in the dark in a water bath at 39° with shaking. Three batches of incubations, corresponding to 12, 24 and 48 hours incubation periods, were conducted separately. A set of three blank fermentations (rumen fluid and buffer only) were run in conjunction with the treatment flasks.

5.2.3. Statistical analysis

Treatments were compared using one way analysis of variance and the modified Fischers LSD (Systat 5.0).

5.2.4. Preparation of calcium soaps from cottonseed oil.

To 100ml of CSO in a 1 l beaker 20g of finely ground NaOH was added with stirring. The mixture was heated to 100°C over a hot plate with stirring until saponification was complete. Saponification was observed to be the change from liquid to solid. 400ml of distilled water was then added to the beaker and the sodium soaps were dissolved with heating. 250ml of 2M $CaCl_2$ solution was then added with stirring causing a white precipitate to collect at the surface of the solution. The white precipitate was washed with 400ml of distilled water with heating and stirring. The water was decanted and the process repeated. The water was again decanted and 400ml of 70% ethanol was added to the white precipitate with stirring. The ethanol solution was decanted and the residue dried at 60°C. The residue was then ground to a fine powder in a mortar and pestle and the resulting product was referred to as calcium soap.

5.2.5. Sample analysis

Dry matter loss of feed sample and rumen fluid inoculum from treatment and blank flasks following incubation was determined according to the procedure of Tilley and Terry (1963) with the following changes. Fermentation was stopped by cooling the flasks on ice. The entire contents of the digest were then placed in 200ml centrifuge tubes (polypropylene screw cap) and centrifuged at 1800g for 15 min. A 20ml sample of supernatant was stored as for rumen fluid for analysis for volatile fatty acid (VFA) and N content (see section 4.3.3 and 4.3.6). The residue was washed with 50ml of distilled water followed by centrifugation (as above). The supernatant was discarded and the residue was transferred with washing to a 150ml conical flask containing 50ml of freshly prepared pepsin HCl solution (pH 2). The flasks were incubated in a water bath at 39°C for 48 hours with shaking. Incubation of the residue with acid pepsin solution was performed to bring about the acid hydrolysis of adherent calcium soap and microbial dry matter. The contents were quantitatively transferred to a 9cm Buchner funnel containing Watman No. 41 (ashless) filter paper and the liquor removed using vacuum filtration. The residue was washed sequentially with 100ml of distilled water, 50ml of 70% ethanol, 50ml ethanol and 50ml of hexane and then dried at 85°C. The weight of this final residue was used to calculate the weight of dry matter loss from the sample + inoculum. OM content was determined on the residue and filter paper from one sample from each digest. N content of the residue was determined on the remaining two samples from each treatment using a micro Kjeldhal technique (see section 4.3.6).

The fermentation 'blanks' were sampled at zero time from the stock incubation media + inoculum and at the end of each incubation period for determination of VFA, N and DM of the residue (see section 4.3.3 and 4.3.6). All other treatments were sampled at the end of the incubation period only. pH was determined on filtrate equilibrated to a carbon dioxide and nitrogen gas mix (1:1v/v) (see section 4.3.3). OM, N and DM content of oaten chaff determined as outlined in sections 4.3.5 and 4.3.6.

Dry matter digestibility (DMD) from the oaten chaff sample + inoculum (substrate) was estimated:-

DMD (mg/g) = (mg dry matter in substrate + mg dry matter in blank at 0h - mg dry matter in final residue at x h) ÷ (mg dry matter in substrate + mg dry matter in blank at 0h) x 1000.†

Organic matter digestibility (OMD) from the substrate was estimated:-

OMD (mg/g) = DMD (mg/g) x (1 − (mg dry matter in final residue on dry ashing ÷ mg final residue prior to ashing)).†

Fractional rate of digestion (FRD) of substrate from x to y hours of incubation was estimated:-

FRD (mg/h) = (DMDy - DMDx) ÷ (y - x) x (g dry matter in substrate + g dry matter in blank at 0h).†

Microbial nitrogen uptake (MNU) was estimated:-

MNU mg/flask = (mg N in filtrate in blank at 0h - mg N in filtrate in treatment at xh) + (mg N in oaten chaff + mg N in residue in blank at 0h - mg N in residue in treatment at xh)‡.

VFA production (VFA) was estimated:-

VFA mmol/flask = (mM VFA in treatment flask at xh - mM in blank flask at 0h) x 0.05l/flask. †

† where xh and yh = hours of incubation at time x and time y respectively.

‡assumes that pepsin HCl digest releases only microbial N and not substrate N.

5.3. Results

5.3.1. VFA production, pH and N content of fermentation liquor

Values for total VFA production and pattern for each treatment are presented in Table 5.2. The control produced an increase in the concentration of total VFA of 32mM over 24 hours of incubation. Inclusion of CSO reduced the final concentration of the total VFA to 25.4mM ($p < 0.01$). The concentration of total VFA was not significantly altered by any of the other treatments.

Table 5.2. The change in total VFA and N concentration, molar proportion of the major VFA's and pH in fermentation liquor in response to inclusion of calcium salts, cottonseed oil or calcium soaps of cottonseed oil LCFA's following 24 hours of fermentation of oaten chaff by mixed rumen microorganisms *in vitro*.

Treatment	$^{3}\Delta$ [Total VFA] mM	Acetate molar %	Propionate molar %	Butyrate molar %	$^{3}\Delta$ [N] mM	pH
Control	32.0bc	57.8b	31.7c	7.2ab	−0.1	6.8a
$CaCl_2$	29.6b	58.7b	30.5bc	7.6d	0.1	7.0c
$Ca(OH)_2$	30.5bc	59.1b	30.2b	7.5cd	0.3	7.1d
^{3}CSO	25.4a	54.8a	34.0d	7.2b	7.7a	6.8a
CSO + $Ca(OH)_2$	32.8c	58.4b	31.0bc	7.1a	0.3	6.9b
CSO + $CaCl_2$	32.2c	55.1a	34.2d	6.9a	−0.2	6.8a
^{3}CaLCFA	32.4c	60.1b	28.1a	8.1e	1.1	6.8a
Standard Error	0.8	0.5	0.4	0.1	0.5	0.03

1. n = 3.
2. Figures in a column with a different subscript are significantly different at the 5% level of probability.
3. CSO refers to cottonseed oil, CaLCFA to calcium LCFA, Δ [Total VFA] and Δ [N] to the changes in the concentrations of the VFA and OMN in fermentation liquor.

The molar proportion of acetate in the control and blank fermentations was 57.8% and 75.6% of the total VFA content, respectively. Inclusion of CSO or CSO + $CaCl_2$ reduced the proportion of acetate by 0.05 ($p < 0.05$) compared to the control. The molar proportion of propionate in the fermentation liquor for the control was 31.7% while the corresponding value for the blank was 15.8%. Inclusion of $Ca(OH)_2$ or calcium soaps decreased the proportion of propionate by 0.05 and 0.11 ($p < 0.05$) respectively while

inclusion of CSO or CSO + $CaCl_2$ increased the proportion of propionate by 0.07 and 0.08 ($p < 0.05$) respectively. The proportion of butyrate for the blank and control flasks was 3.2% and 7.2% respectively. Inclusion of $Ca(OH)_2$, $CaCl_2$ or calcium soap increased the molar proportion of butyrate by 0.04 ($p < 0.05$), 0.06 ($p < 0.05$) and 0.13 ($p < 0.01$) respectively. The increase in butyrate produced by the calcium soaps was also significantly larger ($p < 0.05$) than that produced by the $Ca(OH)_2$ or $CaCl_2$ treatments.

The concentration of N in the liquor from the control fermentation had decreased by 0.1mM after 24 hours of incubation (Table 5.2). Inclusion of CSO in the fermentation caused the concentration of N to increase by 7.7mM ($p < 0.05$) over the same period. The concentration of N in flasks was not significantly altered by any of the other treatments.

The pH of the fermentation liquor for the control was 6.8 after 24 hours of incubation (Table 5.2). Inclusion of CSO, CSO + $CaCl_2$ or calcium soaps did not alter pH. Inclusion of CSO + $Ca(OH)_2$, $CaCl_2$ or $Ca(OH)_2$ increased pH of the filtrate by 0.01 ($p < 0.05$), 0.03 ($p < 0.01$) and 0.04 ($p < 0.01$) units respectively.

5.3.2. Dry matter digestibility

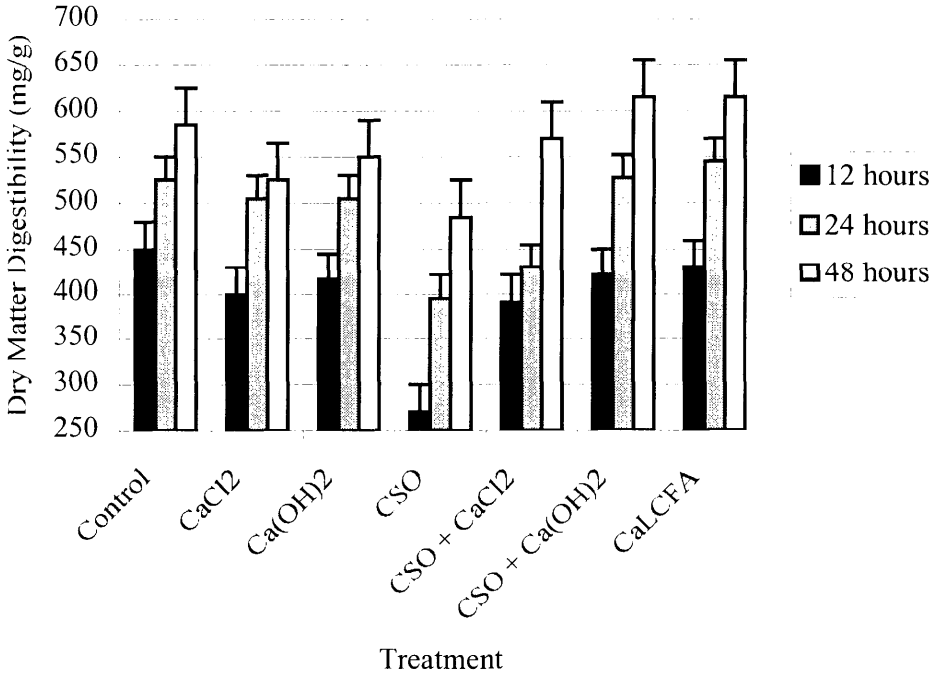

Figure 5.1. The digestibility of oaten chaff by mixed rumen microorganisms *in vitro* in response to inclusion of calcium salts, cottonseed oil or calcium soaps of cottonseed oil LCFA's over 12, 24 or 48 hours of incubation. Bars are the standard error of a mean.

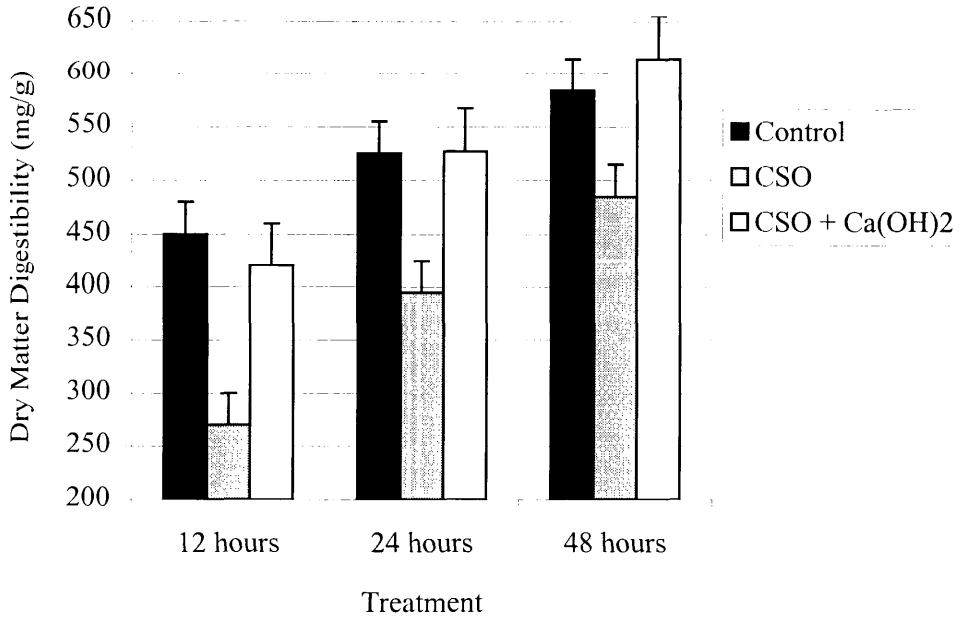

Figure 5.2. The digestibility of oaten chaff by mixed rumen microorganisms *in vitro* after 12, 24 or 48 hours of incubation in response to inclusion of cottonseed oil or cottonseed oil + calcium hydroxide. Bars are the standard error of a mean.

Dry matter solubility estimated at 0 hours incubation was 180mg/g dry matter. DMD for each treatment at 12, 24 and 48 hours of incubation is presented in figures 5.1a,bandc respectively. After 12 hours incubation the DMD for the control was 453mg/g. This had risen to 523mg/g at 24 hours and 583mg/g at 48 hours of incubation.

Inclusion of CSO reduced DMD by 0.41 ($p < 0.01$) after 12 hours, by 0.23 ($p < 0.01$) after 24 hours and 0.17 ($p < 0.05$) after 48 hours of incubation. While the overall rate of digestion of substrate was reduced by the CSO treatment over any time period, the fractional rate of digestion of substrate was less than the control value only from 0 to 12 hours of incubation (Figure 5.3). The fractional rate of digestion for the CSO treatment was 0.88 ($p < 0.01$) and 0.36 larger ($p < 0.05$) than the control from 12 to 24 hours and 24 to 48 hours incubation respectively (Figure 5.3).

The $CaCl_2$ treatment reduced DMD over 12 hours of incubation by 0.12 ($p < 0.05$) compared to the control (Figure 5.1). Over 24 hours of incubation there was no statistically significant difference between the control and the $CaCl_2$ treatments, however over 48 hours of incubation DMD for the $CaCl_2$ treatment was 0.10 ($p < 0.05$) below the control value (Figures 5.1).

DMD for the CaLCFA treatment was not significantly different to the control at 12 hours of incubation (Figure 5.1). Over 24 hours of incubation, inclusion of the calcium soap had

increased DMD compared to the control by 0.04 ($p < 0.05$) (Figure 5.1). There was no difference between the control and this treatment at 48 hours of incubation.

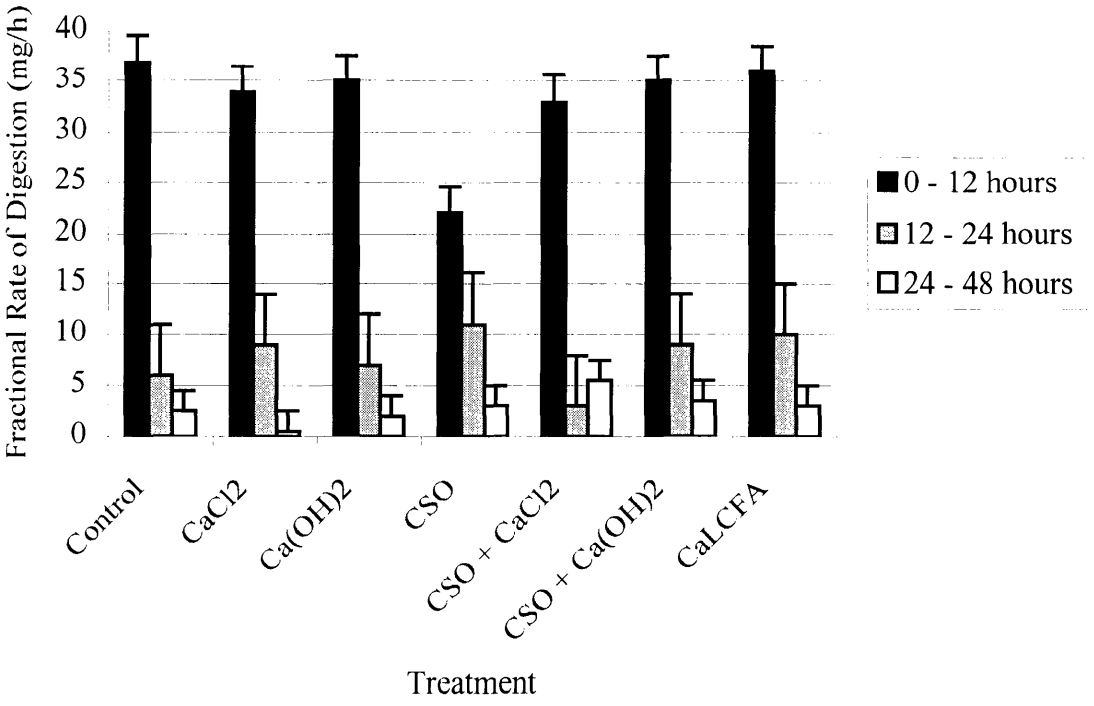

Figure 5.3. The fractional rate of digestion of oaten chaff by mixed rumen microorganisms *in vitro* in response to inclusion of calcium salts, cottonseed oil or calcium soaps of cottonseed oil LCFA's over a) 0 - 12 hours incubation, b) 12 - 24 hours of incubation and c) 24 - 48 hours of incubation.

Inclusion of $Ca(OH)_2$ reduced DMD by 0.08 ($p < 0.05$) after 12 hours of incubation and by 0.04 ($p < 0.05$) after 24 hours of incubation (Figure 5.1). There was no significant difference between the control and this treatment after 48 hours of incubation (Figure 5.1).

Inclusion of both CSO and $CaCl_2$ reduced DMD by 0.13 ($p < 0.05$) after 12 hours of incubation and by 0.17 ($p < 0.05$) over 24 hours of incubation compared to the control (Figures 5.1). There was difference between this treatment and the control after 48 hours of incubation (Figure 5.1).

Inclusion of both CSO and $Ca(OH)_2$ reduced DMD by 0.07 ($p < 0.05$) over 12 hours of incubation compared to the control (Figure 5.1). There was no effect of this treatment over 24 hours of incubation however over 48 hours of incubation DMD for this treatment was 0.05 ($p < 0.05$) higher than that of the control (Figure 5.1).

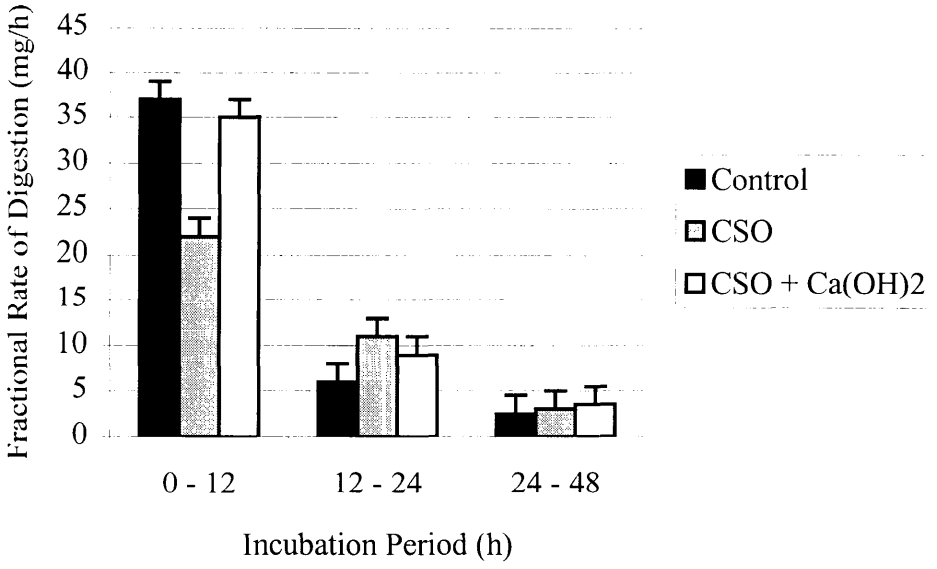

Figure 5.4. The fractional rate of digestion of oaten chaff by mixed rumen microorganisms *in vitro* in response to inclusion of cottonseed oil or cottonseed oil + calcium hydroxide. Bars are the standard error of a mean.

5.3.3. Uptake of nitrogen by microorganisms

The total uptake of N as microbial cells for the control was 4.13mg (Table 5.3). Inclusion of CSO reduced this by 0.35 ($p < 0.01$). Inclusion of $Ca(OH)_2$ with CSO returned microbial N uptake to control levels. Microbial nitrogen uptake on inclusion of $CaCl_2$ with CSO was 0.30 ($p < 0.01$) greater than the CSO treatment but was 0.16 ($p < 0.05$) less than the control. Inclusion of $Ca(OH)_2$, $CaCl_2$ or calcium soaps in the fermentation had no effect on microbial nitrogen uptake.

Microbial nitrogen uptake per unit of total OM available for fermentation in the control was 7.8mg/g over 24 hours of incubation (Table 5.3). Inclusion of CSO or CSO + $CaCl_2$ reduced this measure by 0.35 ($p < 0.01$) and 0.16 ($p < 0.05$) respectively. The difference between the CSO and the CSO + $CaCl_2$ treatments was also statistically significant ($p < 0.01$). The CSO + $Ca(OH)_2$, $Ca(OH)_2$ and calcium soap treatments did not affect this measure. Microbial nitrogen uptake per unit of OM digested for the control was 13.5mg/g (Table 5.3). Inclusion of CSO reduced this value by 0.16 ($p < 0.05$). The CSO + $Ca(OH)_2$, $Ca(OH)_2$, $CaCl_2$ and calcium soap treatments did not affect this measure. The uptake of N by microbes per unit of VFA (C2 to C5 inclusive) was 2.6g/mol for the control (Table 5.3). Inclusion of CSO or CSO + $CaCl_2$ reduced microbial nitrogen uptake by 0.18 ($p < 0.05$) and by 0.16 ($p < 0.05$) respectively.

Table 5.3. The total uptake of organic matter N and energetic efficiency of nitrogen uptake by microorganisms in response to inclusion of calcium salts, cottonseed oil or calcium soaps of cottonseed oil LCFA's following 24 hours of fermentation of oaten chaff by mixed rumen microorganisms *in vitro*.

	Total MNU	[1]MNU/OM	MNU/DOM	MNU/VFA
Treatment	mg/flask	mg/g	mg/g	g/mol
Control	4.13c[2]	7.8c	13.5bc	2.6b
$CaCl_2$	4.01c	7.5c	13.5bc	2.7b
$Ca(OH)_2$	3.96c	7.4c	13.5bc	2.6b
CSO	2.67a	5.0a	11.4a	2.1a
CSO + $Ca(OH)_2$	4.08c	7.7c	13.2b	2.5b
CSO + $CaCl_2$	3.48b	6.5b	13.8c	2.2a
CaLCFA	4.20c	7.9c	13.2b	2.6b
Standard Error	0.09	0.2	0.1	0.1

1. n = 3.
2. MNU refers to microbial nitrogen uptake, OM to organic matter and DOM to digestible organic matter.
3. Figures in a column with a different subscript are significantly different at the 5% level of probability.

5.4. Discussion

A reduction in the rate of digestion of substrate for the CSO treatment was apparent only for the first 12 hours incubation. Sutton *et al.* (1983) established that a reduction in DMD of a diet high in structural carbohydrate in response to LCFA is primarily the result of a loss of cellulolytic (β 1-4 glucanase) activity. It is likely then that CSO caused a reduction in cellulolytic activity in this experiment, at least in the first 12 hours. The decrease in the proportion of acetate and increase in propionate also supports this hypothesis (Wolin and Miller 1983). Over 12 to 24 and 24 to 48 hours of incubation however, those flasks containing CSO digested oaten chaff at a faster rate than the control although DMD had not matched control levels by 48 hours of incubation.

The antimicrobial effect of cottonseed oil therefore occurred early in the fermentation process and did not completely remove fermentative capacity. There may also have been some recovery of fermentative capacity in response to the continued growth and activity of non susceptible microorganisms, the return of activity of susceptible species following natural amelioration of toxicity of the LCFA or both. Reduction of toxicity of

LCFA can occur where the LCFA associates with microorganisms either intra or extracellularly (Demeyer *et al.* 1978, Bauchart *et al.* 1990), the surface of plant fibre (Harfoot et. al. 1974), by precipitation with alkali earth metals (El Hag and Miller 1972) and as a result of biohydrogenation (Kemp *et al.* 1984 a and b). While it is possible that all these processes took place, the shift in production of acetate and propionate over 24 hours of incubation suggests a change in the species mix in flasks in response to CSO rather than recovery of the susceptible microorganisms.

The inclusion of CSO also reduced the total microbial nitrogen uptake and efficiency of production of microbial nitrogen. This suggests that the LCFA either reduced microbial synthesis without affecting the release of nitrogen from the substrate or increased microbial nitrogen turnover through cell death and lysis. The reduction in efficiency of microbial nitrogen uptake per unit of OM available for fermentation for this treatment is largely the result of the reduction in DMD. These data therefore suggest that inclusion of CSO will reduce the amount of microbial nitrogen (cells) produced per unit of OM available for fermentation and provides support for the former hypothesis. Net microbial nitrogen uptake by microorganisms per unit of digestible OM however remained below the control for the CSO treatment. This indicates that the production of cells per unit of OM truly digested was less efficient on inclusion of CSO, supporting the latter hypothesis. A reduction in production of cells per unit of VFA may occur where cells are actively fermenting substrate but where energy gain has been uncoupled from growth (Livesey 1985). The comparatively high rate of digestion of substrate from 12 to 24 hours of incubation for this treatment suggests that microbes are actively fermenting substrate. This however did not result in an increase in nitrogen appearing in cells and also supports a hypothesis that inclusion of CSO has lead to the uncoupling of fermentative processes from the production of cells.

The inclusion of preformed calcium soaps had minimal effect on the fermentation of oaten chaff as measured by DMD and production of cells. DMD was slightly higher over 24 hours of incubation for the calcium LCFA treatment, however this effect was not apparent over 48 hours of incubation. While the molar proportion of acetate for this treatment was not significantly different to the control, the proportion of propionate decreased and that of butyrate increased. The shift in the production of these two VFA's suggests that butyrate producing organisms have been favoured at the expense of propionate producing organisms and indicates the calcium soap used in this experiment is not inert.

It is likely that small amounts of glycerol, soluble calcium, sodium, hydroxide and chloride may have been present in the soap and/or there has been dissociation of the

soap to calcium ion and LCFA. This may have affected the fermentation pattern. Sukhija and Palmquist (1990) observed that calcium soaps comprised of low to medium melting point fatty acids dissociated to a limited extent at pH 6.0 (0.10 dissociated) but dissociation was negligible at pH 7.0. The pH of the filtrate for this treatment was 6.8 after 24 hours of incubation suggesting that little dissociation of calcium soaps occurred. Glycerol would be quickly fermented to VFA or incorporated into cells. Sodium and chloride would be expected to have minimal effect on the course of the fermentation given their high concentration in the buffer solution (177 and 17 mM respectively) and the buffering action of the media would minimise the effect of the hydroxide ion. The change in VFA pattern however matches that observed for those treatments containing $CaCl_2$ and $Ca(OH)_2$ suggesting an effect of calcium ion on fermentative processes.

The inclusion of both $Ca(OH)_2$ and $CaCl_2$ appeared to alter the fermentation pattern in a similar way. After 24 hours of incubation, DMD, total VFA production and VFA pattern and the measures of efficiency of microbial nitrogen uptake were for both treatments. A reduction in the digestibility of the substrate on inclusion of these salts however suggests that inclusion of high levels of soluble calcium may initially suppress cellulolytic activity but not microbial yield in fermentations. Inclusion of $Ca(OH)_2$ with CSO resulted in all measures of fermentative capacity returning to control values by 24 hours of incubation. This suggests that addition of $Ca(OH)_2$ acts to assist in the reversal of the antimicrobial action of the LCFA. The mode of action of $Ca(OH)_2$ may be either to correct a deficiency of calcium brought about by precipitation of the available calcium as calcium soap or by reversing the antimicrobial activity of the LCFA's by converting them to calcium soaps.

The relative importance of either the formation of calcium soaps or the correction of a calcium deficiency on addition of $Ca(OH)_2$ is difficult to determine. The fractional rate of digestion of oaten chaff for the CSO + $Ca(OH)_2$ treatment was intermediate to the control and CSO treatments at 12 hours of fermentation. This indicates that at least in the early stages of fermentation the addition of $Ca(OH)_2$ had not fully reversed the effects of the CSO LCFA's although no calcium ion deficiency would exist. The antimicrobial action of LCFA's early in the fermentation period is therefore most likely the result of a direct toxic action against rumen microorganisms rather than the result of a deficiency of calcium ion. The initial reduction and later recovery of the rate of fermentation of oaten chaff for the CSO + $Ca(OH)_2$ treatment may then result from the kinetics associated with the hydrolysis and subsequent release of the LCFA's into solution. The triglyceride added to each flask was quickly adsorbed onto the oaten chaff substrate. While it is unlikely that coating of the oaten chaff substrate had inhibited colonisation of plant fragments by bacteria (see section 1.3.1) lipolysis and the release of the LCFA would

occur in the immediate vicinity of microorganisms colonising or attempting to colonise the substrate. Bacterial surfaces are observed to bind LCFA's and would therefore compete with the calcium ion for binding of LCFA's (Harfoot *et al.* 1974). So while LCFA's were added to the flasks at a rate of 7.5g/l they would not necessarily disperse and achieve this concentration in solution. In the early stages of fermentation (0 to 12 hours) it is likely that the LCFA's remain at an antimicrobial concentration in the immediate vicinity of the microbes. Later in the fermentation period more of the LCFA may have been removed as calcium soap or other inert form.

The CSO + $CaCl_2$ treatment did not reverse the antimicrobial action of the LCFA's over 24 hours of fermentation. The low ratio of acetate to propionate produced by this treatment is similar to that produced by the CSO treatment. DMD and efficiency of microbial nitrogen uptake were however intermediate to the control and CSO treatments. These observations therefore suggest inhibition of cellulolytic microorganisms as a result of cottonseed oil LCFA's remaining at antimicrobial concentrations in these flasks. The amount of calcium ion available from the CSO + $CaCl_2$ treatment and the pH of the fermentation liquors were similar to fermentations containing $Ca(OH)_2$ + CSO. The lower fractional rate of digestion of substrate for this treatment from 0 to 12 and 24 to 48 hours of incubation and apparent suppression of cellulolytic activity from 12 to 24 hours of incubation suggests that one or more factors were acting transiently to inhibit cellulolytic activity. Bailes *et al.* (1978) reported that calcium ion became toxic to rumen microorganisms fermenting sorghum stalk at a concentration of 4.75mM when measured *in vitro* and that this may relate to inhibition of uptake of magnesium or other cations by microorganisms. $CaCl_2$ and Ca(OH)2 were added to flasks at 0.44 and 0.30g/l respectively and inclusion of either salt could potentially raise the concentration of calcium ion in the fermentation liquor from around 0.5mM to 4.5mM. It is possible that $CaCl_2$ with a solubility of 830g/l went into solution more quickly than $Ca(OH)_2$ with a solubility of 1.2g/l. A rapid but transient increase in the concentration calcium ion in flasks containing $CaCl_2$ may then have inhibited microbial growth and promoted the precipitation of calcium ion with phosphate or carbonate. The difference between the effectiveness of $Ca(OH)_2$ as compared to $CaCl_2$ could then reflect the rate at which the two go into solution. The transient suppression of the digestion of substrate observed with the CSO + $CaCl_2$ treatment may therefore relate to the time required for the cellulolytic microorganisms to recover sufficient activity to actively ferment the substrate.

5.5. Conclusion

The presence of CSO at 20% (w/w) of dry matter suppressed the rate digestion of oaten chaff by mixed rumen microorganisms *in vitro*. This was accompanied by a

reduction in the ratio of acetate to propionate and the total production of VFA and is indicative of a reduction in cellulolytic activity within the fermentation in response to the release of LCFA's from CSO. The presence of CSO also reduced both the amount of microbial cells produced per unit of OM available for fermentation and the amount of microbial cells produced per unit of VFA that appeared in the fermentation liquor. The inclusion of $Ca(OH)_2$ but not $CaCl_2$ with CSO fully reversed the effect of CSO on the fermentation of oaten chaff by 24 hours of incubation although a reduction in the rate of fermentation of oaten chaff was apparent over the first 12 hours of incubation. This suggested that the rate of formation of associations between calcium ion and the LCFA arising from the CSO were slower than the rate of hydrolysis of the CSO to LCFA. The likely difference in the kinetics of calcium ion and LCFA in a fermentation system *in vivo* would therefore need to be taken into consideration. The inclusion of larger amounts of calcium ion may increase the rate of formation of associations between it and LCFA. Calcium soaps of CSO LCFA's had no affect on the pattern of fermentation of oaten chaff but appeared to increase the rate of digestion of dry matter over 24 hours of incubation. This increase was not apparent after 48 hours of incubation. Inclusion of $CaCl_2$ and $Ca(OH)_2$ without CSO, which brought the concentration of calcium ion to around 4.5mM, reduced the rate of digestion of oaten chaff but did not alter the yield of microbial N per unit of OM digested or per unit of VFA produced compared to the control. This suggests that calcium ion is toxic to certain rumen microbial species when present at high concentrations.