THE USE OF *IN VITRO* TECHNIQUES TO EXAMINE THE EFFECT OF ENSILING ON THE RUMINAL DIGESTION OF PERENNIAL RYEGRASS

by

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Declaration

I hereby declare that the work embodied in this thesis is my own and that this thesis, or any part of it, has not previously been submitted as an exercise for a degree to the National University of Ireland or any other University.

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LIST OF ABBREVIATIONS USED

β-HB	beta-hydroxybutyrate
AA	Amino acid
ADF	Acid detergent fibre
ADFN	Acid detergent fibre nitrogen
ADIN	Acid detergent insoluble nitrogen
ADOM	Apparently digested organic matter
ADP	Adenosine diphosphate
AED	Apparent extent of digestion
AEP	Aminoethylphosphate acid
ATP	Adenosine triphosphate
BCFA	Branched chain fatty acids
C2	Acetate
C3	Propionate
C4	Butyrate
CH_4	Methane gas
CHO	Carbohydrate
CO_2	Carbon dioxide
CP	Crude protein
CW	Cell wall
D	Dilution rate
DAPA	Diaminopimelic acid
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
DOMD	Digestible organic matter digested
DP	Degrees polymerisation
ES	Silage synthetic substrate without the organic acids
F	Fibre
F20	Structural carbohydrate fraction isolated by aqueous extraction at 20 ^{0}C
F70	Structural carbohydrate fraction isolated by aqueous extraction at 70 ^{0}C
FD	Freeze dried
GS	Grass synthetic substrate
H_2	Hydrogen gas
H_2O	Water
H_2S	Hydrogen sulphide gas
HCL	Hydrogen chloride acid
K_d	Rate of digestion
K_p	Rate of passage
LA	Lactic acid
LDR	Liquid dilution rate
M	Stage of maturity
MP	Microbial protein
N	Nitrogen
N_2	Nitrogen gas
NAN	Non ammonia nitrogen
NaES	Neutralised silage synthetic substrate

NDF	Neutral detergent fibre
NDFN	Neutral detergent fibre nitrogen
N_e	Excess nitrogen supplementation
NH_3	Ammonia
N_l	Limited nitrogen supplementation
NSC	Non-structural carbohydrate
O_2	Oxygen gas
^oC	^{0}Celsius
OM	Organic matter
OMADR	Organic matter apparently digested in the rumen
OMD	Organic matter digestibility
OMI	Organic matter intake
PCW	Primary cell wall
Pi	Inorganic phosphate
RDP	Ruminal degradable protein
RE	Real extent
SC	Structural carbohydrate
SCFA	Short chain fatty acids
SDR	Solid dilution rate
TAA	Total amino acids
TN	Total nitrogen
TVFA	Total volatile fatty acids
V	Volume
VFA	Volatile fatty acid
W	Water-soluble fraction
W_E	Water-soluble fraction isolated from perennial ryegrass silage post-extensive preservation
W_G	Water-soluble fraction isolated from fresh perennial ryegrass
W_R	Water-soluble fraction isolated from perennial ryegrass silage post-restricted preservation
WSC	Water-soluble carbohydrates

ABSTRACT

THE USE OF *IN VITRO* TECHNIQUES TO EXAMINE THE EFFECT OF ENSILING AND MATURITY ON THE RUMINAL DIGESTION OF PERENNIAL RYEGRASS.

The objective of this study was to examine the effect of ensiling and maturity on the *in vitro* digestion kinetics of the perennial ryegrass cell wall fraction. Preliminary methodological studies concluded that (i) *in vitro* cell wall digestion profiles were optimised when fermentation tubes were horizontally incubated, (ii) perennial ryegrass cell wall isolation by neutral detergent extraction but not by aqueous extraction (70 ^{0}C) adversely affected *in vitro* digestion kinetics (iii) method of inoculum preservation (untreated and frozen at – 20 ^{0}C, with or without cryoprotectant, with or without pre-incubation) did not affect the rate but all imposed a lag ($p<0.05$) and altered the extent of dry matter (DM) digestion, when compared with fresh inoculum. Pre-incubation was beneficial in the absence of a cryoprotectant only ($p<0.05$) and the digestion kinetics of the frozen un-treated inoculum were similar to preservation with a cryoprotectant. A dual flow semi-continuous culture was established. *In vitro* protozoal numbers were less than *in vivo* ($p<0.001$) and *in vivo* ruminal diurnal trends for volatile fatty acid (VFA), ammonia and lactate were qualitatively simulated. When the fresh forage was incubated *in vitro,* ensiling reduced ($p<0.001$) the apparent extent of digestion (AED) of a late season perennial ryegrass cell wall fraction. Ensiling had no effect on the AED of the fractionated cell wall fraction, removed from the whole forage by aqueous extraction. There was a maturity x forage interaction for the cell wall digestion of fresh ($p<0.01$) and fractionated ($p<0.05$) perennial ryegrass ensiled at different maturities. Maturity ($p<0.001$) but not ensiling adversely affected the digestion of the isolated cell wall fraction. Ensiling *per se* decreased the microbial protein production ($p<0.001$) from the water-soluble fraction but did not affect VFA production. The AED of the isolated cell wall fraction from an extensively preserved perennial ryegrass forage was increased when supplemented with the water-soluble component of the fresh herbage (W_G) ($p<0.05$) or with W_G and nitrogen ($p<0.05$). The AED of the isolated cell wall fraction from the restrictively preserved forage was not influenced by supplementation. The biochemical alterations in the W_G fraction due to ensiling did not influence cell wall digestion of the fresh or extensively preserved forage nor did it influence protozoal numbers, microbial protein or VFA production in the rumen semi-continuous culture.

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Agriculture in Ireland accounts for approximately 33 % of national gross outputs, with in excess of two thirds of agricultural outputs based on the bovine animal (beef and dairy industries, CSO 1991). To support this industry, approximately 90 and 95 % of the annual feed requirements of a spring calving dairy cow and a beef animal respectively, are provided in the form of grazed grass and conserved forages (Stakelum, 1993). Approximately 22 million tonnes, or more than 90 %, of the conserved forage is grass silage (Keady, 1996) where 'the main objective in the conservation of a crop is to preserve it at the optimum stage of growth for use during those seasons when the crop is unavailable' (McDonald *et al.,* 1991). Forage 'use' refers to the ingestion of a forage by the ruminant for subsequent metabolism and nutrient extraction, which are described biologically as the forage nutritive value. Chesson (1988) defined carbohydrate 'nutritive value' as 'the potential of the ingested polysaccharide to contribute directly to the nutrition of livestock... it is dependent on the extent to which its component monosaccharides are released and the manner of their subsequent utilisation', which are biological processes influenced by the rumen.

The rumen, a physiological adaptation on behalf of the ruminant to extract fibre as a nutrient source, is one of the ruminant 'four stomachs' which maintains a mixed anaerobic microbial ecosystem surviving on the nutrients extracted from ingested feed. Retention of feeds in the rumen for prolonged periods of time will allow microbial enzymatic hydrolysis of fibre. Fermentation pathways convert nitrogen and energy to microbial protein, volatile fatty acids, peptides and ammonia. Rumen microbes have requirements for energy, nitrogen, growth factors and environmental conditions. Alterations in any of these variables due to the modifications in diet or feeding regimes will affect the ruminal and post-ruminal fermentation of the ingested feed. The rumen is therefore the controlling link in nutrient extraction from ingested feed and subsequent supply to the ruminant host.

The nutritional dynamics of the rumen are influenced by the voluntary dry matter intake (**DMI**) and biochemical composition of ingested feed, which in turn define the feed value (production responses / unit of intake) of the forage. Though ensiling can increase the gross energy content of the forage by 10 %, animal production in both dairy and beef systems (Keady and Murphy, 1993) can often be inferior when compared to production levels maintained on fresh herbage. Such an apparent contradiction is

attributed to the poor feed value of the ensiled herbage. Steen *et al.* (1998) stated that control of food intake is quite complex, influenced by both the animal (physiological status and control) and feed characteristics (palatability, degradability, digestibility, rate of passage, physical and chemical form). It is argued that digestibility is one of the more important factors affecting DMI (Keady and Murphy, 1993). Rumen digestibility of forage dry matter can be negatively influenced by poor preservation (Keady and Murphy, 1993) and maturity (Baker *et al.*, 1991, Givens *et al.*, 1993, Keady *et al.*, 1998). Therefore, production responses in dairy (Gordon, 1980) and beef (Steen, 1992) systems can also deteriorate with forage maturity. Biochemical alterations due to maturity and ensiling may influence the rate and extent of carbohydrate and protein fermentation in the rumen (Keady and Murphy, 1993), thus altering the subsequent supply of nutrients to the lower intestine and liver (Chamberlain and Quig, 1987).

Current feed evaluation research strives to attain sufficient knowledge on ruminant feedstuffs to accurately predict individual nutrient supply to the animal and their subsequent utilisation in production, thus allowing the dietary manipulation of product quality within a production system (Tamminga and Williams, 1998). To understand, and perhaps correct for the nutritional inadequacies of the ensiled forage in ruminant nutrition, it therefore becomes important to describe the impact ensiling can have on the ruminal fermentation of soluble and insoluble nutritional components of the herbage. Such issues have been addressed using *in vivo* and *in situ* studies, however studies incorporating the functional rumen are subject to the many interactive biological processes of the ruminant animal. Therefore the use of *in vitro* techniques provides a controlled environment, removing the unwanted variation that can be found with *in vivo* or *in situ* techniques, to assess the implications of intrinsic alterations in feed components for rumen fermentation.

Since the conception of the simple batch fermentation technique in the 1950s, more elaborate and specific techniques have been developed which are supported by improvements in chemical analysis. Batch systems can be used to monitor both soluble and insoluble substrate disappearances over time, while continuous or semi-continuous systems simulate more closely the dynamics of the rumen and results can be analysed using suitable mathematical models, which generate kinetic parameters describing the dynamics of the fermentation system.

1.2 PERENNIAL RYEGRASS - BIOCHEMICAL COMPOSITION OF FRESH AND ENSILED FORAGE

1.2.1 Introduction to plant function and metabolism

To sustain daily function, growth and reproduction, plants have a requirement for three nutrients, water, minerals and CO_2. Root absorption accounts for the plants procurement of the first two nutrients, with CO_2 absorbed by the leaves. Water is the main component of the functional plant accounting for approximately 75–85 % of fresh weight. Biochemically it is important as, in conjunction with CO_2 it is one of the building blocks of all plant constituents. The two main physiological roles of plant water may be defined as transport and cooling, as a large proportion of water absorbed from the roots is lost in transpiration through the leaves in a process necessary to prevent thermal death of leaves by heating from solar radiation (Butler and Bailey, 1973).

The mineral content of the soil will dictate that available to the plant with greatest requirements for nitrogen, potassium and sulfur. Sanderson and Wedin (1989a) found that the nitrogen yield of all fractions increased with nitrogen application (230 kg N/Ha increased nitrogen content by 71 % TN) but there was no effect on the overall distribution ratio, with approximately 11 % of TN present in the cell wall. Photosynthesis is an important cellular metabolic process, which is fundamental in the provision of carbohydrate precursors through the Calvin cycle and is generally represented by the equation

$$6CO_2 + 6H_2O \xrightarrow{\text{light}} C_6H_{12}O_6 + 6CO_2$$

This biochemical process can be divided into two phases. The first is the capture of solar energy by light absorbing pigments, such that hydrogen is removed from water to reduce $NADP^+$ to NADPH leaving behind molecular oxygen (a byproduct of plant photosynthesis) and simultaneously ADP is phosphorylated to ATP. This energy capture (through molecular excitation post energy absorption) occurs in the photosynthetic pigments (chloropylls, carotenoids and phycobilins) located in the membrane of the thylakoids, which in turn are found in the chloroplasts. The basic elements of a plant cell are described in Figure 1.1. In the second phase, the energy rich bonds are used to reduce carbon dioxide to glucose units and structural polysaccharides, via the carboxylation of ribulose 1,5-diphosphate with the regeneration of $NADP^+$ and ADP (Calvin cycle, see Lehninger, 1976).

Perennial ryegrass is described as a flowering monocot C_3 herbaceous plant which may be simply segregated into root, stem and leaf tissue, functioning mainly in nutrient absorption, transport and support, and metabolic energy regulation (photosynthesis and respiration) respectively. It is suggested that all plant tissue cannot be fully characterised on any single criterion such as structure, function, location or mode of origin (Keeton, 1980). It is hence broadly divided into two main categories: meristematic and peristematic tissue. The former is a region of active cell division, composed of immature meristem cells. These cells generally have thin cell walls, are rich in cytoplasm with newly formed meristem cells differentiating as components of other tissues. The latter is composed of more mature differentiated cell types: surface tissue (epidermis), fundamental tissue (parenchym, collenchyma, sclerenchyma and endodermis) and vascular tissue (xylem and phloem).

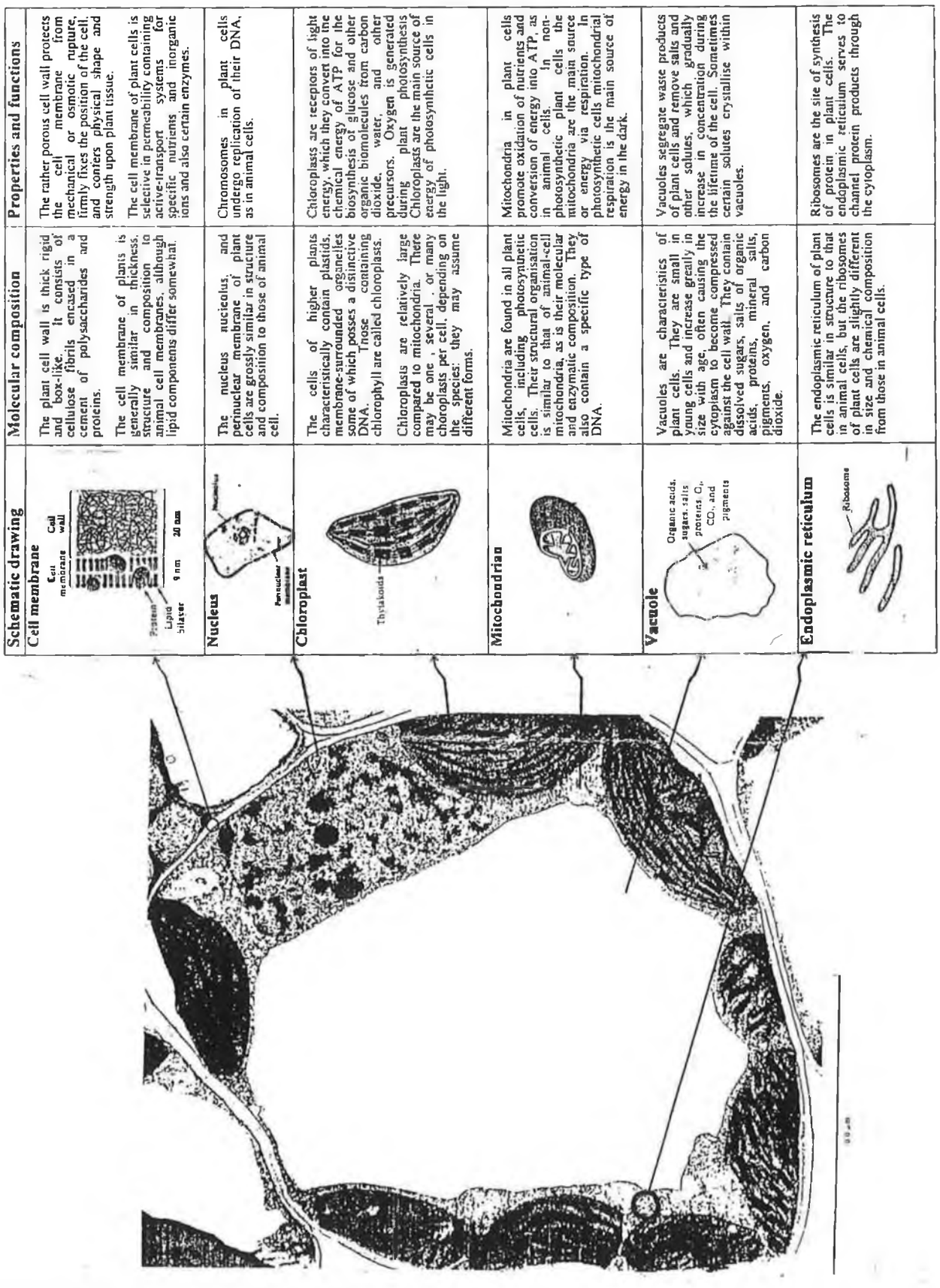
The epidermis is the principal surface cell tissue on leaves. These cells can secrete a waxy, water resistant cuticle on the outer surface and develop thick outer walls, often impregnated with cutin to ultimately protect against water loss, mechanical injury and invasion of parasitic organisms. The parenchyma cells are capable of cell division and most of the choloroplasts of leaves are in the tissue of parenchmya cells. They can be involved in nutrient storage and at later stages of development in plant support and shape. Collencyma and schlerenchyma cells function mainly in plant support, with the latter dying during plant growth (with disintegration of cytoplasm and nucleus), giving strength to the plant body through their uniformly very thick lignified secondary walls. The vascular tissue is more complex in nature, composed of cells associated with differentiation and/or support, and functioning as ducts through which water and dissolved solutes move. Sap carried upward in the plant in a continuous path running to the leaf tip in the xylem represents mainly water and nutrients absorbed from the roots. Its secondary function is plant support. The phloem is largely responsible for the transport of biochemical metabolites such as carbohydrates and amino acids up or down in the plant.

1.2.2 Non-structural carbohydrates

The monosaccharides glucose and fructose (reducing sugars), the disaccharide sucrose (non-reducing) and the storage polysaccharide fructan are the predominant non-structural carbohydrate (**NSC**) found in temperate grass plant tissue and all are water soluble (Moore *et al.,* 1994). Under Irish conditions water soluble carbohydrates (**WSC**) averaged 20 % DM, with fructans accounting for 70% of the WSC fraction and fructan levels 50 % higher in the stem than leaf (McGrath, 1988). Fructans are fructose polymers that normally contain terminal glucose residues and appear to be formed by the addition of fructose molecules to sucrose (Nelson and Spollen, 1987). Levan, a β-(2$\rightarrow$6) linked polymer of fructose with a terminal glucose, is the fructose polysaccharide present in grasses and concentrated in the stem. They can achieve degrees of polymerisation (DP) of 26 in bromegrass to

260 in timothy grass (Nelson and Spollen, 1987) and random branching may occur. Trace sugars identified in perennial ryegrass were

Figure 1.1. The main cellular components of a plant cell (Lenhinger, 1976)



melibiose, raffinose and stachyose (Butler and Bailey, 1973). There is diurnal variation in WSC concentration (2 % increase from early morning to mid-day, which subsequently decreases). The main factors influencing WSC concentration are species type (Humphreys, 1989), environmental conditions (higher concentrations of WSC are normally found at cool temperatures), nitrogen application (increasing application can decrease WSC concentration) and maturity (**Table** 1.1) (Butler and Bailey, 1973, McDonald *et al.*, 1991). The fructan concentration will increase initially with maturity due to its location but as cell wall development and lignification proceeds its concentration will drop. Starch is another storage polysaccharide, which is normally not present, or present in insignificant amounts, in temperate grasses (Butler and Bailey, 1973). It is composed of two polysaccharide types, amylose (linear, α-1-4 linked glucan) and amylopectin (highly branched, α-1-4 glucan chains with α1→6 links).

Table 1.1 Change in the composition (g kg^{-1} DM) of perennial ryegrass cut at four stages of growth (taken from McDonald *et al.*, 1991)

Date cut	Average height (cm)	Leaf + stem ratio (dry weight)	Crude protein	Ether soluble	WSC	Hemi-cellulose	Cellulose	Lignin	Ash
22 April	10.5	10.0	209	80	158	113	170	30	101
14 June	23.3	1.1	61	36	221	127	217	33	59
19 July	52.3	0.1	34	27	177	183	284	72	42
13 Sept.	56.3	0.1	31	28	42	210	331	100	39

1.2.3 Structural carbohydrates

The structural polysaccharides (**SC**) involved in cell wall development maybe divided into two main classes (**Table** 1.2): the fibre (cellulose) and the matrix (hemicellulose and pectin) polysaccharides. Cellulose is a glucan (β-(1,4)-linked glucose units), with a DP of 7,000-10,0000 glucosyl units. It is present in plant tissues as fibres composed of microfibrils which are held together by strong intermolecular and intramolecular hydrogen bonds. Hemicellulose is based on a back bone of xylose units (β-(1,4)—D-xylopyranose) and may have single unit side chains or terminal units of arabinose, glucuronic acid or their derivatives. On average, the ratio of xylose:arabinose:uronic acid is 80:15:5 (Butler and Bailey, 1973). The hemicellulose fraction may also have other pentosans (arabinogalactan) and hexosans such as the mannans, glucomannans or galactoglucomannans and β-glucans. The combined quantity of cellulose and hemicellulose is referred to as the neutral detergent fibre fraction (**NDF**) and NDF less the hemicellulose fraction is referred to as the acid detergent fibre fraction (**ADF**). Pectic substances are a group of amorphous polysaccharides (pectin, galactan and araban) which may or may not be water-soluble (Van Soest *et al.*, 1991). Pectin consists largely of unbranched chains of α-(1,4)-D-galacturonic acid units with small amounts of L-arabinose and D-galactose

substitution and is closely associated with homogenous galactans and araban.

Polysaccharides can therefore be defined and classified in terms of the monosaccharides present, ring structure (furanose or pyranose), glycosidic bonds (1→2, 1→3,1→4, 1→6), configuration (α or β) and polysaccharide structure. Digestion of these carbohydrates begins with hydrolysis of these structures in the rumen, to their oligo and mono- units and is dependent on specific enzyme activities of ruminal microflora. In lignin however, some twenty different types of linkages are involved which are based on ether linkages (Chesson and Forsberg, 1988). Hydrogen bonding dictates the strength of polysaccharide interactions and depends on the conformation of the individual molecules. The stable configuration of cellulose, mannans etc. allows for extensive intramolecular and inter chain H bonding of sugar residues giving microfibrils of highly ordered crystalline molecular aggregates (Rees *et al.*, 1982). Amorphous regions will develop where the glycan conformation does not allow stringent H bonding or where regions of sugar heterogeneity will disrupt the crystalline structure i.e. xyloglucans and mixed β-glucans (Hatfield, 1989). Covalent interactions are mainly mediated through glycosidic, ester and ether linkages and cross linking wall polymers and are predominant in amorphous structures (Hatfield, 1989). There is no evidence of covalent linkages of cellulose to other polysaccharide units (Jung, 1989).

Table 1.2 Biochemical components of forages.

Structural Units		
Hexose	**Pentoses**	**Sugar acids and amines**
Glucose (glc)	Arabinose (ara)	Galacturonic acid (gal A)
Mannose (man)	Xylose (xyl)	Glucuronic acid (glc A)
Galactose (gal)	Ribose (rib)	Glucosamine (glc NH_2)
Fructose (fru)		
Rahmnose (rha)		

Non-structural		*Structural*	
Substance	Structure	Substance	Structure
Monosaccharides		**Cellulose**	β(1→4) glucan (crystaline)
Glucose	D-glucopyranose	(fibres)	(crystalline)
Fructose	D-fructofuranose		
		Hemicellulose	
Disaccharides		(cell wall matrix) pentosans	β(1→4) xylan with some arabinose and uronic acid side chains
Sucrose	Glc α1 → 1 fru		
Maltose	Glc α1→ 4 glc		
Melibiose	Glc α1 → 6 glc	**Hexosans**	β(1→3) β(1→4) glucan (linear)
Lactose	Gal β1 → 4 glc		β(1→4) glucomannans (linear)
		Xyloglucan	β(1→4) glucan with β(1→6) lined xylose side chains
Trisaccharides			
Raffinose	α(1→6) galactosyl sucrose	**Pectic complex**	
Maltotriose	α(1→4) glucosyl maltose	(intracellular component) pectin	β(1→4) galacturonan (methylesters)
			β(1→4) galactan and mixed linked arabinan
Tetrasaccharide			
Stachyose	α(1→6) galactosyl raffinose		
		Others	
Polysaccharides		Glucan	β(1→3) glucan
Starch : amylose	α(1→4) glucan (linear)	Chitin	β(1→4) acetyl 2-amino deoxyglucan
Amylopectin	α(→) α(1→6) glucan (branched)		
Fructans : inulin	β(1→2) fructan		
Levan	β(2→6) fructan		
Dectran	α(1→) fructan		
Galactomannans	β(1→) mannans with α (1→) gal side chains		

1.2.4 Maturation

At a cellular level, cell growth or elongation is defined by the development of the primary cell wall, which is separated form adjacent cells by the middle lamella. The primary cell wall is mainly composed of hemicellulose polysaccharides, proteins, pectins and xylans. Cellulose is also present in smaller amounts (25-30 %, Butler and Bailey, 1973) and is amorphous in nature (Chesson and Forsberg, 1989). Both the middle lamella and the primary cell wall are rapidly digested in the rumen (approximately 12 h). Phenolic compounds (non-core lignins) are also deposited in the primary cell wall and may represent initiation sites for lignification, though p-coumaric acid is not thought to be involved (Chesson, 1988). Phenolic compounds are present in small amounts (< 1 % cell wall DM) and are readily metabolised by rumen bacteria (Chesson *et al.*, 1982) but they maybe selectively inhibitory of fungal cellulolytic activity (Gordon *et al.*, 1995). Their role in cross-linking would explain a positive correlation between the release of phenol compounds from cell walls and increased microbial and enzymatic degradation (Hatfield, 1989). Engels (1989) showed that where thin cross sections of stem and leaf are exposed to digestion, giving microbes immediate access to all wall layers, extensive digestion of lignified secondary cell wall is observed with little digestion of the middle lamella/primary cell wall even after 3 weeks. This maybe attributed to the higher lignin concentration in the middle lamella/primary cell wall or the composition of the lignin structure. Gordon *et al.* (1995) have provided evidence that only ferulic acid is present in primary cell wall and is covalently linked to polysaccharides through ester linkages. Such an association would affect the rate of digestion only (Jung and Allen, 1995). Digestion of the primary cell wall may be limited by the presence of an undisrupted external cuticle layer (Chesson and Forsberg, 1989). The immature cell wall tissue describes undifferentiated cells in the primary cell wall and cells which never develop lignified secondary cell wall (mesophylls and phloem present mainly in the leaf).

When cell elongation ceases, a secondary cell wall is laid down for structural support of the cell. The secondary cell wall is laid down inside the primary cell wall and becomes progressively thicker as it grows towards the centre of the plant cell (Bacic *et al.*, 1988). The polysaccharide deposited is richer in crystalline cellulose than in xylan, pectins are no longer incorporated into the cell wall and lignification begins (Chesson, 1988). Lignification is the covalent interaction of guaiacyl, syringyl and hydroxyphenyl units into large molecular polymers, which are capable of molecular association with the matrix polysaccharides (core lignin). It commences in the cell corners and proceeds progressively through the middle lamella and primary cell wall to the SCW. As lignification proceeds the lignin that is deposited shifts from a guaiacyl type lignin to a lignin richer in syringyl units and is not thought to be chemically bound to the cellulose fraction (Chesson and Forsberg, 1989). Fry (1986) and Iiyama *et al.* (1990) suggested that a cross link is formed with a single ferulic acid residue which bonds with the

polysaccharide (arabanoxylans) and lignin moieties, through ester and ether linkages respectively. P-coumaric acid may only be associated with lignin, through ether linkages (Lam *et al.,* 1992) and will therefore only act as a physical hindrance in digestion. Lignin-carbohydrate complexes are soluble at rumen pH but are not digestible in the anaerobic environment, as ether linkages require oxidative enzymes or oxidising agents for disruption. The mature cell wall implies lignified material, mainly sclerenchyma and vascular tissue.

In isolated form all hemicellulose and cellulose polysaccharides are fully digestible (Wilson, 1994) but lignification of the cell wall can have a linear or curvilinear effect on digestibility (Jung and Vogel, 1986). Removal of lignin via chemical treatment has been shown to increase rumen degradability of barley straw by 21-28 units (Morrison, 1988). Digestion rates vary with cell type (Gordon *et al.,* 1985) and cell wall digestion is negatively affected by lignification, chemical interactions and the physical hindrances within these components (Buxton, 1989, Jung and Deetz, 1993, Jung and Allen, 1995). Lignin, substitution of the amorphous regions and extensive bonding of linear polysaccharides to the crystalline region of cellulose may exert a negative impact on the rate of fermentation by shielding cellulose or hemicellulose from enzymatic hydrolysis (Hatfield, 1989, Jung and Deetz, 1993). The insufficient porosity of lignified cell walls to allow the free diffusion of microbial enzymes from the surface may affect the rate of digestion. Accumulation of lignin on the exterior of a fibre particle, forming an impenetrable microbial layer, will affect the extent of digestion (Gordon *et al.*, 1983). Lignification can therefore affect both the rate and extent of cell wall digestion and its effect on digestion may be more accurately described in terms of extent of ether linkages (Jung and Allen, 1995). The negative relationship between digestible organic matter digested (**DOMD**) and lignin (Givens *et al.*, 1993a, Givens *et al.*, 1993b) does not hold for primary and secondary regrowths (Givens *et al.*, 1993a, Givens *et al.*, 1993b, Van Soest, 1978) as it is suggested that the lignin-polysaccharide structure may be different between spring and autumn material (Givens *et al.*, 1993a) thus altering the kinetics of rumen fermentation.

Bosch *et al.* (1992a) explained the faster rates of ADF degradation when compared to NDF degradation, by stating that NDF is a mixture of cellulose, hemicellulose and lignin, of which particularly hemicellulose is encrusted with lignin. This raises the argument that hemicellulose may (Morrison, 1983) or may not (Jung and Vogel, 1986) be selectively protected by lignin indicated by increased concentrations of xylose in the residue. Discrepancies in results may be attributed to the analytical procedures used (Jung and Vogel, 1986, Wilson, 1994), the degree of arabinose substitution which can physically hinder the activity of the arabinofuranoside enzyme in xylan digestion or substrate preferences, as Chamberlain and Choung (1995) concluded that xylose was not used

preferentially by rumen microbes when greater microbial protein production was obtained by supplementation with various other sugars.

1.2.5 Cellular nitrogen

Forage proteins can be enzymatic or structural in nature and are concerned with the growth and biochemical functions of the cells. Approximately 75 - 90 % of total nitrogen in fresh grass is present as protein (Oshima *et al.*, 1979) and the amino acid composition of proteins does not vary greatly within plant species (Hatfield, 1989). The remaining nitrogen content of herbage is primarily composed of amino acids, amides, peptides, amines, and nitrates (Oshima *et al.*, 1979).

Soluble protein increases with crude protein (**CP**) content but decreases with maturity (Sanderson and Wedin, 1989b, Van Vuuren *et al.*, 1991). Soluble cytoplasmic proteins account for > 80 % of total cellular nitrogen and 4 – 38 % of total plant protein (Sanderson and Wedin, 1989b). Ribulose-diphosphate carboxylase, responsible for carbon fixing during photosynthesis, can often constitute up to 50 % of the total soluble protein (Butler and Bailey, 1976). Leaf protein is situated mainly in the chloroplasts and chlorophyll (Butler and Bailey, 1976). Theodorou *et al.* (1996) suggest that robust cellular enzymes, described by a broad pH (5 – 8), temperature optima and substrate specificities and which are intimately associated with controlled cell death, may play a very important role in ruminal proteolysis of grazing animals, via internal plant cell proteolytic activity. They emphasis the recognized importance of this cellular proteolytic process during the ensiling process and that *in vitro* and *in sacco* studies, examining herbage digestion kinetics may overlook this contribution due to the dried and mill nature of the substrate. This argument is supported by the findings of Zhu *et al.* (1999) who found proteolytic breakdown of plant proteins when fresh herbages were incubated *in vitro* without rumen micro-organisms present.

Extensin, the main structural protein, is a hydroxyproline based protein with extensive substitution of arabinose and galactose (Butler and Bailey, 1973) and is present only in the primary wall. There is an inverse relationship between CP and NDF content, and the nitrogen associated with the cell wall increases with maturity (van Vuuren *et al.*, 1990, van Vuuren *et al.*, 1991). Bosch *et al.* (1994) found no significant relationship between cell wall content and the rumen degradation rate of CP, though corrections were not made for microbial protein (**MP**) contamination in the *in sacco* technique. The neutral detergent fibre nitrogen (**NDFN**) fraction of leaves and stems was found to be 6.4 and 2.4 g/kg NDF respectively, with ADF nitrogen (**ADFN**) accounting for 21 and 49 % of cell wall nitrogen respectively (Sanderson and Wedin, 1989b). This is attributed to the greater percentage of primary cell wall and thus extensin, in the leaf material (Sanderson and Wedin, 1989b). Sanderson

and Wedin (1989a) found that the nitrogen yield of all fractions increased with nitrogen application (230 kg N/Ha increased nitrogen content by 71 % TN) but there was no effect on the overall distribution ratio, with approximately 11 % of TN present in the cell wall. Nitrogen application was found to increase herbage CP, increase in the digestion rates of organic matter (**OM**) and CP but decrease OM content (van Vuuren *et al.*, 1990).

1.2.6 Ensiling

Forage preservation should avoid adverse changes in the biochemical composition of the herbage, which would minimise nutrient losses, and thus changes in herbage nutritive value (McDonald *et al.*, 1991). Optimisation of the ensiling process has been positively associated with improvements in forage digestibility and animal production (Harrison *et al.*, 1994) but Zimmer (1980 as cited by McDonald *et al.*, 1991) from a review of 504 trials, concluded that unavoidable energy losses could be restricted to 7 % with good management practices (**Table** 1.3).

Table 1.3. Energy losses during ensiling and causative factors (taken from McDonald *et al.*, 1991)

Process	Classification	Approx. loss %	Causative factors
Residual respiration	Unavoidable	1-2	Plant enzymes
Fermentation	Unavoidable	2-4	Micro-organisms
Effluent or	Mutually	5- >7 or	DM content
Field loss by wilting	unavoidable	2- >5	Weather, technique, management, crop
Secondary fermentation	Avoidable	0- >5	Crop suitability, environment in silo, DM content
Aerobic deterioration during storage	Avoidable	0- > 10	Filling time, density, silo, sealing, crop suitability
Aerobic deterioration after unloading	Avoidable	0 ->15	As above, DM content, silage, unloading technique, season
Total		7- >40	

These unavoidable losses occur through plant and microbial enzymatic activities. Preservation by ensiling relies on the rapid development and maintenance of an anaerobic environment of reduced pH, to minimise the oxidative and pH-sensitive catabolic enzymatic activities of plant and microbes (McDonald *et al.*, 1991). The buffering capacity of a herbage will resist a fall in pH and can be attributed to the anions present (organic acid salts, orthophosphates, sulphates, nitrates, and chlorides) and the activity of plant proteins (10-20 % of total buffering capacity, McDonald *et al.*, 1991).

1.2.5.1 Plant and microbial enzymatic activity during preservation

Plant respiration can be defined as the oxidative degradation of organic compounds to yield utilisable energy (McDonald *et al.*, 1991) and will occur in the harvested forage until WSC and/or oxygen are

depleted. Wilting can also affect respiration and all catabolic energy released is assumed lost in heat production due to the lack of biosynthetic pathways (McDonald *et al.,* 1991). Plant proteolytic activity pre-ensiling is associated with conditions and duration of the wilting period of the forage (Carpinterno *et al.,* 1979, Brady, 1960), while plant proteolysis post ensiling can decrease protein nitrogen from 800 to 40 g/kg N after 16 days (Kemble, 1956). The proteolytic activity of plant enzymes will decrease with increasing DM (McDonald, 1982). The low environmental pH may be sufficient to reduce or inhibit plant proteolysis (McDonald *et al.,* 1991). A low pH may also promote acid hydrolysis of the hemicellulose fraction (Dewar *et al.,* 1963), thus providing more fermentable WSC for microbial fermentation.

The dominant microbial population during ensiling will influence the biochemical composition of the preserved forage (McDonald *et al.,* 1991). The majority of the indigenous microbial population present on the forage at ensiling (10^6 - 10^8 bacteria /g DM, Lindgren *et al.,* 1983) are strict aerobes which do not survive the rapid development of anaerobic conditions in a well sealed silo. They are succeeded by the growth of facultative anaerobic (Lactic acid bacteria, Enterobacteriaceae, Bacillus and yeasts) and obligate anaerobic species (Clostridium) which are present as spores on the forage (McDonald *et al.,* 1991). In a favorable progression of microbial domination (**Table** 1.4), the clostridia and enterobacteria, with pH optima of pH 7.0 to 7.4, are inhibited by a rapidly decreasing pH due to the proliferation of thelactic acid bacteria (Woolford, 1984). Strains of Pedicoccus, Enterococcus and Leuconostoc should become dominant in the first two days of fermentation, and subsequently be superseded by the more acid tolerant Lactobacllus and Pediococcus strains (Shiels, 1999). The lactic acid bacteria can be homofermentative or heterofermentative, where carbohydrates are mainly fermented to lactate or lactate, acetate and ethanol respectively (McDonald *et al.,* 1991). The lactic acid bacteria are mainly non-proteolytic, with a poor ability to ferment amino acids (McDonald *et al.,* 1991). The excessive energy losses with clostridial fermentations can be attributed to the production of energy wasteful products (CO_2 and hydrogen), and the deamination and decarboxylation of amino acids to produce ammonia. This can increase the buffering capacity of the forage, with a subsequent clostridial fermentation of the lactic acid to butyric acid.

Table 1.4 Dry matter and gross energy losses calculated from some important fermentation pathways (taken from McDonald *et al.*, 1991)

	Loss (%)	
	DM	**Energy**
Lactic acid bacteria		
Homofermentative		
Glucose (or fructose) + 2 ADP + 2 P_i → 2 lactate + 2 ATP + $2H_2O$	0.0	0.7
2 citrate + ADP + Pi → lactate + 3 acetate + 3 CO_2 + ADP	29.7	+1.15
malate → lactate + CO_2	32.8	+1.8
Heterofermentative[a]		
Glucose + ADP + Pi→ lactate + ethanol + CO_2 + ATP + H_2O	24.8	1.7
3 fructose + 2 Pi → lactate + acetate + 2 mannitol + CO_2 + 2 ATP + H_2O	4.8	1.0
Clostridia		
2 lactate + ADP + Pi → butyrate + 2 CO_2 + 2 H_2 + ATP + H_2O	51.1	18.4
Enterobacteria		
Glucose + 3 ADP + 3 Pi → acetate + ethanol + 2 CO_2 + 2 H_2 + 3 ATP + 2 H_2O	41.1	16.6
Yeasts		
Glucose + 2 ADP + 2 Pi → 2 ethanol + 2 CO_2 + 2 ATP + 2 H_2O	48.9	0.2

[a]Citrate and malate fermentation are the same as for the homofermentative lactic acid bacteria

1.2.5.2 Effect of extensive and restricted preservation on forage composition

The composition of the resulting silage can vary with preservation technique (Fox *et al.*, 1972, Steen *et al.*, 1998) but in general, plant and microbial activity will result in an increase in forage DM due to effluent loss, and a variable extent of microbial fermentation of the WSC and hemicellulose components to volatile fatty acid (**VFA**) and organic acids (McDonald *et al.*, 1991). Though CP can remain relatively constant, up to 66 % of the protein content (Carpintero *et al.*, 1979, Heron *et al.*, 1986) can be degraded to peptides, amino acid and ammonia, giving silages a greater protein degradability in the rumen when compared to grasses (Lopez *et al.*, 1991, Petit and Tremblay, 1992, Cushnahnan and Gordon, 1995). Grass silage which has under gone a good fermentation, would be typified by a pH of <4.5, a predominance of lactic acid versus acetic acid, ammonia-N content of <1 % of DM and <0.5 % butyric acid in DM (Harrison *et al.*, 1994).

The addition of sugar at ensiling, as a complementary carbohydrate source, reduces the risk of prematurely arresting the lactic acid fermentation due to depletion of the indigenous sugars. Forages can be well preserved in this way but are extensively fermented. Keady (1996) concluded from literature that in general, an accelerated growth of the lactic acid bacteria due to increased availability of substrate gave a more rapid development of acid conditions than the untreated forage, while Leibensperger and Pitt (1988) modelling the effects of sugar addition on ensiling, proposed that for different forage DM and rates of application, there was little effect of sugar addition on pH and proteolysis when compared to the untreated herbage, as the time required for pH reduction was not short enough to prevent extensive proteolysis. Varying degrees of losses can occur during extensive fermentations, due to effluent production, conversion to gas or undesirable fermentation products such as acetic and butyric acids (Fox *et al.*, 1972) and the proliferation of clostridias and yeasts, particularly at low rates of addition (10 g WSC /kg fresh weight, Weise, 1969). Fitzgerald (1995) recommended the addition of 4.2 - 8.4 g WSC/ kg forage DM. A variable application rate is necessary to address the fact that grasses harvested at early stages of growth are more highly buffered than those cut at later stages and thus have a greater capacity to resist a fall in pH. An extensively fermented but well preserved silage will therefore be characterised with extensive fermentation of the WSC and fermentable hemicelluloses fractions and some degree of proteolysis (Keady, 1996).

In contrast, the addition of an acid to the forage pre-ensiling, to immediately reduce pH, to act as an anti-microbial agent (Woolford, 1975, McDonald and Henderson, 1974) and to inhibit plant respiration (Henderson *et al.*, 1972), should result in a well preserved silage where fermentation and proteolysis of the forage components have been severely restricted. Formic acid is the strongest of the organic acids but much weaker than the mineral acids (HCl and sulphuric) and application rates to reduce silo pH to

a minimum of pH 4 normally range from 2 – 5 l/tonne fresh weight. Carpintero *et al.* (1979) examined the effects of increasing formic acid application on the fermentation process in laboratory silos. The results outlined in **Table** 1.5, show a greater retention of the WSC and protein components, and a reduction in the production of VFA with increasing application rate of formic acid. These results are supported by Barry *et al.* (1978), O'Kiely (1993) and Jaakkola *et al.* (1991). High levels of formic acid addition (> 4 l/t) may cause acid hydrolysis of the hemicellulose fraction (Dewar *et al.*, 1963) but may also be necessary to prevent yeast and enterobacterial proliferation (Chamberlain and Quig, 1987).

Increasing maturity of the ensiled herbage will also affect the fermentation profile of the formic acid treated herbage. Rinne *et al.* (1997a, 1997b) ensiled a mixed sward at 4 stages of maturity, from pre-bloom (29 May) to late bloom (25 June). There was a reduction in the NDF concentration during ensiling that was attributed to acid hydrolysis and a loss of NDF-N (**Table** 1.6). The hemicellulose fraction lost during ensiling decreased with maturity (32 %, 26 %, 18 %, and 12 % DM) which may reflect the more resilient lignified cell wall of the herbage. The organic acids, ammonia and non ammonia-N concentrations of the silage also decreased with maturity. Keady *et al.*, (1995) and Jaakkola *et al.*, (1991) found that the decrease in the hemicellulose content by formic acid addition (mainly acid hydrolysis) was accompanied by WSC retention and ammonia concentration reduction, compared to the untreated forage. Cushnahan *et al.* (1995) found that the urinary nitrogen losses were greater for extensively preserved silages when compared with grass, with the restrictive preservation being intermediate.

From a review of literature, Keady and Murphy (1993) concluded that when forage preservation is good, a restricted fermentation will improve the nutritive value of the silage, as the production response obtained from molasses treated silage (15.8 l/ton) was only 29 % that of formic acid treated silage (3.03 l/ton). Fox *et al.* (1972) found that DMI was greater for the restricted but not extensive preservation. It could be suggested that the superiority of restrictively fermented silage is attributed to the lower content of fermentation acids (**Table** 1.7). The preserved WSC component is suggested to behave similar to that of supplemented WSC, by supporting an increase in the butyrate proportion in the VFA pool (Jaakkola *et al.*, 1991).

Though Chamberlain *et al.* (1982) decreased the non-protein nitrogen of silage by increasing the application rate of formic acid, no significant differences were observed in ammonia concentration or microbial protein synthesis in the rumen of sheep. Formic acid therefore may inhibit microbial and plant enzyme, retains a fraction of the WSC and protein content of the herbage, and may cause acid hydrolysis of the hemicellulose fraction.

Table 1.5 The effect of different levels of formic acid (g kg^{-1} fresh weight) on the composition of ryegrass-clover silages after a 50 day ensiling period (taken from Carpintero *et al.* 1979)

	Formic acid (85 % w/v) [a]						
	Grass	0	0.4	1.0	2.0	4.1	7.7
Composition of DM (g/kg)							
WSC	203.0	12.0	33.0	72.0	124.0	211.0	250.0
Total nitrogen (TN)	19.3	18.2	17.8	18.5	19.3	19.2	18.6
Acetic acid		28.8	24.1	18.9	13.3	4.5	3.1
Propionic acid		0.18	0.27	0.22	0.36	0.28	0.19
Butyric acid		0.19	0.04	0.04	0.16	0.23	0.03
Lactic acid		122.0	153.0	115.0	117.0	66.0	5.0
g/ kg TN							
Protein-N	819.0	265.0	285.0	325.0	358.0	401.0	462.0
Ammonia-N		95.0	79.0	59.0	46.0	12.0	12.0

[a] Containing 850g formic acid kg^{-1}

Table 1.6. Chemical composition of grasses and corresponding silages harvested at different stages of grass maturity (taken from Rinne *et al.*, 1997a)

Date of harvest	May 29		June 6		June 15		June 25	
	Grass	Silage	Grass	Silage	Grass	Silage	Grass	Silage
Dry matter (DM) (g/kg fresh weight)	271	261	231	226	198	217	278	267
Composition of DM (g/kg)								
Neutral detergent fibre	464	409	555	497	600	579	648	623
Acid detergent fibre	202	229	242	264	277	313	311	326
Ash	71	82	72	77	68	68	66	69
WSC	238	57	152	42	158	70	117	65
Total N (TN)	29.3	29.9	25.0	26.7	18.9	18.7	17.0	17.4
Soluble N (g/kg TN)	388	745	349	728	355	641	406	589
Total Volatile fatty acid		102		96		75		59
Acetate		25		16		14		10
Propionate		1.2		2.3		0.1		1.2
Butyrate		0.2		1.9		0.4		0.2
Lactate		75		76		60		47

Table 1. 7. Effect of ensiling and pattern of silage fermentation on the chemical composition of herbage (g/kg alcohol-corrected toluene dry matter (DM) unless stated otherwise) (taken from Jaakola *et al.*1991)

	Fresh grass	Extensively fermented silage	Restricted fermented silage
DM (g/kg fresh weight)	154.2^{a}	168.0^{ab}	182.3^{b}
Composition of DM (g/kg)			
Neutral-detergent fibre	5730	547.0	582.0
Acid-detergent fibre	267.0^{a}	299.0^{ab}	307.0^{b}
Hemicellulose	306.0^{b}	249.0^{a}	278.0^{ab}
Water soluble carbohydrate	189.0^{c}	34.0^{a}	112.0^{b}
Ash	100.0	98.0	94.0
Nitrogen (N)	30.6	28.8	30.2
Ammonia N (g/kg N)	19.5^{a}	43.5^{ab}	70.7^{b}
Acetic acid	ND	12.4^{b}	6.4^{a}
Propionic acid	ND	1.3^{a}	3.5^{b}
Butyric acid	ND	0.2	0.9
Ethanol	ND	11.7^{a}	21.6^{a}
Lactic acid	ND	109.9^{b}	24.1^{a}
Buffering capacity (meq/kg DM)	801^{a}	1182^{b}	627.0^{a}
Gross energy (MJ/kg DM)	18.9	19.3	18.5

[a,b,c] Within a row values with a common superscript are not significantly different ($p>0.05$)

ND = Not determined

1.3 THE RUMEN

The ruminant animal has evolved a complex digestion system to maximise nutrient extraction from fibrous carbohydrate-based forages (**Figure** 1.2). Feeding preferences among ruminants define three groups which differ in rumen function, namely the concentrate selectors, intermediate types and the grazers (Church, 1988, Lechner-Doll *et al.,* 1991). Cattle and sheep are both grazers, but differ in intake (Keady and Murphy, 1993), chewing activity (Faichney, 1986) and particle mean retention time (Prigge *et al.,* 1984, Lechner-Doll, 1991). They have similar rumen particle distributions (Sutherland 1988) and rumen mass: body weight (Lechner-Doll *et al.*, 1991). Prigge *et al.* (1984) in a comparative study of wethers and steers, maintained on forage based diets, found significant species/forage and species/level of intake interactions ($p<0.05$) for dry matter digestibility (**DMD**) but there was no difference in liquid dilution rates due to species.

The ruminant has four stomachs, of which the rumen (reticulo-rumen) representing 85 % of the total stomach capacity, is the most important (Moss, 1994). It supports a mutualistic relationship between the host and an anaerobic microbial population, responsible for 30 to 100 % of apparent feed digestion (Rode *et al.,* 1985, Murphy *et al.,* 1994), supplying 70-100 % of amino acid requirements to the ruminant animals and 70-85% of the energy supply through the absorption of VFA (see Sinclair *et al.,* 1995). Church (1988) has detailed the biological function of the remaining stomachs. The acid stomach and large intestine are the secondary sites of feed digestion. Site of digestion is influenced by level of intake (Beever *et al.,* 1972, Todorov and Djouvinov, 1994), particle size and feed composition (**Table** 1.8) but not frequency of feeding (Robinson and Sniffen, 1985).

The lower intestines can compensate for poor rumen digestibility due to increased turnover rates but not decreased forage quality (Bowman *et al.,* 1991, Todorov and Djouvinov, 1994). Rumen, small intestine and large intestine digestibilities are approximately 56.2 to 64.4, 26.3 to 33.7 and 4.2 to 16.7 % of total organic matter digested (**OMD**) (Galyean and Owens, 1991), while starch digestion in the small intestine can be 40-70 %. Digestion in the large intestine is inefficient due to reduced retention times and excretion of MP in the faeces (Orskov, 1994) though acidic hydrolysis of the fibre component may increase the rate of digestion (Mertens and Ely, 1979). Absorption of nutrients occurs in the omasum and SI. Microbial nitrogen, feed nitrogen and purine disappearance in the small intestine can be 68, 73 and 88 % respectively (Owens *et al.*, 1984). Schonhusen *et al.* (1999) concluded that 78 % of RNA disappearance occurs between the proximal duodenum and the terminal ileum, with 24 % of this from endogenous sources.

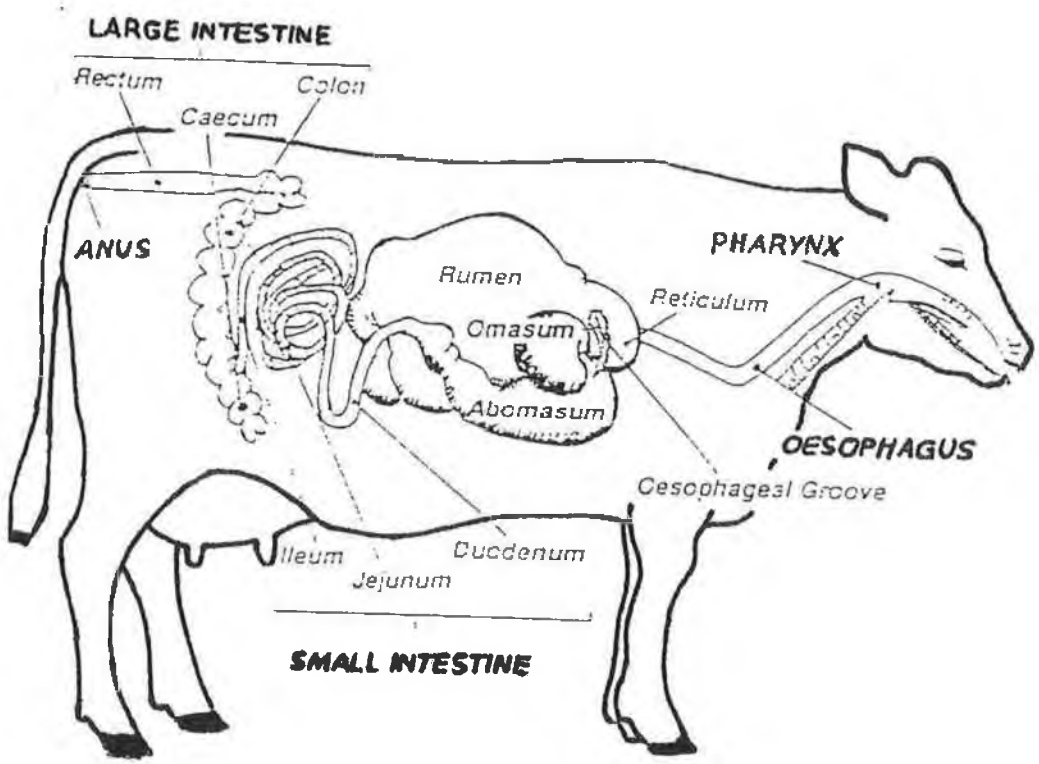


Figure 1.2 The specialised digestive tract of the ruminant animal.

Table 1.8 General effect of dietary factors on site and extent of organic matter digestion in ruminants (adapted from Church, 1988)

Diet	*Factor*	*Ruminal extent of digestion*	*Total tract digestibility*	*Relative shift in site of digestion*
Roughage	↓ particle size	←→ or ↓	↓	Intestines
	↑ concentrate intake	←→ or ↓	←→ or ↓	Intestines
	↑ intake	←→ or ↓	↓	Intestines
Concentrates	↓ particle size	↑	↑	Rumen
	↑ fibre intake	↓	↓	Intestines
	↑ intake	↓	↓	Intestines
Dietary fats		↓	←→	Intestines
Defaunation		↓	↓	Intestines

1.3.1 Rumen environment

Inoculation of the rumen begins after birth and is thought to develop through the passing of saliva directly between animals or indirectly in aerosols, foodstuffs, or communal drinking water (Eadie, 1962, Hobson, 1971), with rumination in calves occurring from 3-10 weeks of age, depending on DMI and VFA concentration in the rumen (Church, 1988).

The rumen, which can be 40 to 100 l and 3 to 15 l in volume in cattle and sheep respectively (Weimer, 1992), has a relatively constant temperature range of 38-42 ^{0}C and a gas composition of approximately 65 % CO_2, 27 % CH_4, 7 % N_2, 0.6 % O_2, 0.2 % H_2 and 0.01 % H_2S (Weimer, 1992). There is a requirement by the cellular tissue of the rumen wall for oxygen. Oxygen entering the rumen environment due to transfer from blood, feeding and rumination was estimated to be 38 l O_2/day in sheep (Czerwaski and Breckenridge, 1969). The anaerobic environment is maintained by the 'oxygen uptake' ability of the rumen fluid, where Newbold *et al.* (1993) calculated that, in sheep, a rumen with a volume of 6 litres has the oxygen uptake capacity of 11.5 to 16 l/d. Dissipation of oxygen occurs through microbial organelles called hydrogenosomes (Prescott *et al.*, 1993) which may be indigenous to the rumen or supplemented via probiotics (Newbold, 1996) thus maintaining an 'anaerobic' environment. Diurnal variations and variations in feeding regimes and diet compositions can alter the redox potential (-250 to –400 mV), osmolarity (250 to 420 mOsmol/kg rumen contents) (Carter and Grovum, 1990), pH (pH 5.8 -7) (Church, 1988) and liquid and solid turnover rates of the rumen.

1.3.2 Rumen function

The contents of the rumen (approximately 12 % DM) are not homogenous. A bouyant solid fibrous mat is maintained at the longitudinal pillar and the retention capacity of this mat is thought to increase with true fibre content of the diet (Weidner and Grant, 1994). Microbial sequestration in the mat, by species

(protozoan) with generation times greater than the liquid flow rate enhances microbial survival and propagation (Hungate, 1966).

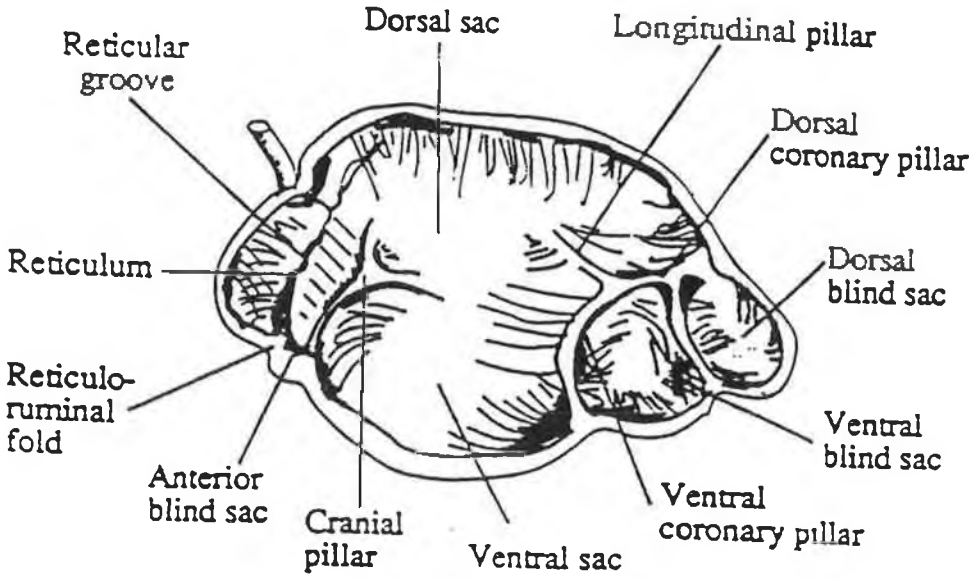
Within the rumen there exists further partial compartmentation created by muscular pillars projecting into the rumen (**Figure** 1.3a) and necessary to facilitate rumen motility. This results in a passive mixing of contents (**Figure** 1.3b), which helps rumination and eructation of gases, promoting a continuous turnover of the contents and assisting feed passage (Church, 1988). Excessive acid production and microbial dominance may cause the ruminal pH to decrease well below 6 causing a condition of acidosis, which can be fatal. Buffering of rumen pH occurs through saliva production, which contains bicarbonates and phosphates (McDougall, 1948) and deamination of amino acids with ammonia production.

The inner wall is also covered with small projections of papillae which increase the internal surface area thus enhancing nutrient absorption (Church, 1988). The absorption rates of most nutrients are sensitive to lumen pH (Dijkstra, 1994). Propionic and butyric acids are absorbed more rapidly than acetic acid at lower pH (McLoed and Orskov, 1984). The molar proportion of VFA can influence VFA absorption from the rumen (**Table** 1.9), while interactions between a low pH and high levels of lactic acid and osmolality can reduce absorption (Gaebel *et al.*, 1987). Due to the lipophilic nature of the rumen epithelium, it is suggested that VFA are absorbed in the un-dissociated form (Gabel and Martens, 1991). The pk value for VFA (pk 4.8) would suggest that at normal rumen pH 6.2-6.8, VFA exist and are absorbed in the dissociated form, with the un-dissociated form reformed after absorption (Orskov, 1994). Microbial activity, absorption and liquid flow from the rumen will therefore influence the concentrations and ratios of VFA and ammonia concentration in the rumen.

Table 1.9. The effect of initial pH and individual concentration of experimental solutions introduced into the rumen of dairy cows on fatty acid fractional absorption rates (/h) (taken from Dijkstra, 1994)

	pH				***Concentration (mM)***		
	4.5	**5.4**	**6.3**	**7.2**	**100**	**50**	**20**
Acetic	0.35	0.35	0.33	0.21	0.32^{ab}	0.43^{a}	0.18^{b}
Propionic	0.67^{a}	0.54^{ab}	0.51^{ab}	0.35^{bc}	0.44	0.51	0.6
Butyric	0.85^{a}	0.53^{b}	0.46^{a}	0.28^{b}	0.54	0.45	0.6

^{a,b,c} Means within rows and treatments with different subscripts are significantly different ($p<0.05$)



Right side of the reticulo-rumen.

Figure 1.3a Recticulo-rumen

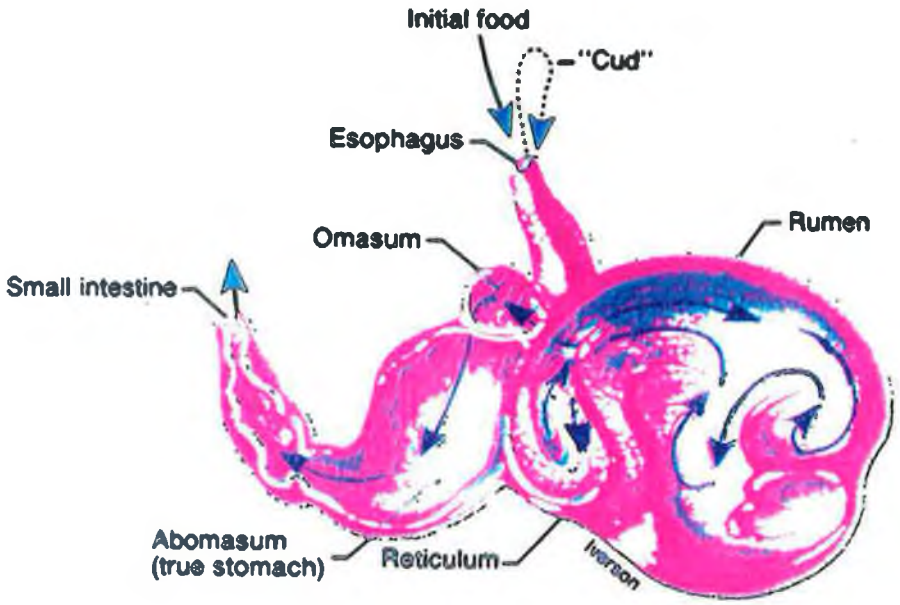


Figure 1.3b Flow patterns in the reticulo-rumen

1.3.3 Feed retention in the rumen

The feed value of a forage is influenced by DMI, which in turn is influenced by the retention time of ingested feed in the total digestive tract. The retention time of feed in the complete stomach can represent up to 80 % of the total mean retention time of feed particles in the entire digestive tract. Retention time in the rumen is significantly higher than residential times in other stomachs (Peyraud and Mabrini, 1992) and is influenced by many factors.

1.3.3.1 Particle size reduction

It was suggested that feed left the rumen when the particle size was reduced to a critical particle size, sufficiently small to pass through the omasal orifice. Ulyatt *et al.* (1986) states that the critical particle size for sheep and cattle is 1-2 mm and 2-4 mm, respectively. Faecal particle size does not differ greatly from the profile of sizes found in the reticulum (Ulyatt *et al.*, 1986), suggesting that particle size reduction is a reticulorumen process (**Table** 1.10). Rumen retention time was found to be inversely related to particle size (Rode *et al.*, 1985, Mambrini and Peyraud, 1992). A significant interaction between critical particle size and feed passage rate is discussed as a controlling factor for DMI (Van Soest, 1982, Orskov *et al.*, 1988, Madsen *et al.*, 1994). Increased rumen mean retention time will therefore increase the rumen fill value of a forage and thus reduce its DMI (Poppi *et al.*, 1981).

Table 1.10. Particle size distributions in the stomachs of sheep fed chaffed hay (% particulate DM retained on sieve) (taken from Ulyatt *et al.*, 1986)

Sieve size (mm)	*Rumen*	*Reticulorumen*	*Omasum*	*Abomasum*
4.0	16.5^{a}	10.7^{b}	0.0^{c}	0.0^{c}
2.0	8.6^{a}	8.6^{a}	0.6^{b}	0.6^{b}
1.0	14.6^{a}	15.3^{a}	3.4^{b}	4.0^{b}
0.5	17.4^{a}	18.6^{a}	15.7^{b}	19.4^{a}
0.25	11.9^{a}	12.8^{a}	26.0^{b}	22.7^{c}
<0.25	31.0^{a}	34.0^{a}	54.4^{b}	53.3^{b}

^{a,b,c} Between organs means with different superscripts are significantly different ($p<0.001$)

Chai *et al.* (1984) suggest that chewing activity accounts for the greatest percentage of particle size reduction by physically breaking and weakening plant cell walls. This is important as ruminating time is thought not to exceed 9-10 h/d (see Bosch *et al.*, 1992a) – 12 h/d (Kennedy and Doyle, 1993) after which intake will decrease.

Microbial digestion of feed particles is thought to be responsible for 20 % of feed particle size reduction (McLeod and Minson, 1988a, McLeod and Minson, 1988b). Increased DMI is associated with increased NDF digestion (Oba and Allen, 1999). Mertens and Ely (1979) reported a 0.6 %

increase in DMI with a 1 % increase in digestion rate while DMI increased by 17 % as the rate of cellulose digestion increased from 0.061 to 0.102 /h (Gill *et al.*,1969). The importance of digestion as an influential factor on DMI and production is discussed by Van Soest (1982), Nandra *et al.* (1993) and Oba and Allen (1999). The latter found that the voluntary intake of organic matter (**OMI**) was more closely related to *in sacco* degradability at 24 h (r^2 = 0.88) than to the *in vivo* digestibility (r^2 =0.70).

1.3.3.2 Interaction of rumen flow dynamics and particle size

Peyraud and Mabrini (1992) found that the time spent chewing hay and the transition time of the bolus through the stomachs was 5.9 h and 41 h respectively. This suggests a long retention time in the stomach independent of the critical particle size. Luginbuhl *et al.* (1990) reported that 88 % of particles were sufficiently small to pass through the omasum after 12 h, while Bosch *et al.* (1992b) found that 70 % of rumen contents on a silage based diet, were less than the critical particle size. Particle breakdown may not be the only limiting factor in rumen fill. Faichney (1986) classifies rumen feed particles as those which have a low probability of leaving the rumen (1.18 mm), those readily removed from the rumen (<1.18 mm), and those which should behave as solutes (<.0.15 mm). The fibrous mat, previously assumed to selectively retain large particles for size reduction, is suggested to retain particles < critical particle size. This would result in a quantity of fine particles moving at a slower rate than the liquid dilution rate (**LDR**) (Faichney, 1986). This is supported by the work of Luginbuhl *et al.* (1994), who estimated the total mean retention time of fluid, leaves, stems and faeces of coastal bermudgrass hay placed in the rumen over a range of DMI to be 34, 81.7, 91.5 and 65.2 h respectively. The rumen mean retention time of particles is influenced by the LDR and solid dilution rates (**SDR**) of the rumen (Faichney, 1986), which vary from 0.055 to 0.155 /h and 10 to 35 h respectively, with possible extremes due to production systems (0.02 to 0.33 /h and 5 to 50 h respectively, Crawford *et al.*, 1980). This can be confounded by the physiology (Lechner-Doll *et al.*, 1991, Kabre *et al.*, 1995) of the animal and environmental conditions (Kennedy, 1985). The LDR can be influenced by diet composition even among forage sources. Mambrini and Peyraud (1992) suggest that ensiling may decrease the rumen LDR and increase the mean retention time of rumen particles. Holden *et al.* (1994) found an increase in LDR with pasture feed when compared to hay and silage diets though it was not significant.

1.3.3.3 Particle density

Rumen mean retention time is also inversely related to particle density, as particles of low density (0.8 g/ml) are retained longer (52-91 h total mean retention time) than particles with a high density (1.5 g/ml) (19-44 h total mean retention time, Evans *et al.*, 1973). Kaske and von Englehardt (1990) found that 1-mm plastic particles with a density of 1.44 g/ml left the reticulorumen of sheep 24 times faster

than those with densities of 0.92 g/ml and 1.03 g/ml. The increasing density of a particle is important, as it will pull the particle from the mat to the lower dorsal area, where it can be pulsed to the reticulorumen for passage. The density of a feed particle will increase as digestion proceeds and size decreases, due to the release of gas from internal spaces and/or liquid absorption (Lechner-Doll *et al.*, 1991). Wattiaux *et al.* (1992) found that the specific gravity of feed particles might decrease with the earlier stages of digestion, due to entrapment of fermentation gases and gases nucleating on the outer surfaces of feed particles. Other authors have found no link between sedimentation rate of small particles and particle passage (Dardillat and Baumont, 1992, Kennedy, 1995) and Wilson and Kennedy (1996) state that erroneous conclusions can be made from such results if they are considered in isolation.

1.3.3.4 Dry Matter Intake

Increasing intake may negatively affect the rumen mean retention time of particles, if not compensated by increased rumen fill (Kabre *et al.*, 1995) and the relationship can be linear (Luginbuhl *et al.*, 1994) or curvilinear (Kabre *et al.*, 1995). Decreasing intake from 99 to 50 % lengthened the rumen mean retention time of the fluids, leaves, stems and feaces particles by 12, 22, 27 and 18 h respectively, thus increasing exposure to the microbial environment of the reticulorumen, though whole tract passage rate did not differ suggesting a shift of fermentation to the lower intestine at higher intakes (Luginbuhl *et al.*, 1994). Increased intake of forage in the diet will also increase the LDR (Rode *et al.*, 1985), which may negatively affect the efficiency of MP synthesis in the rumen (mg N/g organic matter fermented in the rumen) but also increase the total microbial nitrogen flow to the duodenum. Murphy *et al.* (1994) suggests that reduced microbial nitrogen flows may be related to reduced growth in the rumen and/or futile recycling of MP. Processing of feeds (i.e. reducing initial feed particle size) can increase DMI. This can decrease rumination time and increase the intake of digestible energy. The former may lead to low ruminal pH (Heinrich *et al.*, 1999) while the latter may shift the site of fermentation from the rumen to the large intestine rendering less microbial nitrogen available to the host (Oskov *et al.*, 1970).

1.3.4 Rumen microbial populations

1.3.4.1 Protozoa

The protozoa are present at 10^5 - 10^6 cells/ml and are 5-250 μm in size (Hobson, 1988). Protozoa can represent 2 % of the weight of rumen contents, 40 % of microbial N and 60 % of the end-products formed (Church, 1988). The protozoal population can be influenced by the host animal, its geographic location, the nature of the feed and frequency of feeding (see Williams and Coleman, 1988, Jouany *et*

al., 1988) and is dominated by the ciliates, which consist of two main groups, the holotrichs and the entodiniomorphs (**Table** 1.11).

Table 1.11. Main protozoal genera found in the rumen (adapted from Hobson, 1988)

Entodiniomorphs	Holotrichs
Entodinium	*Dasytrichia*
Polyplastron	*Isotrichia*
Diplodinium	*Oligisotricha*
Epidinium	*Polymorpehella*

In forage diets holotrich protozoa may only represent 20 % of the protozoal population as they are mainly involved in the utilisation of NSC and soluble sugars. Substrate utilisation is genus-dependent (Williams and Coleman, 1988). They can have long generation times (2.86, 0.72, 1.45, 2.86 and 0.33 d for *Polyplastron, Epidinium, Dasytrichia, Isotricha* and *Entodinium* respectively) relative to the liquid turnover in the rumen and therefore must sequester themselves in amongst the fibrous mat of the rumen for survival (Czerkawaski, 1987). As a result of sequestration approximately 10 % of microbial crude protein entering the abomasum is protozoal in origin (Church, 1988). Optimum protozoal pH for activities of cellulase, amylase and protease were 5.0-7.5, 6 and 3.5 respectively (Williams and Coleman, 1988). *Dasytricha, Isotricha spp.* and some entodiniomorphid ciliates possess internal organelles called hydrogenosomes which consume oxygen by respiratory activity. NADH oxidase, peroxidase and catalase are also involved in oxygen scavenging (Yarlett *et al.*, 1983). This activity has both a protective and energy-producing role in protozoal survival and helps maintain the low redox potential of the rumen. Jouany *et al.* (1988) reviewing defaunation (by animal isolation, dietary or chemical manipulation of the rumen) of the rumen stated that there it generally increases the number of amylolytic bacteria, decreases the cellulolytic populations and increases fungal numbers. Cell wall digestion in the total tract can decrease by 5 – 15 % with defaunation, with the greatest impact when measured at the duodenum (28 %). Defaunation can also decrease the concentration of rumen ammonia to 50 mg/l which is less than required for optimum bacterial growth and the contribution of protozoal storage polysaccharide to the lower intestine could be significant enough to reduce blood sugar levels.

1.3.4.2 Fungi

The strictly anaerobic rumen fungi population is found in all the major sites of the digestive tract. They are most numerous in the rumen, omasum and large intestine ($1.17x10^5$, $1.82x10^5$, $4.9x10^4$ tallus forming units /g DM, Davis *et al.*, 1993). Eight species of anaerobic fungi have been isolated from the rumen consisting of polycentric and monocentric fungi, which differ in respective life cycles. Anaerobic fungi culturing, biochemistry and ecology have been reviewed by Theodorou *et al.* (1996).

Cultures exhibited cellulase, pectinase, esterase, saccharolytic, and proteolytic activities. Esterase activity, absent or expressed at low levels in bacteria, may be important in polysaccharide digestion when dealing with the physical hindrance of esterified phenols (Akin, 1993). Due to the restriction of their substrate niche, fungal species are thought to fulfil similar roles in the rumen (Forano *et al.,* 1996) and inoculation with polycentric fungi (Phillips and Gordon, 1995) was associated with a decrease in monocentric numbers. They have mixed acid fermentation profiles producing formate, acetate, lactate, ethanol, CO_2 and H_2 though little is known of the fermentation pathways utilised by the microbes (O'Fallon *et al.,* 1991). Their survival *in vivo* is pH dependent (Grenet *et al.,* 1989).

1.3.4.3 Bacteria

Most morphological forms are represented in this bacterial population, normally present at 10^{10} - 10^{11} cells/ml rumen fluid, with facultative anaerobes present at 10^7 -10^8 cells/ml (Hobson, 1988). The bacteria can range from 1-50 μm in size. Liquid associated bacteria and solid associated bacteria vary in composition (Merry and McAllan, 1983, Craig *et al.*, 1987b) and liquid associated bacteria may constitute only 20-30 % of ruminal organisms (Craig *et al.,* 1987a). Sheep receiving all roughage diets (Faichney, 1980) and cattle receiving roughage: concentrate diets (Wolstrup and Jensen, 1978) had a solid associated bacteria fraction of 90 and 77 % respectively. Microbial populations in the rumen can be described as cellulolytic, amylolytic, saccharolytic, pectinolytic and proteolytic depending on substrate preferences (**Table** 1.12).

Cross feeding of intermediate end products is the basis of many bacterial interactions and end products such as succinate, lactate, ethanol, formate, and H_2 often seen in pure cultures are replaced by acetic, propionic and butyric acid in mixed interactive cultures of the rumen (**Figure** 1.4).

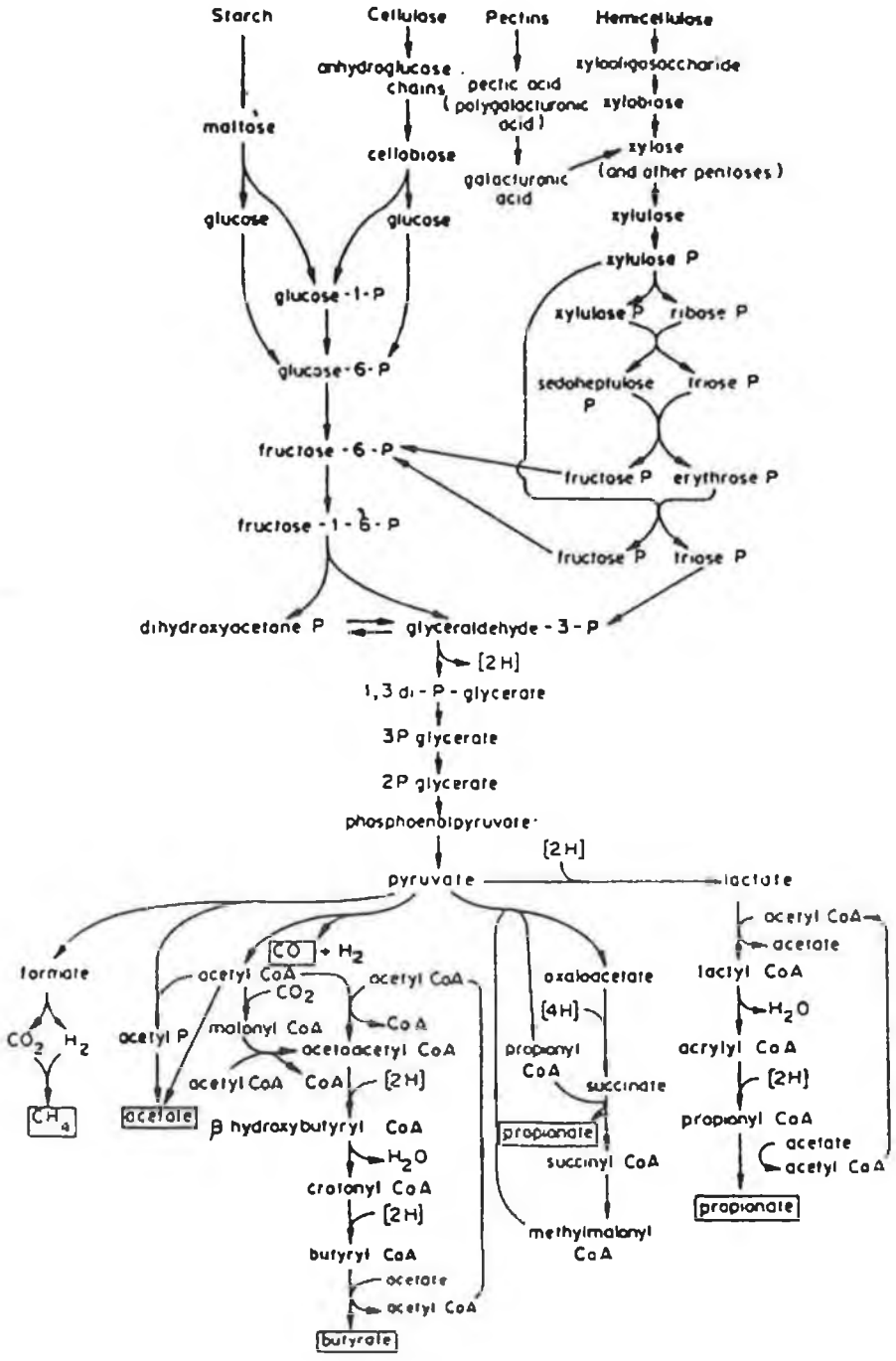


Figure 1.4 The biochemical breakdown of carbohydrate nutrient fractions to volatile fatty acids and methane

Table 1.12 Grouping of rumen bacterial species according to the type of substrates which are fermented (taken from Church, 1988)

Major celluloytic species
Bacteroides succinogenes
Ruminococcus flavefaciens
Ruminococcus albus
Butyrivibrio fibrisolvens

Major Hemicellulolytic species
Butyrivibrio fibrisolvens
Bacteroides ruminicola
Ruminococcus spp

Major Pectinolytic species
Butyrivibrio fibrisolvens
Bacteroides ruminicola
Lachnospira multiparus
Succinivibrio dextrinosolvens
Treponema bryantii
Streptococcus bovis

Major Amylolytic species
Bacteroides amylophilius
Streptococcus bovis
Succinimonas amylolytica
Bacteroides ruminicola

Major Ureolytic species
Succinivibrio dextrinosolvens
Selemonas spp
Bacteroides ruminicola
Ruminococcus bromii
Butyrivibrio spp
Treponema spp

Major Methane-producing species
Methanobrevibacterium ruminantium
Methanobacterium formicicum
Methanomicrobium mobile

Major Sugar -utilising species
Treponema bryantii
Lactobacillus vitulinus
Lactobacillus ruminus

Major Acid-utilising species
Megasphaera elsdenii
Selemonas ruminantium

Major Proteolytic species
Bacteroides amylophilius
Prevetello ruminicola
Butyrivibrio fibrisolvens
Streptococcus bovis

Major Ammonia-producing species
Prevetello ruminicola
Megasphaera elsdenii
Selemonas ruminantium

Major Lipid-utilising species
Anaerovibrio lipolytica
Butyrivibrio fibrisolvens
Treponema bryantii
Eubacterium spp
Fusocillus spp
Micrococcus spp

Cellulose digestion by ruminal microbes has been shown to be a first order kinetics with respect to cellulose concentration implying that the rate of degradation is limited by the amount of substrate available rather than the cellulolytic capabilities of the microbial population (Waldo *et al.,* 1972, Russell, 1987). This makes survival within such a competitive nutritive niche difficult. Substrate competition may limit the number of cellulolytic bacteria as non-cellulolytic microbes such as *Prevetello ruminicola* and *Selemonas ruminantium* can compete for and dominate the utilisation of cellodextrins and other products of cellulose hydrolysis (Russell, 1985, Lou *et al.*,1996). When cellulose is limited, population dominance depends on a microbes ability to adhere to (Chesson *et al.,* 1986, Shi *et al,* 1997) and hydrolyse (Gylswyk and Schwartz, 1984) the substrate, to utilise hydrolytic

products, to temporarily store polysaccharide and to promote energy efficient cell yield (Shi *et al.*, 1997). Rumen bacteria may also produce growth inhibitors restricting the growth of substrate competing organisms (Pwionka and Firkins, 1993).

The three main bacterial cellulolytic species (*Bacteroides succinogenes*, *Ruminocccus flavefaciens* and *Ruminococus albus*) are non-proteolytic, with a limited ability to incorporate amino acids (Weimer, 1992) and therefore have a requirement for ammonia and a dependency on cross feeding interactions from proteolytic and ureolytic microflora. Urease activity in the rumen is found in epithelium-associated bacteria, which are involved in the conversion of blood urea to ammonia and CO_2, during *in vivo* nitrogen recycling which can be approximately 60 g N/d in cattle (Church, 1988). Wallace (1996) reviewed the proteolytic systems of the rumen and concluded that

- the proteolytic activity and spp. involvement are animal and diet dependent
- the proteolytic ability is present in many microbial spp.
- protozoa and bacterial spp. mainly ingest particulate and soluble feed protein, respectively.
- hydrolysis of the resulting dipeptides is mainly dominated by *P. rumincola*
- amino acid deamination can be carried out by either/and a microbial population of low and high specific activity (**Table** 1.13).

The role of anaerobic fungi in protein utilisation is unclear (Hoover and Stokes, 1991).

Table 1.13 A summary of the properties of ammonia producing bacteria from the rumen

High numbers (> 10^9 cells/ml)	Low numbers (10^9 cells/ml)
Low activity (10-20 nmol NH_3 min^{-1}/mg protein)	High activity (300 nmol NH_3 min^{-1}/mg protein)
Butyrivibrio fibriosolvens *Megasphaera elsdenii* *Prevetello rumincola* *Selemonas ruminantium* *Streptococcus bovis*	*Clostridium aminophilium* *Clostridium stickland* *Peptostreptococcus anaerobius*

Fatty acids are not metabolised in the rumen but can be hydrogenated (Williams, 1982). Rumen bacteria modify fatty acids in a two stage process, firstly hydrolysis and then hydrogenation with complete saturation dependent on a mixed microbial population (Church, 1988). Entodiniomorphid protozoa, bacteriodes, and ruminococci are very active in hydrogenation but the hydrolysis of fatty acids is often the rate limiting step (Church, 1988, Abaza *et al.*, 1975). *Anaerovibrio lipolytica, Megasphaera esldenii* and some strains of *Selenomonas ruminantium* can ferment glycerol (Russell

and Wallace, 1998). Synthesis of microbial fatty acid is low as dietary lipids are readily incorporated into cells (Church, 1988). Holotrichs can take up long chain fatty acids and directly incorporate them into phospolipids, thus protecting them from hydrogenation (Demeyer *et al.*, 1978). Composition of the *de novo* microbial fatty acid component will reflect the anabolic substrates, which are often branched, non-branched, odd or even VFA (Church, 1988).

1.3.5 Ruminal cellulolytic activity

Celluloytic activity is dominated by the bacterial species but all microbial populations are capable of cellulose degradation (**Table** 1.14). The specific enzyme activity expressed by organisms can be growth related while expressions of enzymatic activity can be substrate dependent (Williams *et al.*,1989). The cellulolytic activities in batch cultures increased to a maximum with exponential and stationary phase cultures, while chemostat cultures showed lower activities in rapidly growing cells (see Williams *et al.*, 1989).

Table 1.14 Cellulolytic microorganisms of the rumen (taken from Weimer, 1992)

Bacteria Predominant species *Bacteroides succinogenes* *Ruminococcus flavefaciens* *Ruminococcus albus*	**Bacteria** Less predominant *Butyrivibrio fibrisolvens* *Clostridium longisporum* *Clostridium lochheadii* *Eubacterrium cellulosolvens* *Micromonospora ruminantium*
Protozoa *Diplodinium pentacanthum* *Enoploplastron caudatum* *Epidinium caudatum* *Entodinium caudatum* *Eudiplodinium bovis* *Eudiplodinium maggii* *Ophryoscolex caudatus* *Ophryoscolex tricoronatus* *Ostracodinium dilobum* *Polyplastron multivesiculatum*	**Fungi** *Anaeromyces muronatus* *Caecomyces communis* *Neocallimastix frontalis* *Neocallimastix joyonii* *Neocallimastix patriciarium* *Orpinomyces bovis* *Piromyces communis* *Ruminomyces elegans*

Protozoa encode enzymes for cellulose and hemicellulose digestion, with activities and specificity differing among species. The cellulase and endopectate lyase activity of entodiniomorphid protozoa can be 80 to 94 % higher than that of holotrichs (Jouany *et al.*, 1988). The presence of protozoa in the rumen appears to have a positive effect on bacterial celluloytic activity and cell wall digestion in the rumen general (Jouany *et al.*, 1988, Jouany and Martin, 1997). The ciliate population may be responsible for up to 30 – 40 % of fibre digestion in the rumen (Demeyer, 1981), though their close

relationship with symbiotic bacteria makes accurate quantification of celluloytic activity difficult. The anaerobic fungi rapidly colonize fibre material (Bauchop, 1981, Grenet *et al.,* 1989), their numbers proliferate on fibre diets and they have an ability to enhance the degradation of lignocellulosolic material (Davies, 1991, Sijtsma and Tan, 1993). However, the high specific activity of fungal extracellular cellulolytic enzymes (Wood *et al.*, 1986) is thought to be strictly regulated (Weimer, 1992) and produced in small amounts. Windham and Akin (1984) found that the bacterial cellulolytic activity was greater than that of rumen fungal activity. The sensitivity of fungal enzymatic activity to concentration of soluble carbohydrates and end products of fermentation may be supported by microbial interaction (Bernalier *et al.,* 1991, Zhu *et al.,* 1996, Theodorou *et al.,* 1996). Coculturing with bacterial species (*S. ruminantium*) can improve cellulose degradation while some ruminococci spp. can exhibit competitive or antagonistic activity towards rumen fungi (Irvine and Stewart, 1991). The greatest contribution of rumen fungi to cellulose digestion may be in the disruption of recalcitrant material for bacterial colonisation. Disrupted plant material is colonized much faster than intact material by all microbial species (Windham and Akin, 1984) and a reduction in particle size will improve the kinetics of fermentation.

1.3.6 Mode of cellulolytic activity

Digestion of the plant cell wall requires a consortium of enzymes (polysacharidases, glycoside hydolyases, xylansese, esterases, etc) to hydrolyze the varied chemical bonds of cellulolytic and hemicellulolytic polysaccarides and to subsequently metabolize the mono-, di-, and oligisaccharides released (Forano *et al.,* 1996). Some of these enzymes may be synthesised by a single microorganism or active through a close synergistic relationship between bacterial species, whose simplified enzyme systems complement each other. Adhesion of the main celluloytic species to the fibre matrix maybe a prerequisite to cellulose digestion and survival (Akin, 1993). Structural carbohydrate and NSC fermenting bacteria can utilize the products of cell wall breakdown. Therefore it is suggested that the processes of adhesion may help to localize the products of cellulolytic fermentation, thus preventing them from solubilising into the general rumen environment (Mitsumori and Minato, 1997).

The specific and non-specific mechanisms of bacterial adhesions are dominated by ligands or physiochemical (van der Waals, hydrogen bonding, ionic attraction) forces respectively. Initial attractions to the substrate surface may be mediated through weak van der Waal forces, gravity, diffusion, taxis, motility or convection. Irreversible adhesion is specific in nature and is associated with cellulosomes, and cellulose binding domains (Pell and Schofield, 1993). The cellulosomes, present on the cell surface of solid associated microbes, are responsible for mediating cell attachment to fibre matrix through a non-catalytic protein called cellulsomes-integrating protein. These complexes

aggregate the necessary enzymes responsible for the extensive hydrolysis of polysaccharides to mono or disaccharides through specific receptor domains, and mediate attachment to the substrate through the cellulose binding domain (Mitsumori and Minato, 1997). B. *succinogenes* species, a predominant cellulolytic microorganism, can contain cellulosome genetic coding for 14 endo-glucanases, together with β-glucanases, cellodextrinases and comprehensive xylanases (Forano *et al.,* 1996). Non-specific, specific exoploysaccharide interactions and some cellusome/ cellulosome integrating protein interactions can be disrupted by methodological procedures (Pell and Schofeld, 1993). Protozoal association with fibre matrix can be species specific (Pell and Schofield, 1993) and may be mediated through attachment via their oral cavity (Weimer, 1992). Fungal adhesion has been proven through electron microscopy (Weimer, 1992) and is necessary for fungal survival in the rumen. Within a 28 h life cycle rhizoids of vegetative thalli attack cell walls by penetrating through stomata and cracks in the epidermal layer. Adhesion occurs rapidly (70 % of bacterial adhesion occurred within 1 minute, Shi *et al.,* 1997) and exhibits structural preferences (Latham *et al.,* 1978). Adhesion may also be substrate dependent as highly lignified material such as xylem cells appear to 'inhibit' microbial attachment (Akin, 1989).

1.3.7 Factors influencing celluloytic activity

1.3.7.1 pH

pH is an important regulator of cellulolytic activity (Hiltner and Dehority, 1983) and species adaptation (Mackie and Gilchrist, 1979). The optimum pH for the growth of cellulolytic microbes is 6.5 (Van der Linden *et al.* 1984). *In vivo* pH may be below 6.2 for 17 – 19 h daily (Robinson *et al.*, 1986, Dillon *et al.*, 1989).

The ability of microbes to survive in environments of fluctuating pH was demonstrated when rumen liquor adjusted to 5.5, stored for 1 h and then readjusted to pH 6.9 with sodium carbonate, did not lose its original digestive capacity (Terry *et al.*, 1969). Slyter (1976) found that inoculum cultured at pH 5.5 had a pH dependent cellulolytic activity (13, 45 and 1 % NDF digestion pH 5.5, 6.5 and 5.0 respectively).

Cellulolysis is inhibited at pH below 6.0- 6.2 *in vivo* and *in vitro* (Terry *et al.,* 1969, Orksov and Fraser, 1975). Russell (1987) suggests that the negative effect of lower pH may be caused through the disruption of fundamental cellular metabolic processes (e.g. proton motive force) rather than enzyme inactivation. Mould *et al.* (1984) suggested that the pH effect is a biphasic one. pH reduction from 6.8

to 6.0 is moderate in effect and may be due to microbial associative effects with fibre (Shiver *et al.*, 1986) as isolated fibrolytic enzyme activity remains high in this pH range (Groleau and Forsberg, 1981). pH reduction below 6.0 is more severe and may be due to a combination of attenuated associative effects and transmembrane proton fluxes (Russell, 1987). This is supported by Shriver *et al.* (1986) who found that the NDF digestibility in chemostat culture was unaffected by pH variations from pH 7.0 to 6.2 (32 and 33.1 % respectively) but decreased dramatically at pH 5.8 (8.1 %).

Grant and Mertens (1992) and Grant and Weidner (1992) examined the effect of pH 5.8 and 6.8 and pH 6.8, 6.5, 6.0, 5.8, and 5.5 respectively, on NDF digestion. The results show a definite negative impact on digestion of forage types due to decreased pH. Considering the significant interaction of forage and pH, a general conclusion was made that below pH 6.2 the lag and rate of fermentation of all forages are significantly increased and decreased respectively. It was suggested that pH 5.5 was the lower practical limit for fibre digestion as the rate had become minimal. It has been demonstrated that the optimum pH for fibre digestion is pH 5.5-6.2 (Orskov and Fraser, 1975). The NSC fermenting group is more acid tolerant (Hungate, 1966). Studies with *P. rumincola* (Russell *et al.*, 1979) showed no effect on growth rate as pH decreased to pH 5.8 but subsequently decreased linearly with falling pH. Hungate (1966) states that the digestion rate of lactate utilising bacteria reaches zero at pH 4. The microbial yield of the NSC fermenting group is 50 % and 0 % at pH 5.5 and 4.5 respectively (Russell and Domobrowski, 1980). Therion *et al.* (1982) found the net growth rate of *M. elsdenii* on lactate to be optimum at pH 6 (0.58 /h) but growth continued over a pH range of 4 to 7.5.

A decrease in pH is associated with a concomitant production of VFA, which can inhibit microbial fermentation (see section 1.4.4.4). At low pH values, undissociated acids can pass through the microbial cell wall, dissociating in the more alkaline environment, causing an accumulation of anionic species and resulting in finally intracellular disruption (Russell and Diez-Gonzalez, 1998). High VFA concentrations can also increase the osmolarity level in the rumen which can negatively affect digestion (See section 1.4.4.4, Faverdin, 1999).

1.3.7.2 Microbial interaction

The metabolic activity of the methanotrophic bacteria (methanogensis) utilizes hydrogen and carbon dioxide, formate, acetate or methanol for the production of methane and shifts the bacterial end products of fermentation from the reduced ethanol, succinate and lactate to acetate and H_2 production (Fonth and Morvan, 1996), while that of the fungi is shifted away from ethanol and lactate towards acetate and formate (Bernalier *et al.*, 1991). It is seen as a wasteful diversion of 4-10 % of bovine metabolic energy (Orskov and Fraser, 1975, Vermoral, 1995). Approximately 70 % of total

methanogensis (Krumholz *et al.*, 1983) is attributed to the interactive relationship of the methanogenic population with the hydrogen producing ciliate protozoa (Miller and Hobbs, 1994) and defaunation can result in a 30 to 45 % decrease in methanogenesis. Coculture studies with methanogenic bacteria, have highlighted the importance of interspecies hydrogen transfer for celluloytic activity. The maintenance of a low partial pressure of hydrogen (10^{-4} atm, Fonty and Morvan, 1996), promotes greater yields of ATP during fermentation (Russell and Wallace, 1988) thus improving growth yields and cell mass. Cellulolytic digestion for the hydrogen producing cellulolytic bacteria is improved with this microbial interaction (Van Nevel and Demeyer, 1988). Reductive acetogenesis is an alternative and more beneficial fermentation pathway for the utilisation of hydrogen ($2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$), but though these species *(A. ruminis, E. limosum and C. pfennigii)* have been isolated in the rumen (Leedle and Greening, 1988, Fonth and Morvan, 1996) their contribution to H_2 utilisation is low (Nollet *et al.*, 1998) and may be due to their ability to utilise numerous other substrates (Fonth and Morvan, 1996) and/or lack of ability to compete with methanogenic bacteria for H_2 (Lopez *et al.*, 1999).

Protozoa have no urease enzymes (Onodera *et al.*, 1977) and therefore cannot use urea or ammonia in the synthesis of amino acids. Their main protein source is bacterial nitrogen with evidence that scavenging can be as high as 30-40 % of the bacterial population and can be species specific with an increase in Gram negative and *Selemonas*-like bacteria with defaunation (Coleman, 1986). Uptake is pH sensitive being optimum at pH 6.0, and 0, 75 and 30 % of optimium uptake at pH 5, 7 and 8.0 respectively (Coleman, 1986) and can be as high as 90 % of bacterial DM/day in the rumen of sheep (Coleman, 1975). Ciliates utilise only 50 % of ingested nitrogen, the rest expelled as short chain peptides and amino acids (Coleman, 1975). Proliferation of the protozoa in the rumen will therefore increase microbial nitrogen recycling , thus reducing microbial flow to the duodenum.

Entodiniomorphs can prey on zoospores and engulf the mycelium of fungi (Jouany and Martin, 1997). Protozoa can also help to stabilise environmental pH of the rumen by engulfing rapidly digestible substrates, maintaining it as a storage polysaccharide (amylopectin) and fermenting it slower than bacterial populations. This reduces the immediate bacterial lactate production, thus preventing a severe pH drop (Faichney *et al.*, 1997). Lactate fermentation in the rumen may also be 15 times greater for protozoal populations than bacterial (0.133 - 1.12 g/g protozoal protein/h), with metabolism associated only with entodiniomorphid species (Newbold *et al.*, 1987). Protozoal populations could be responsible for 30 % of VFA production from lactate (Newbold *et al.*, 1987, Newbold *et al.*, 1986), producing mainly acetic and butyric acids, while propionic acid can be inhibitory to protozoal growth (Jaakkola *et al.*, 1991, Jaakkola and Huhtanean, 1992).

1.3.8 Energetic efficiency of rumen microbial fermentation

The fermentation pathways of carbohydrate material by rumen microbes have been described in detail (Baldwin and Allison, 1983, Russell and Wallace, 1988). The survival and growth of microorganisms is influenced by many factors (**Table** 1.15) but ultimately dependent on an efficient storage and transfer of energy during microbial anabolic and catabolic reactions, through intermediate high energy phosphate bonds (Russell and Wallace, 1988).

Yields of adenosine triphosphate (**ATP**) and reducing equivalents will vary with the fermentation pathway used (**Table** 1.16). The anaerobic degradation of carbohydrate components in ruminal fermentation yield very low levels of ATP when compared with aerobic oxidation (2 vs. 36 ATP moles / mole respectively, Prescott *et al.*, 1993). This 'inefficiency' is essential for energy retention in the end products of fermentation which is later released during oxidation in the Krebs cycled or stored for subsequent host utilisation (Prescot *et al.*, 1993).

Table 1.15. Factors influencing the physiological growth characteristics of rumen bacteria (taken from Russell and Wallace, 1988)

Growth characteristic	Influencing factors
Maximum growth rate (k_{max})	Type of substrate Availability of growth substances Presence of toxic substances
Substrate affinity (k_S)	Type of substrate Attachment Maximum growth rate
Theoretical maximum growth yield (Y_G)	Type of substrate Availability of growth factors Presence of toxic compounds Uncoupling of growth
Maintenance (m)	Type of substrate Availability of growth factors Presence of toxic compounds Uncoupling of growth
Death rate (d)	Availability of substrate(s) Presence of toxic compounds Protozoal predatation
Passage rate (p)	Attachment Animal factors

Table 1.16. Enzymatic reactions producing ATP (~P) or reducing equivalents (2H) and the balance of these reactions in various fermentations[a] (taken from Russell and Wallace, 1988)

Enzyme	Final product					
	Lactate	Acetate	Propionate[b]	Butyrate	Ethanol	Valerate
Glucokinase	-1	-1	-1	-1	-1	-1
Phosphofructokinase	-1	-1	-1	-1	-1	-1
Glycerate kinase	2	2	2	2	2	2
Pyruvate kinase	2	2	2	2	2	2
Acetate kinase	-	2	-	-	-	-
Fumarate reductase [c]	-	-	2	-	-	-
Butyrate kinase	-	-	-	1	-	-
Total (~P)	***2***	***4***	***4***	***3***	***2***	***2***
Glyceraldehyde-3-phosphate dehydrogenase	2	2	2	2	2	2
Lactae dehydrogenase	-2	-	-	-	-	-
Pyruvate oxidoreductase	-	2	-	2	2	1
Alcohol dehydrogenase	-	-	-	-	-4	-
Malate dehydrogenase	-	-	-2	-	-	-1
Fumarate reductase	-	-	-2	-	-	-1
β-Hydroxybutyrate dehydrogenase	-	-	-	-1	-	-
Butyryl-CoA dehydrogenase	-	-	-	-1	-	-
β-Hydroxyvalerate dehydrogenase	-	-				-1
Valeryl-CoA dehydrogenase	-	-				-1
Total (2H)	***0***	***4***	***-2***	***2***	***0***	***-1***

[a] From 1 molecule of hexose via Embden-Meyerhof-Parnas pathway

[b] The randomiszing pathway employing succinate as an intermediate. If the non-randomizing pathway via acrylyl-CoA reductase were used, the (2H) balance would be the same, but the ~P is thought to be only 2.

[c] Assumes an ATP-linked fumarate reductase reaction : *M. elsdenii,* the predominant organism making valerate, does not have this enzyme since it uses the acrylate pathway to make propionyl-CoA.

Rumen bacteria have a superior growth yield when compared to that of other anaerobic systems (Hespell and Byrant, 1979). *S. ruminantium* and *Streptococcus bovis* in pure culture can yield 29-100 g cells/mol hexose (Russell and Baldwin, 1979), where the aerobic and anaerobic yield of *Escherichia coli* is 26 and 83 g cells /mol hexose, respectively. The cellulolytic bacteria can have growth rates of 11 - 32.4 g cells/ mol CHO consumed, higher than the average anaerobic yield of 5.4 - 10.8 g of cells/mol CHO consumed (Weimer, 1992). Fungi, however, appear to have a lower cell yield (Borneman *et al.,* 1989). Inferior Y_{ATP} (15 -23 and 25 -34 g microbial cells/mol ATP for *in vitro* and theoretical situations, respectively) may suggest possible inaccuracies in biochemical summations (Hespell and Byrant, 1979, Russell and Wallace, 1988) and limitations of the *in vitro* technique used. Theoretical estimations of fermentation balances (Groot *et al.,* 1998) are limited in their application to *in vitro* situations as it is assumed that all carbon and reducing equivalents are incorporated into microbial cells, acetate, propionate, butyrate, CO_2 and methane only.

Reductions in maintenance energy (energy and nutrients used for non-growth purposes), energy spilling (uncoupling of anabolic and catabolic reactions) or extracellular recycling processes would also increase Y_{ATP} (Moss, 1994). It is also important in an environment where energy sources may only be occasionally abundant, that microbes can store sufficient energy not only to remain viable, but also to respond rapidly and effectively to the subsequent influx of available energy. In situations of energy excess, intracellular storage polysaccharide (α-dextran), which requires 0.3 times the energy of protein production, can increase by 75 % (Stewart *et al.,* 1981). The ratio of acetate: propionate: butyrate (VFA molar proportion ratio) from the fermentation of this stored CHO is approximately 68:20:12 (Thompson and Hobson, 1971) compared to 65:25:10 and 50:40:10 (Church, 1988) from storage CHO in forage and concentrate respectively, though the ratios can be pH dependent (Kaufmann *et al.,* 1980). The efficiency of microbial growth may also be affected by the composition of microbial cells, which can vary dramatically (Russell and Hespell, 1981).

At a rumen LDR of 0.06 /h, 32 % of the energy generated is dissipated as maintenance energy (Harrison *et al.,* 1980) but it is affected by species type, growth rate and cell composition (Russell and Wallace, 1988). A decrease in rumen dilution rate (increasing residence time) will increase the maintenance energy requirements of the microbial population and extent of (digestible) substrate degradation (Owens *et al.*, 1984). Increasing the dilution rate will increase the Y_{ATP} (19 % increase when D increased from 0.068 to 0.115 /h, Kennedy and Milligan, 1978) but decrease rumen digestibility. It is important to note that microbial efficiency (Y cells/ 100 g organic matter truly or apparently digested) is independent of microbial yield in the rumen (Church, 1988) and ruminal situations which will improve yield (i.e. low mean retention time and high LDR) may decrease microbial efficiency.

Amino acids (**AA**) can also be degraded to VFA, CO_2, ammonia and branched chain fatty acids (**BCFA**) (Baldwin and Allison, 1983) but they are a poor source of energy for microbial growth, yielding only 0.9 moles ATP/mole AA compared with 3.98 /mole for soluble sugars (Glyswyk and Schwartz, 1984). Few microbial species can utilise protein alone as an energy source (Baldwin and Allison, 1983), but *M. esldenii* and *P. rumincola*, two of the more active deaminating bacteria, can not supply their respective cellular requirements with sufficient maintenance energy from proteins alone due to limitations in the rate of AA uptake (Russell and Wallace, 1988). The fermentation of protein is regulated by availability of carbohydrate and is extensive if the solubility and availability of AA exceeds that of the carbohydrate fraction.

Substrate preferences do exist for microbes and growth rates on these substrates vary but Y_{ATP} is more influenced by growth rate and cell composition than substrate (Russell and Wallace, 1988). Changing growth rates and substrate availability can also affect the end product formation (**Table** 1.17) and energy yield (propionate production via the acrylate pathway can dominate at high rates of fermentation, **Table** 1.16). In cellulose-limited conditions a metabolic shift to acetate production, with increasing the LDR is characteristic of the cellulolytic bacterial species (Pavlostathis *et al.*, 1988, Weimer *et al.*, 1991).

Table 1.17. Fermentation products and ATP yields for the growth of *Streptococcus bovis* in glucose-limited chemostat[a] (taken from Russell and Wallace, 1988)

	Fermentation products (mM)			ATP yields	
Dilution rate (h^{-1})	Lactate	Acetate	Ethanol	M ATP per m glucose fermented	M ATP h^{-1}
0.807	9.22	0.47	0.30	2.09	16.85
0.423	8.39	2.35	0.86	2.40	11.89
0.315	6.89	2.95	1.34	2.53	8.91
0.245	5.42	3.80	1.76	2.69	7.24
0.228	4.33	3.70	1.84	2.75	6.19
0.195	1.95	4.08	2.22	2.99	4.81
0.168	1.56	4.31	2.41	3.04	4.23
0.127	1.64	5.06	2.74	3.07	3.68
0.088	1.92	4.95	4.00	2.91	2.78

When energy and growth requirements are in excess cellular growth will be dependent on the availability of a suitable nitrogen source and in optimum conditions 25-30 g microbial nitrogen/100 g organic matter fermented, are expected (Hoover and Stokes, 1991). Using continuous culture, Hoover and Stokes (1991) showed an increase in carbohydrate digestion and microbial production efficiency in response to increasing levels of degradable protein supplementation with responses up to and greater than 20 % degradable intake protein. The theoretical shape of the energy/protein response curve is thought to be sigmoidal (Wallace, 1997). Hoover and Stokes (1991) suggest that the optimum ratio for dietary NSC:ruminal degradable protein (**RDP**) for maximum MP yield is 2. Herrerra-Saldana *et al.* (1990) suggest a ratio of 1.5-2.5 for rumen degradable starch:RDP. To predict the nitrogen composition and quantity required for the potential energy availability in a diet can be difficult (Russell and Wallace, 1988), as can the successful matching of protein/energy supply patterns (synchronisation). Energetic uncoupling (asynchrony) may result in low MP production per unit of carbohydrate digested (Chamberlain and Choung, 1995). MP production may be restricted due to an exhaustion of peptides postfeeding (peptide concentration was 200 mg/l and <25 mg/l, 0 and 2 h post feeding respectively,

(Broderick *et al.*, 1991) or a lack of fermentable carbohydrate, in diets high in NSC and soluble nitrogen respectively. Synchronisation of microbial fermentation of silage diets is discussed later.

1.3.9 Physiological importance of end products of fermentation

The hosts nutrient supply is obtained through the absorption of VFA, MP, VFA and minerals from the digestive tract, through the metabolically active gut wall into the portal drained viscera (Church, 1988). The liver is the communicating link between the digestive tract and the peripheral tissues, and is supplied with blood from the portal vein and the hepatic artery and drained via the hepatic vein into the vena cava (Danfear, 1994). The metabolic energy necessary for ruminant maintenance, growth and production is derived from *in vivo* hormonal control and substrate regulation of these nutrients (McDowell and Annison, 1991). The liver serves to regulate the metabolic activities of the host, modifying the nutritional blood profiles as required by a range of physiological processes (Danfear, 1994). The nutrient partitioning of the absorbed profile of fermentation end products can influence animal production (Thomas and Martin, 1988, Dijkstra, 1994) and the nutrient requirements by peripheral tissues is dependent on the physiological state of the animal i.e. growth, fattening, embryo development and lactation (Orskov and Ryle, 1990). When required the host can mobilise internal reserve tissue to fulfil nutrient deficits (McDowell and Annison, 1991, Orskov and Ryle, 1990).

The gut mucosa can alter the proportion and conformation of the absorbed VFA profile. A review of net portal absorption data concluded that 30, 50 and 90 % of acetate, propionate and butyrate respectively were metabolised by stomach tissue (Britton and Krehbriel, 1993). The butyrate content is converted to the ketone β-hydroxybutyrate (**β-HB**). All VFA can be used by the host to generate ATP in intermediary metabolism (Orskov, 1994). The main VFA are utilised with equal energetic efficiencies (Orskov, 1994). Acetic acid though often produced in greatest quantities contributes a small proportion of the total energy derived from nutrients due to its low calorific value (**Table** 1.18). Approximately 80 % of acetate reaching the liver escapes oxidation (Church, 1988) and reaches the peripheral tissues, where it is absorbed from the blood and becomes the main precursor for lipogenesis.

Table 1.18. Volatile Fatty Acids in mixtures expressed as molar % and as percent of total energy (taken from Orskov and Ryle, 1990)

				Molar %		
Acetic acid	35	45	55	65	75	85
Propionic acid	55	45	35	25	15	5
Butyric acid	10	10	10	10	10	10
				% of energy		
Acetic acid	22	30	39	48	59	72
Propionic acid	62	53	43	33	21	7
Butyric acid	16	17	18	19	20	21

Propionate can reduce the capacity of the liver to detoxify ammonia via the urea cycle, with the result that ammonia spills over into the peripheral blood leading to effects on insulin secretion, with implications for the partitioning of nutrients (Chamberlain and Choung, 1995). The ruminant liver, unlike non-ruminants, is a net producer of glucose (85 % of requirements) as there is little net glucose absorption across the portal drained viscera from dietary sources in dairy cattle and steers (Hungtington, 1990). Glucose is required as a direct energy source for tissue metabolism and synthesis, and is also a necessary source of NADPH, which is required for fat synthesis. NADPH is formed by glucose oxidation via the hexose monophosphate pathway. Propionic acid is a glucogenic VFA and can be used as a precursor to glucose synthesis (gluconeogensis) *in vivo* along with glycogen and some amino acids (excluding lysine, leucine and taurine) (Church, 1988). Glucogenic energy obtained from VFA is therefore dependent on the molar ratio (**Table** 1.19). Of the lactate absorbed in the liver, formed through the anaerobic fermentation of glucose in tissue, or in rumen fermentation, 10 to 20 % can be converted to glucose, with a significant proportion of the remainder metabolised to CO_2 (Gill *et al.*, 1986, Church, 1988). Glycerol, the glucogenic precursor of the fatty acid complex, represents only 4-5 % of the total molecular energy (Orskov and Ryle, 1990) and therefore will make a small contribution to gluconeogenesis on the molar basis of fatty acid oxidised, considering also that approximately one third of this is used for glucose synthesis (Church, 1988).

Table 1.19. Effect of Molar proportions of Volatile Fatty Acids on glucogenic energy, expressed as percent of total energy in the mixture (taken from Orskov and Ryle (1990)

Molar %			
Acetic acid	**Propionic acid**	**Butyric acid**	**Glucogenic energy (%)**
45	45	10	53
55	35	10	48
65	25	10	36
75	15	10	21

In ruminants, VFA are normally absorbed in the free form from the digestive tract. Post absorption they are converted to triglycerides for incorporation into chylomicrons, which are transported to the blood via the lymph system draining the digestive tract (Danfear, 1994). They are required for adipose tissue development and arachidonic acid (an essential fatty acid) is a precursor for prostaglandin synthesis (Church, 1988). Fatty acids of less than 14 carbons, enter the blood directly and are transferred to the liver where they are rapidly oxidised (Church, 1988). *De novo* fatty acid synthesis is predominantly from β-HB and acetate, with a small percentage glucose based. Butyrate is the preferred substrate for mammary fatty acid synthesis, while acetate and lactate are utilised in adipose tissue development (Church, 1988). The metabolic activity and requirements of 80 % of the ketones formed (an energy reserve for

peripheral tissue use) are obtained from butyrate with the balance obtained from acetate and acetoacetate.

There are three sources of protein for ruminant absorption in the small intestine, that of microbial origin (50-80 % of the total, Harrison *et al.*, 1994), undigested feed protein which has escaped fermentation and endogenous protein, while ammonia for recycling can be absorbed at most stages of the digestive tract. All sources supply AA and peptides to the ruminant, which are necessary for *in vivo* protein synthesis. Essential AA must be supplied through absorption, as they cannot be synthesised *in vivo*. The AA profile of MP, rich in methionine and lysine, is closely related to that of the requirements of growing ruminants (**Table** 1.20) (cited by Chamberlain, 1987).

Table 1.20. Amino acid components of rumen bacteria, milk, meat and wool compared with the amino acid requirements of a ruminant (expressed as percent of lysine) (Cole and van Lunen, 1994)

Amino acid	*Rumen bacteria*	*Milk*	*Lamb*	*Beef*	*Wool*	*Requirement*
Lysine	100.0	100.0	100.0	100.0	100.0	100.0
Methionine + cystine	50.9	47.6	39.8	44.0	386.7	48.7
Tryptophan	19.2	17.1	13.3	14.3	56.7	13.7
Threonine	66.3	61.0	46.9	50.5	216.7	55.3
Leucine	93.5	124.4	73.5	87.9	313.3	96.9
Valine	65.7	90.2	49.0	58.2	170.0	66.3
Isoleucine	61.9	68.3	46.9	56.0	113.3	62.9
Phenylalanine + tyrosine	114.3	120.7	74.5	91.2	336.7	91.3
Histidine	26.8	36.6	32.7	40.7	26.7	36.4
Arginine	55.4	48.8	62.2	73.6	336.7	33.8

In a review of AA and peptide absorption, Webb and Bergman (1991) stated that regions of the digestive tract were selectively predisposed to AA absorption, proportional uptake of essential AA was greater than non-essential AA, competition for absorption exists between AA and peptide absorption into the portal and mesentric blood systems occurs with absorption rates greater than AA. Approximately 50 % of the energy stored in some AA can be glucogenic in nature (Church, 1988) and may provide up to 20 % of the ruminants glucose requirements. Alanine and glutamine are mostly hepatic glucogenic in nature, while glutamate and aspartate are predominate in renal gluconeogensis (Church, 1988).

1.4. *IN VITRO* SYSTEMS IN STUDIES OF RUMEN FERMENTATION

1.4.1 Role of *in vitro* techniques

Ruminant digestion can be examined *in vivo* by measuring total tract digestion, measuring the appearance of endproducts, the disappearance of substrate or measuring the retention time of digesta in compartments of the digestive tract. *In vivo* measurements can be subject to technical error as quantification of flow rates may be inaccurate due to the liquid/solid phase markers used (Tamminga *et al.,* 1989a, Tamminga *et al.,* 1989b), nitrogen or carbohydrate disappearance may be under- or over-estimated due to endogenous contamination of samples (Orskov *et al.,* 1986, Illg and Stern, 1994) and animal variation within species can be quite significant (Mehrez and Orskov, 1977, Michalet-Doreau, 1992). It may also be erroneously assumed that all soluble material is immediately digested (Mahadevan *et al.,*1980, Broderick *et al.,* 1992) and that a time point VFA ratio is representative of the true VFA ratio produced and absorbed into the portal blood (Britton and Krehbiel, 1993). *In vivo* techniques can be expensive, time consuming and labour intensive with concerns that the welfare of fistulated experimental animals may be compromised by the need for invasive surgery. The *in situ* technique can also be used to measure rumen (and total tract) digestion of feed components over time. Using a rumen fistulated animal, sealed nylon bags containing a defined amount of feed are suspended in the rumen, and removed at defined times relative to the start of the period. Calculations are done on a weight basis and the technique may encounter some of the difficulties highlighted for the *in vivo* procedures (Huntington and Givens, 1995, Jouany *et al.,* 1998, Vanzant *et al.,* 1998).

In vitro systems can be cheap and versatile and are well-controlled methodologies (Stern *et al.*, 1997). The ranking of substrate kinetic coefficients is similar between *in sacco*, *in vitro* gas and *in vitro* DM disappearance techniques (Huhtanen and Jaakola, 1994, de Smet *et al.,* 1995, Cone, 1996) but numerically techniques will differ. Varel and Kreikemeier (1995) used the *in sacco* and *in vitro* technique (Goering and Van Soest, 1970) to estimate the NDF digestion kinetics of a fibre diet and found that the lag was significantly lower and the rate and extent were significantly higher than *in vitro*. These results were supported by Bach *et al.* (1999). *In vitro* systems can be used to accurately quantify VFA production (Sutton, 1968) as batch and continuous systems generally operate in absolute terms i.e. absolute profiles of rumen fermentation which assumes no absorption of end products and no protozoal recycling of microbial nitrogen, due to defaunation of the continuous systems.

The specific research objectives and practical limitations of the experimental study will govern the methodological method used. *In vivo* techniques are necessary to highlight any substrate/animal interactions but only the controlled *in vitro* systems can be used to examine the influence of intrinsic

properties on the subsequent digestion of the substrate (Mertens, 1993). Tamminga and Williams (1998) concluded that ' *in vitro* methods have proven their value (in the area of) mechanistic modelling...the role of *in vitro* methods in the prediction of nutrient supply lies probably more in helping to elucidate the mechanisms underlying digestive processes than in giving straight forward predictions of nutrient supply'.

Systems may be batch or continuous or semi-permeable in nature (Czerkawski, 1986, Stern *et al.*, 1997), allowing for short (120 h) and long term studies (weeks) respectively. The *in vitro* system should not be limited or altered by any experimental parameter other than that under examination. Care must be taken to avoid biased estimates of the intrinsic fermentation kinetics, which may arise due to inoculum variation, inoculum preparation, fermentation environments (anaerobiosis, nutrients, temperature, end product inhibition, and agitation) pH control or substrate preparation (Mertens, 1993).

1.4.2 Batch systems

In batch systems buffer, substrate, nutrients and inoculum are added together (time zero). Only short term experiments are possible (120 h maximum). Time series sampling is used to obtain kinetic data necessary to characterise digestion curves or end product formation. Systems are described as 'destructive' when experimental units must be removed at predefined times to describe fermentation profiles (Goering and Van Soest, 1970) or alternatively 'non-destructive' (gas production). The former technique requires a large number of experimental units over any time period. Gascoyne and Theodorou (1988) detail a consecutive batch system, where through sequential inoculations a stable microbial population (including small protozoa) can be maintained for up to 12 days.

The kinetics of substrate fermentation are described using mathematical models of varying degrees of complexity (Fisher *et al.*, 1989, Singh *et al.*, 1992, Mertens, 1993), which will describe the lag (h, time before initial fermentation begins), rate (/h, rate of substrate disappearance) and extent (%, maximum disappearance of substrate) of the substrate/component fermentation. Waldo *et al.* (1972) defined different ruminal retention times for forages of varying NDF content, which will influence estimations of effective rumen degradability and may (Lopez *et al.*, 1991) or may not be taken into account (Tamminga *et al.*, 1991, Hoffman *et al.*, 1993) when calculating such.

Indirect measurements of the kinetics of component digestion can be estimated using a technique of curve subtraction, which is applied in situations where the fraction is not easily isolated for independent assessment. Schofield and Pell (1995) have used the technique to estimate the kinetic

parameters of the soluble NDF component by subtracting the digestion curve of NDF from that of the whole forage, while Stefanon *et al.* (1996) used this technique to estimate the fermentation parameters of the insoluble component of bromegrass, by subtracting the gas profile of the water soluble component, from that of the whole forage. Two assumptions were stated 1) that the component extraction procedure does not cause significant structural changes in the extract and 2) that the microbial population responsible for the degradation of the extract is not significantly different from that responsible for fibre digestion in the unfractionated forage. The latter curve subtraction was based on ten time point measurements over 48 h. When expected and observed values were compared, deviations of 0-10 % were partially attributed to an interaction between the soluble and insoluble component during digestion. Organic matter (**OM**) digestion can be predicted from the stoichiometry of the VFA (Church, 1988).

1.4.2.1 Modified Tilley and Terry system

The Tilley and Terry technique (1969) describes a two stage *in vitro* estimation of total tract forage digestibility. The dried milled substrate is incubated anaerobically at 39 ^{0}C with rumen fluid and buffer for a defined period, normally 24 h, after which the residue is then subjected to an acid/pepsin hydrolysis step simulating rumen and abomasum digestion/hydrolysis respectively. The modified Tilley and Terry batch system of Goering and Van Soest (1970) has optimised the preliminary stage to describe only the ruminal digestion kinetics of a forage. Substrates are normally dried and milled, and then incubated anaerobically for 72 – 96 h with rumen fluid, thus estimating the periodic and maximum ruminal disappearance of the substrate, which is normally expressed as apparent DM digestion or NDF digestion. Tubes can be sampled periodically for VFA under a stream of CO_2. Fermentation times should be carefully considered and when little is known of the data set, 11 equally spaced time points should be used (Mertens, 1993). Variation between time points is suggested to be greater at the inflexion points of a digestion curve and therefore observations should be taken more frequently in this period. Observations at the start and end of fermentation are also critical in defining lag and extent. A zero time is necessary to distinguish between solubilization and lag in DM and NDF digestibility studies and time points should be recorded to the nearest 0.1 h (Mertens, 1993).

1.4.2.2 Gas production systems

Gas production is a measurement of substrate digestion based on product formation rather than solubility or disappearance. Fermentation gases are released into the headspace above the liquid culture and are predominately CO_2 and CH_4, with 50 % of gas volume arising from fermentation (Blummel and Orskov, 1993). Direct gas production is the endproduct of the microbial fermentation, while indirect gas production results from the release of CO_2 from the carbonate buffer due to the production

of fermentation acids. The fermentation of protein sources produces less gas than OM (Cone and van Gelder, 1999), while the concomitant production of ammonia can interfere with the indirect production of gas volume due to the formation of the ammonium ion (NH^{4+}) in the presence of H^+. The contribution of fat to gas production is negligible (Getachew *et al.,* 1998).

Propionate (P) and acetate (A) are produced by alternative metabolic pathways (Church, 1988)

$C_6H_{12}O_6 \rightarrow 2CH_3CO_2H + 2CO_2 + 8H^+$

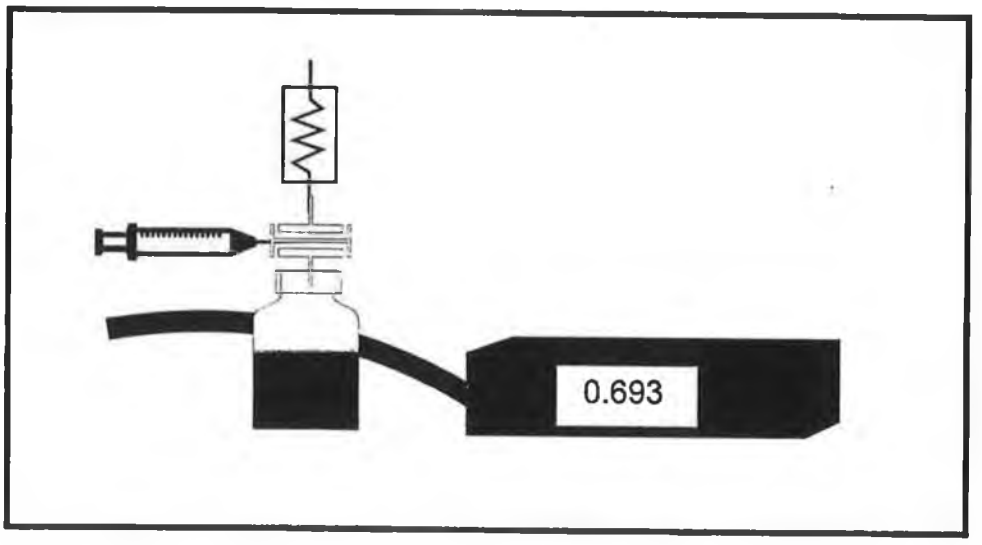
$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CO_2H + 2H_2O$

As propionate produces no direct gas, a comparison of gas curves must only be done when the A:P ratio are similar (Beuvink *et al.*, 1992). Volatile fatty acid ratios may differ between substrates of different chemical composition (Menke and Steingass, 1988, Groot *et al.*, 1998), between forages of different maturities (Stefanon *et al.,* 1996, Cone and van Gelder, 1999) or between different microbial species (Russell and Hespell, 1981).

Data for the description of digestion curves can be collected directly by using calibrated syringes (Menke *et al.*, 1979, Krishnamoorthy *et al.*, 1991), indirectly using liquid displacement systems (Jouany and Thivend, 1986, Beuvink *et al.*, 1992) or calculated from changes in pressure at fixed volumes (Theodorou *et al.,* 1994, Pell and Schofield, 1993). Many systems are now automated to remove the need for intensive periodic sampling (Davies *et al.,* 1995, Pell and Schofield, 1993, Cone, 1989). All gas systems, methodology and applications were reviewed by Getachew *et al.* (1998).

The technique of Theodorou *et al.* (1994) was used in Section 4.2 and Section 6.1 where the methodologies are described in detail. Briefly serum bottles (160ml volume) were used as culture vessels. All components (buffer, reducing solution, substrate and inoculum, see) are added at t=0 under anaerobic conditions. The serum bottle is then crimp sealed and inverted to mix. Culture vessels are incubated anaerobically at 39 ^{0}C without agitation. After a short period of time (approximately 5 minutes) the headspace pressure of each vessel is the returned to 0 psi by withdrawing a sufficient volume of gas by syringe (Figure 1.5). The time is then recorded as the real t=0 for fermentation. Periodically the increase in headspace pressure is recorded, prior to the withdrawal of fermentation gases such that the headspace pressure is returned to 0 psi each time. The serum bottle is then inverted to mix the contents and re-incubated. At the end of a defined fermentation period, culture bottles are sampled for VFA and the residue collected.

Figure 1.5 The gas pressure transducer assembly and digital display unit in use for measurement of headspace pressure (Theodorou *et al.*, 1994).



It is suggested that replicates of three be used in any gas run as ' a very different fermentation pattern for one of the replicates' can develop (Beuvink *et al.*, 1992). Gas volumes released for a given substrate quantity can be affected by temperature (Beuvink *et al.*, 1992), pH and atmospheric pressure, as the ideal gas law states that PV = nRT, where P = pressure (atm), V = volume (l), n= moles of gas, R = the gas constant (.08206L atm/K per mol) and T = temperature (degrees Kelvin) (Kohn and Dunlap, 1998). Lowman *et al.* (1998) found greater gas production with increased sampling times, but there was no effect on DM disappearance or VFA ratio. This may be explained by increased CO_2 saturation of the buffer medium at higher atmospheric pressures. Studies showed that excessive accumulation of fermentation gas (>7 psi) had a negative impact on the linear relationship between gas volume and pressure (Theodorou *et al.*, 1994). These results were not supported by Schofield and Pell (1995) who examined a pressure range of 0 to 0.6 atmospheres. Headspace volume is constant in any study. Rymer *et al.* (1998) concluded that inoculum concentration and the mixing of substrate and medium before incubation altered the resultant fermentation profile. Blending of rumen contents had no effect on gas release. Indirect gas volume is affected by buffer:substrate ratio (Getachew *et al.*, 1998). Agitation was linearly related to indirect gas release up to 45 strokes/min (Rymer *et al.*, 1998). Indirect gas composition can be calculated from the stoichiometric relationships described by Wolin (1960) once

gas volume and VFA ratios are known. This was validated by Blummel and Orskov (1993).

Gas profiles can be assessed using the exponential model of Orskov and McDonald (1979) (Siaw *et al.*,1993, Valentin *et al.*, 1999), the Gompertz model of France *et al.*, (1993), the dual pool model of Schofield and Pell (1995a) or multiphasic models of Cone *et al.* (1996). Where 3 phases are described they are understood to represent the fermentation of the soluble component (phase 1), the fermentation of the insoluble component (phase 2) and the turnover of the microorganisms (phase 3) which is accompanied by an increase in NH_3 concentration and a decrease in microbial biomass (Cone and van Gelder, 1999). Such models require large data sets for accurate predictions.

Gas production profiles may not be linearly related to substrate disappearance (Groot *et al.*, 1998). As there is an indirect relationship between gas production and MP production (Krishnamoorthy *et al.*, 1991, Blummel *et al.*, 1997) quantification of fermentative gas volumes could favour short chain fatty acid (**SCFA**) production rather than MP production. To address this Blummel and Bullerdick (1997) suggest the use of a partitioning factor which is calculated as the ratio of substrate truly digested to gas volume produced and thus reflects variation in microbial yield and enhances the prediction of voluntary feed intake *in vivo* (Getachew *et al.*, 1998). Cone and van Gelder (1999) discuss the need to consider the interference of ammonia production on indirect gas release and the correction of such profiles before respective gas profiles of substrates differing in maturity, and hence protein fraction, are compared.

1.4.3 Continuous or semi-continuous culture systems

A variety of long term rumen simulation cultures have been developed and it is stated that the number of times any system is used is inversely related to the complexity of design (Czerkawski, 1986). Systems have varied in vessel size (0.5-10 l), buffer (McDougall or Weller and Pilgrim), control parameters (pH, LDR, SDR), agitation , feeding rate, particle size and substrate allowances. The three most cited rumen simulation models are the semi-continuous or Rusitec system of Czerkawski and Breckenridge (1977), the single flow semi-continuous system of Slyter *et al.* (1964) which controls only the LDR and the dual flow system of Hoover *et al.* (1976) which controls the LDR and SDR. The function of these systems has remained relatively constant over time, though operational conditions i.e. flow rates, buffers, pH control and feeding regimes may have changed.

1.4.3.1 *In vivo* vs. *in vitro*

For validation most systems have been compared with experimental data from published literature (Abe and Kumeno, 1973, Hoover *et al.* 1976, Czerkawski and Breckenridge., 1977, Estell *et al.*, 1982,

Merry *et al.*, 1987). With concurrent *in vivo* validations the number of experimental parameters which were statistically compared varied (Slyter and Putnam ,1967, Hannah *et al.*, 1986, Mansfield *et al.*, 1994, Prevot *et al.*, 1994). Only variables of similar units i.e. proportions or concentrations can be compared in a study of this nature due to difference in absolute amounts of input and outflow between the two cultures.

In vivo estimations of DM intake/rumen volume for a 50:50 forage : concentrate diet is 14.5 g/100ml (Moloney *et al.*, 1993). In reported studies where LDR and SRT are comparable amongst experiments daily feed input values can vary from 7 (Mansfield *et al.*, 1994) to 3.5 (Hoover *et al.*, 1976) to 2.9 g DM/ 100 ml inoculum (Merry *et al.*, 1987). Other studies have allowed for more interactive processes by allowing feed inputs to be dictated by LDR (Fuchigami *et al.* 1989) and SDR (Crawford *et al.*, 1980, Shriver *et al.*, 1986), which are supported by the *in vivo* studies of Galyean *et al.* (1976), Kennedy and Milligan (1978) and Henning and Pienaar (1983). However interpretation of the results becomes much more complex.

Differences in the microbial ecology between *in vivo* and *in vitro* studies can affect total non-carbohydrate digestion, (Mendoza *et al.*, 1993), bacterial efficiency (Viera, 1986), microbial composition and utilisation of N source (Viera, 1986, Williams, 1986, Schadt *et al.*, 1999). The bacterial profile of the *in vitro* environment is influenced by, and reflects the pH (Hoover *et al.*, 1984), the digestion profiles (Mansfield *et al.*, 1994) and the anaerobic status of the system (Slyter and Putnam, 1967).

Prevot *et al.* (1994) evaluated microbial population shifts in the liquid phase during pre-steady state days of the Rusitec, as operated by Czerwaski and Brenkenridge (1977). Ciliates and bacterial numbers decreased significantly early in the adaptation phase, but there was little effect on total VFA (**TVFA**) or VFA proportions which may highlight the importance of the solid associated populations in the fermentation of high fibre diets. Carro *et al.* (1995) found that the protozoal population decreased in the first days of incubation before reaching a steady state value. The holotrichs were sensitive to pH (<6.5) but in stable environments of low dilution rates (0.03 /h) they were present in proportions similar to *in vivo* results.

Slyter and Putnam (1967) found no significant differences between *in vivo* and *in vitro* bacterial cultures, though only 50 % of organisms could be identified. There were common changes between physiological groups and composition of groups. There is difficulty in maintaining protozoal numbers and populations in continuous systems due to lack of sequestration (Slyter and Putnam, 1967, Abe and

Kumeno 1973, Hannah *et al.,* 1986, Mansfield *et al,* 1994). Holotrichs are normally lost completely from the *in vitro* continuous systems and greater numbers of total viable bacteria cells are found *in vitro* than *in vivo*. A reduction in the protozoal population may support increased microbial efficiencies and viable bacterial counts *in vitro* (Mansfield *et al.,* 1994). This work also found no effect of operational conditions on the fungal population. Attempts to maintain the protozoal population have been made by increasing retention times (Hoover *et al.,* 1976a), reduced substrate input (Merry *et al.*, 1983, Teather and Sauer, 1988), minimising agitation and allowing stratification (Abe and Kuihara, 1984, Teather and Sauer 1988, Fuchigami *et al.*, 1989, Broudiscou *et al.*, 1997), continuous feeding (Teather and Sauer, 1988) and nutritional additions (Broudiscou *et al.,* 1997). Levels of 10^4 to 10^5 cells/ml have been achieved in most cases but holotrich species are nearly always lost (Abe and Kumeno, 1973). Intermittent or slow agitation (100 rpm) appears to be the most advantageous treatment in dual flow continuous cultures.

1.4.3.2 Rusitec semi-continuous system

Czerkawski and Breckenridge (1977) describe a rumen simulation technique (**RuSiTec**) that can maintain a microbial population for long periods of time (49 days). This work is based on that of Aafjes and Nijhof (1967). The system simulates the compartmental nature of the rumen and microbial populations (Czerkwaski, 1984), and consists of four 1litre vessels (**Figure** 1.6). They are closed systems, with liquid leaving the fermentation vessel through a single overflow facility. The LDR is therefore directly related to the rate of saliva input. The system is charged with inoculum, buffer and water. The feeding method of the system is such that each vessel contains a perforated polyethylene container, repeatedly moved up and down through the chamber, which holds two nylon bags, one filled with rumen solid digesta and the other with the experimental substrate. After day 1 the bag of solid digesta is removed and replaced with a substrate bag. Gas volume and composition can be measured daily. Thereafter the system is sampled every day and any bag removed after 48 h incubation.

Differential LDR exists between compartments decreasing as one goes from the liquid to the solid compartment (Czerkwaski and Beckenridge, 1979), but is not thought to influence the *in vitro* DM disappearance (Carro *et al.,* 1995). The feeding regime of the Rusitec introduces diurnal variation into the system and steady state is reached when the daily output of products of fermentation does not change significantly from day to day over a specified number of days. The introduction of rumen digesta into the system on Day 1 optimises the development of a uniform rumen microbial population by introducing solid associated microbes, while the provision of a solid mat matrix enhances the survival of the protozoal population. The system was not operated at a dilution rate greater than 1 volume/day (0.042 /h) as the concentration of the end products would be too low for measurement.

Experimental dilution rates ranged from 0.01 to 0.04 /h (Czerkawski and Brenkenridge, 1977, Czerkawski and Brenkenridge, 1979a, Czerkawski and Brenkenridge, 1979b). The Ruistec lacks pH control and physical factors such as accessibility to food and sequestration may affect the efficiency of feed conversion at different feeding levels. Carro *et al.* (1995) found that the pore size of the nylon bags (40, 100 and 200 μm) affected DMD, NDFD and microbial populations in the system.

1.4.3.3 Single and dual flow continuous systems

The system of Slyter *et al.* (1964) is fed every twelve hours and is a closed system. The LDR is a function of saliva input (**Figure** 1.7) as there is a single overflow. In the absence of pH control, buffering of the system is dependent of the buffer inflow, which may lead to excessive LDR. The dual flow system of Hoover *et al.* (1976) thus gave the continuous system more operator control. The original system of Hoover *et al.* (1976a) consisted of three 4 l fermentation vessels, with a constant working volume of 2277 ml (**Figure** 1.8). The system is charged with filtered inoculum and maintained under a continuous flow of N_2 and therefore is not closed. It allows for solid feed input at variable rates without disruption of fermenter function. Liquid dilution rate and SDR are independent and controlled by buffer input and a filtered withdrawal of vessel liquid. The vessel contents are homogenous, thus allowing for pH control, though Czerkawski and Breckenridge (1977) suggest that the homogenous nature of the Hoover system is not suitable to simulation of the heterogenous rumen due to the lack of compartmentation.

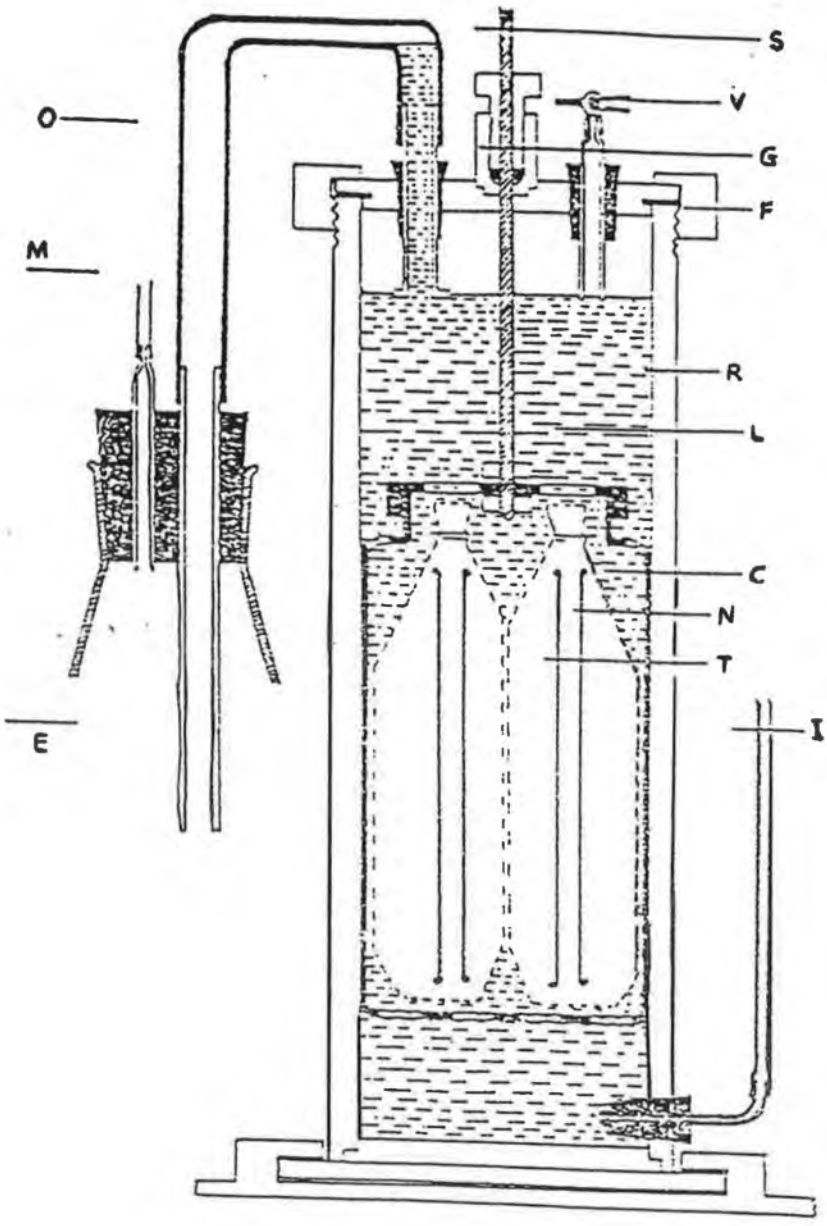


Figure 1.6 The Rusitec *in vitro* fermentation system

(S) Driving Shaft; (V) Sampling valve; (G) Gas tight gland; (F) Flange; (R) Main reaction vessel; (L) Rumen fluid; (C) Perforate food container; (N) Nylon gauze bag; (T) Rigid tube; (I) Inlet artificial saliva; (O) Outlet through overflow; (M) Line to gas-collection; (E) Vessel for collection of effluent.

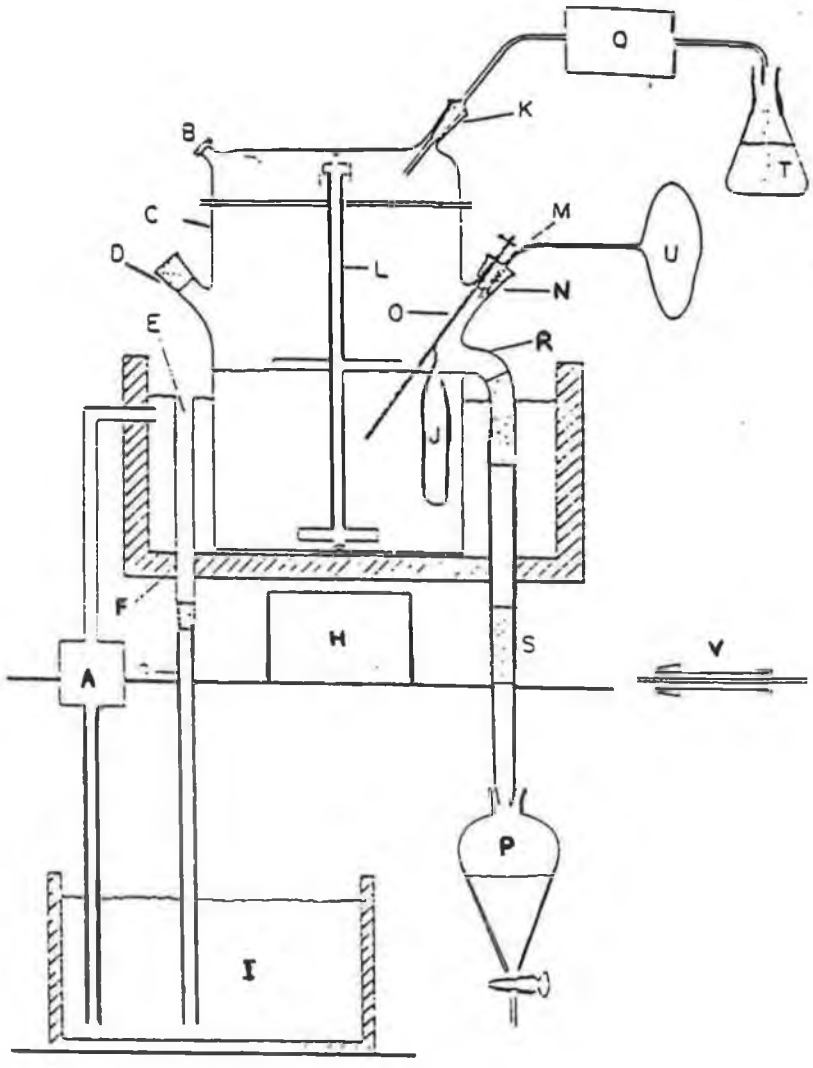


Figure 1.7 The single flow *in vitro* continuous fermentation system

Centrifugal water pump (A); (B) Gas sampling port; (C) Fermenter; (D) Feeding port; (E) Water-drainage pipe; (F) Plexiglas reservoir; (G) Drainage tube; (H) Magnetic stirrer; (I) Water bath; (J) Dialysis sac with cation-exchange resin; (K) Saliva inflow ground glass joint; (L) Fermenter stirring device; (M) Gas-outlet tube; (N) Fermenter port; (O) Sampling glass tube and resin holder; (P) Liquid-effluent collection funnel; (Q) Peristaltic pump; (R) Effluent outlet; (S) Effluent rubber tubing; (T) Saliva-water reservoir; (U) Gas-collection bladder; (V) Feed-input apparatus.

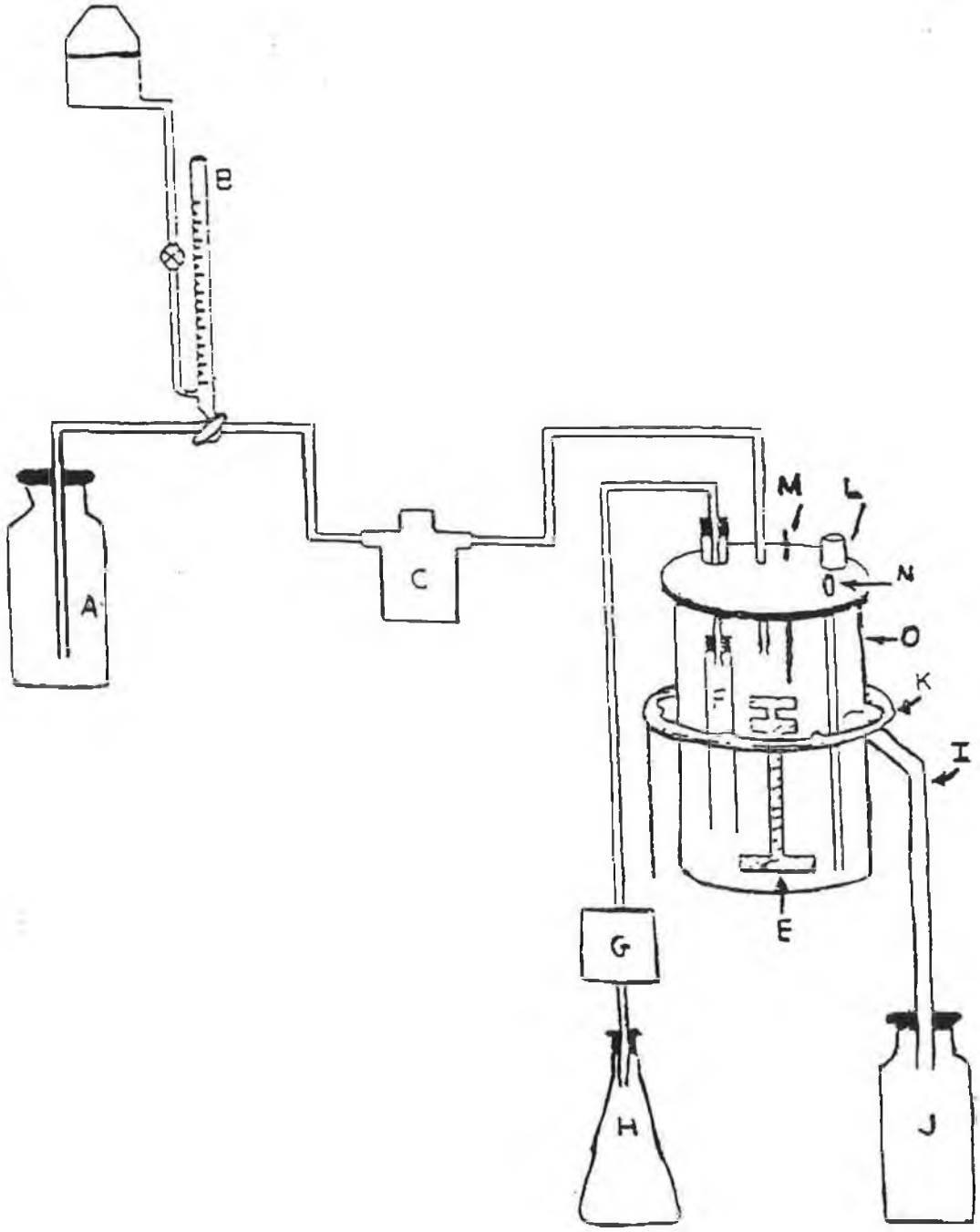


Figure 1.8 The dual flow *in vitro* fermentation system

(A) Buffer reservoir; (B) Buiret; (C) Peristaltic pump; (D) Fermenter; (E) Magnetic Stirrer; (F) Filter; (G) Peristaltic pump; (H) Filtered effluent reservoir; (K) Heated water spray ring; (L) Feed port; (M) Thermister; (N) Nitrogen gas input port.

1.4.3.3.1 Operational conditions of dual flow systems

Operational conditions of the systems and analysis of dietary components are well documented. Time delay in inoculum sampling will not affect experimental results if the donor animal is maintained on a constant diet (Hoover *et al.,* 1976a). Buffers used are based on *in vivo* estimation of mineral contents of saliva (McDougall, 1947) or rumen contents (Aafjes and Nijhof, 1967). Broudiscou *et al.* (1999) examined the effect of mineral salts on the fermentation and gas production rate of mixed microorganism *in vitro* and defined the optimum mineral content of buffer to support *in vitro* maintenance of protozoa and methanogenic species. Steady state is established after 4 days of fermentation (Hoover *et al.*, 1976a, Merry *et al.*, 1987, Miettinen and Setala, 1989a). Sampling period is 3-5 days but Merry *et al.* (1987) found that increasing the number of experimental replicates (n=2 to 4) was more beneficial than increasing the number of sample days (3 or 5 days) for reducing experimental variation of measured parameters. Sampling of effluents and vessel contents is done once or twice daily (Hoover *et al.* 1976). Abe and Kumeno (1973) sampled contents every 4 h after feeding.

Particle size of 1 mm (Fuchigami *et al.*, 1989), pelleted (Hoover *et al.*, 1976, Merry *et al.*, 1987, Mansfield *et al.*, 1994) or in chopped form (Czerkawski and Breckenridge, 1979) is often used. The influences of particle size on fibre and cellulose digestion *in vitro* (Dehority 1961, Dehority and Johnson, 1961) and *in vivo* (Meyer *et al.*, 1965) may not be evident in the RSSC due to a controlled SDR (Hoover *et al.*, 1976a).

Many studies have been completed to explain the effects of LDR (Hoover *et al.* 1984, Meng *et al.,* 1999), SRT (Hoover *et al.,* 1982, Schadt *et al.,* 1999) and LDR and SRT (Shriver *et al.* 1986) in continuous cultures. Digestion coefficients of DM, NDF and ADF increase with increasing SRT and LDR. Total VFA increases with increasing LDR, as does the proportion of propionate, with a general decrease in acetate and butyrate. Increasing SRT increases the proportion of acetate, decreases the propionate and may or may not influence butyrate. Contrary to the results of Schadt *et al.* (1999) and Meng *et al.* (1999) and despite increases in protein and DM digestion at higher SRT, Meng *et al.* (1989) and Hoover *et al.* (1984) found that microbial nitrogen output or efficiency were not affected by decreasing SRT. This was explained by a shift from soluble to structural carbohydrate fermentation at longer SRT. Schadt *et al.* (1999) and Meng *et al.* (1999) found that the effect of SRT and LDR respectively, on the efficiency of MP synthesis was diet dependent, with optimum dilution rates depending on basal diet (Meng *et al.,* 1999). Carro *et al.* (1995) found that increasing LDR (2.3 and 3.5 %/ h) in the Rusitec system decreased TVFA and propionate proportion and increased butyrate with no effect on acetate proportion. The Rusitec system cannot incorporate pH control due to lack of

homogeneity and therefore the pH was significantly higher in systems with higher LDR. Y_{ATP} of microbial cultures increases with increasing LDR (Isaacons *et al.*, 1975, Maeng *et al.*, 1989). This is explained by the dilution of endproducts and nitrogen sources which may be inhibitory, reduction in autolysis, removal of predatory protozoa, and/or a reduction in maintenance requirements of microbes (Crawford *et al.*, 1980). Increasing pH was found to be positively related to acetate production (r^2 =0.57, Hoover *et al.* 1982, Shriver *et al.*, 1986) and total VFA production (Hoover *et al.*, 1984) and negatively related to propionate and butyrate production (Shriver *et al.*, 1986). In dual systems, the decrease in microbial efficiency and fibre digestibility with decreasing pH (Hoover *et al.*, 1984, Shriver *et al.*, 1986) can be partially attributed to the increase in system osmolarity due to buffer addition.

Alternative methods for MP estimation originated due to the difficulty in distinguishing the microbial nitrogen fraction from feed and endogenous nitrogen fractions in *in vivo* samples. Inherent components in the microbial cellular matrix, such as diaminopimelic (**DAPA**) and aminoethylphosphate acid (**AEP**, Czerwaski, 1974) for bacteria and protozoa respectively and purine content (adenine and guanine which are subunits of the ribonucleic acid molecule, Zinn and Owens, 1986), have been used with variable success (Whitelaw *et al.*, 1984, Illg and Stern, 1994, Robinson *et al.*, 1996). The accuracy of any method depends on obtaining a representative relationship between the measured parameter and total microbial nitrogen. Non-representative sampling of the population may give anomalous results (Whitelaw *et al.*, 1984, Illg and Stern, 1994, Robinson *et al.*, 1996) as basic assumptions such as a consistent N:measured parameter ratio may be invalidated (Obispo and Dehority, 1999). *In vitro* ratios are based on the purine, DAPA, AEP content of cells. Garrett *et al.* (1987) compared D-Alanine and DAPA as bacterial markers and found that the coefficient of variation for measured parameter:N ratio was less with D-alanine but concluded that the cellular ratio was not consistent within *in vitro* incubations and between *in vitro* and *in vivo* microbial samples from similar dietary sources. External markers such as N^{15} and P^{32} have also been used (Merry *et al.*, 1984, Calsamiglia *et al.*, 1999).

The ideal microbial marker should 1) not be present in feed, 2) be biological stable, 3) have a relatively simple assay, 4) occur in similar percentages for all microbes, 5) be a constant percentage of the microbial cell at all growth stages and additionally for *in vivo* 6) not be absorbed in the digestive tract. Aminoethylphosphate acid has been found in bacterial cells (Whitelaw *et al.*, 1984) and DAPA may vary with substrate (Schadt *et al.*, 1999). Purine concentration can vary with sample preparation (Ha and Kennelly, 1984), sampling time after feeding (Cecava *et al.*, 1990), microbial species (Firkins *et al.*, 1987) and digestion of feed purines has been found to vary *in vivo* (Djouvinov *et al.*, 1998) but not *in vitro* (Calsamiglia *et al.*, 1996). Broderick and Merchen (1992) recommended the use of purines or N^{15}, while Calsamiglia *et al.* (1996) suggested that feed purines could contaminate the isolated

bacterial pellet making N^{15} a more reliable technique.

Results obtained from simulation models may sometimes be more reflective of the experimental methodology used rather than the experimental treatment. High estimates of total non-structural carbohydrate digestion and low NDF for high starch diets in *in vivo* and *in vitro* comparisons (Shriver *et al.*, 1986, Windschitl and Stern, 1988, Mansfield *et al.*, 1994) may be due to substrate processing since the *in vitro* diets were milled and pelleted. This is suggested to cause gelatinization of the starch due to moisture high pressure and heat, which makes the starch more available for fermentation (Theurer, 1986). Comparative results for the digestion of CP and microbial nitrogen show better agreement when corrected for endogenous protein (factor 3.6 g of endogenous N/kg duodenal DM flow, Brandt *et al.*, 1980). Efficiency of MP production tends to be higher *in vitro* than *in vivo*. Efficiencies greater than 100 % (Mansfield *et al.*, 1994) for *in vitro* microbial synthesis when expressed as a percentage of dietary N digested were explained by suggesting that the urea source of N in the artificial saliva, may be more important source of N when compared with *in vivo* results. The study of Crawford *et al.* (1980b) examined the effect of LDR and SRT on fermentation *in vitro*. The system lacked an automatic pH control and pH drifted upwards with increasing SRT (due to feeding regime). This will confound interpretation of results and most continuous systems now incorporate pH control (Mansfield *et al.*, 1994, Merry *et al.*, 1987). During rapid fermentation, a continual challenge to pH stability will require repeated additions of buffer to maintain uniformity of the system, thus increasing osmolarity of the system. High osmolarity may affect some of the experimental variables.

1.4.4 Experimental methodology

1.4.4.1 Inoculum variation

The 48 h endpoint Tilley and Terry technique shows little effect of inoculum variation on estimations of total tract digestion (Akter *et al.*, 1992, Borba and Ribeiro, 1996, Jung and Varel, 1988) but it is not time dependent. *In situ* results, which are time dependent normally up to 48-72 h, have varied between species, within species and with time of sampling (Hungtington and Givens, 1995) reflecting variation in inoculum activity. Time dependent *in vitro* studies are also sensitive to inoculum variation. *In vitro* Beuvink *et al.* (1992) found significant variation between periods ($p< 0.01$) when pooled inoculum samples were taken from two sheep on four different occasions. Moore *et al.* (1962) suggested that four donor animals should be used to composite the inoculum and reduce potential variation *in vitro* due to inoculum. Mauricio *et al.* (1998) found a greater variation between two donor cows than source of inoculum (fresh inoculum or faeces) when the *in vitro* fermentation of grass and straw was examined.

Faeces is regarded as a more suitable source of microbial inoculum because it does not require invasive surgical procedures of the donor animals and the fermentation in the hindgut is qualitatively similar to that of the rumen (Church, 1988). Mauricio *et al.* (1998) using cows, and Aiple *et al.* (1992) using sheep, as donor animals found that the extent of fermentation was not affected by inoculum source (rumen or faeces) over 72 h but that fresh faeces gave a greater lag in fermentation than fresh inoculum. The altered lag resulted in an inferior fermentation profile but rates of digestion were not characterised. Aiple *et al.* (1992) found that sheep faeces were superior to cattle faeces in total gas produced in 48 h, as anaerobic bacteria were thought to survive better in the pelleted faeces of sheep. None of the above studies attempted to quantify the MP content/ml of inoculum, which can influence fermentation (Aiple *et al.*, 1992) and use this as the basis for inoculum preparation comparisons.

When diets varying in carbohydrate composition were fed to donor animals there was an effect on enzyme activity (Noziere and Michalet-Doreau, 1997), microbial populations (Byrant and Robinson, 1968, Leedle *et al.* 1982, Leedle and Greening, 1988) and microbial cellular composition (Cecava *et al.*, 1990, Hussein *et al.*, 1995) of the inoculum. Many of these effects can be diurnal. Huntington and Givens (1998) found that the basal diet (forage or forage: concentrate) significantly affected the initial phase of fermentation as the time dependent rate differed significantly (0.015 and 0.191 $h^{-0.5}$ respectively, $p< 0.007$), as did final pH, butyrate molar proportion and time to reach the half asymptote. Cumulative gas volume, combined rate and lag were not affected. The effect of donor diet on the earlier stages (0-6 h) of *in vitro* fermentation was also reported by Doreau *et al.* (1993), who found that diets of low forage content gave significantly greater gas production than hay based diets for sucrose and starch substrates. Mertens *et al.* (1998) examined the effect of four donor diets differing in NDF content (24 and 32 %) on gas production from similar substrates in a Latin square experiment. It was concluded that cow donor and its diet significantly affected gas production kinetics. Weimer *et al.* (1999) also concluded in a similar experimental design that animal rather than basal diet had a greater effect on the cellulolytic population present. De Smet *et al.* (1995), when assessing the acidotic effect of feeds, found that the *in vitro* results correlated better with *in vivo* when rumen fluid was sampled after feeding, though many *in vitro* studies detail sampling before feeding in methodology to avoid rapid changes in microbial populations and the difficulty of sampling from freshly ingested digesta, after feeding.

1.4.4.2 Inoculum preparation

Different microbial populations are associated with different fractions of rumen contents. *In vitro* inocula containing whole rumen contents yield faster digestion and VFA patterns similar to *in vivo* than inocula containing only strained ruminal fluid (Barry *et al.*, 1977, Brock *et al.*, 1982) but its use is

impractical as the high percentage of residual DM will lead to problems with dispensing and high background readings with blanks which may be problematic for substrates like fibre.

Strained rumen fluid is normally used with efforts made to isolate solid associated bacteria. Maximum recovery of solid associated bacteria from whole rumen contents is approximately 53-64 % (Merry and McAllan, 1983, Craig *et al.* 1987a, Olubobkun *et al.* 1988, Whitehouse *et al.*, 1994) but many of these treatments are too severe to maintain viable populations for *in vitro* studies. Pell and Schofield (1993) reviewed methodological effects on bacterial adhesion. Senshu *et al.* (1980) showed increasing viable counts of bacteria and protozoa isolated from whole rumen contents when fresh digesta residues were washed up to six times, with improvements in the fermentation of starch and cellulose. Dehority and Grubb (1980) found an increase in viable colony counts with storage at 0 ^{0}C for 8 h but found no significant differences in the percentages of the total population capable of utilising glucose, cellobiose, starch or xylose. Tween 80 significantly increased the total colony count. Craig *et al.* (1984) found that chilling of whole rumen contents before washing, blending of whole rumen contents or addition of the surfactant Tween 80 had no beneficial effects on NDF digestion or rates of protein degradation. Blending has been shown to destroy protozoa (Byrant and Burkey, 1953) and increase gas production in blanks (Pell and Schofield, 1993).

Strained rumen fluid contains many sources of vitamins, protein and growth factors which may be interfer with certain experimental studies. The separation of cells from the liquor by high speed centrifugation, was first reported by McNaught (1951). Since then several workers have washed cell suspensions successfully. Dehority *et al.* (1960) purified the cellulolytic inoculum even further by separating the fractions sedimented at 1,500 and 20,000 g which represents the feed and protozoal fraction and microbial fraction respectively.

The concern with purification techniques is the potential inactivation of the enzymatic activity by both exposure to oxygen (Leedle and Hespell, 1983) and adverse temperatures. Aeration of the inoculum decreased cellulolytic activity when used in a 30 h fermentation (Johnson, 1957) and excessive aeration was thought to inactivate microbial activity after two buffer washes. Cheng *et al.* (1955) used washed suspensions without a loss in activity but used larger aliquots of inoculum than Johnson (1957). Storage of faeces under aerobic conditions and at room temperature negatively impacted gas production (Aiple *et al.*, 1992).

1.4.4.3 Inoculum preservation

Inoculum variation can influence *in vitro* measurements and thus compromise the measurement of any

intrinsic parameter. In industries that can require a consistent source of bacterial inoculum methods of preservation have been developed to preserve a reference batch of the inoculum, from which a sub sample is removed, cultured and used for the industrial processes. It would be hoped that if the preservation and preparation methods are clearly defined and regulated, the inoculum cultured from any sub sample should not vary between fermentations. Much of the exploratory work to assess problems or potentials with these preservation methods has been carried out with pure cultures (Lievense *et al.,* 1994, Castro *et al.,* 1995, Castro *et al.,* 1997, To and Etzel, 1997). The main methods of preservation are freeze drying (lyophilisation), spray drying and freezing.

Freeze-drying is a batch operation in which a solvent is removed from a frozen solution by sublimation. Though drying via sublimation is slow the low temperature process minimises the chemical alterations and cellular disruptions caused by drying procedures (spray drying) operated at higher temperatures (Johnson and Etzel, 1995). However, freeze- and spray-dyers are expensive to build and operate and the viability of stored inocula can be dependent on the humidity and storage atmosphere, with evidence that oxidation of the fatty acid content of membrane lipids can occur if these conditions are not optimum (Castro *et al.,* 1995)).

Microbial survival during freezing, freeze-drying and spray drying is dependent on the strain of the microorganism, growth conditions, age of the culture, nature of the suspended medium and processing conditions (el-Kest and Marth, 1992) and there is evidence that method of preservation can reduce cell viability but not enzymatic activity (cell viability of *Lactobacillus helveticus* was greater for freezing and freeze drying than spray drying (54, 48 and 7.4 % survival respectively) but enzyme activity (galactosidase and aminopeptidase) was significantly higher for spray drying operated at lower temperatures, than freezing or freeze drying and there was no lag in acid production for any of these three treatments (Johnson and Etzel, 1995).

Frozen cultures can suffer cellular injury as the temperature declines due to disruption of the cellular membrane, its composition and its function and dehydration of the cell due to the formation of ice crystals, with the cell susceptible to osmotic shock on thawing and disruption of protein structures and functions, which are often temperature sensitive (el-Kest and Marth, 1992). Duration of storage at – 20 ^{0}C was found to affect the extent of cell viability of *Lactobacillus* species (Moss and Speck, 1963, el-Kest *et al.,* 1991, el-Kest and Marth, 1992), though Johnson and Etzel (1995) stated they found no effect of storage duration up to 4 weeks when studying *Brevibacterium linens*. The dehydration of the cell during freezing, will result in the subsequent concentration of intracellular solutes. Long term exposure of the bacterial cell to what may be toxic levels of any solute may cause viability to decrease

(MacLeod and Calcott, 1976). The deterioration of the *Lactobcillus* cultures appears to be reduced at much lower storage temperatures (-198 ^{0}C, el-Kest *et al.*, 1991).

Damage due to freezing can be reduced or alleviated by controlled reductions in temperature and/or the use of cryoprotectants. Cryoprotectants are often low molecular weight compounds (glycerol, dimethylsulfoxide, sugars) that can protect the cells from damage incurred during freezing and/or storage, though larger compounds and a complex of undefined substances such as blood, extracts of malt or bacteria can also be used (el-Kest and Marth, 1992). The freeze-thaw damage is generally minimised in biological cultures, by reducing the formation of intra- or extra-cellular ice crystals thus minimising cell disruption, while penetration of the cell membrane by the cryoprotectant can reduce the fraction of electrolytes both inside and outside of the cell. To and Etzel (1997), however found that the addition of glycerol did not improve the survival of *B. linens* after freezing and thawing. Metabolic disruptions of the cell can be overcome by supplying the microbes with their nutritional requirements during fermentation or in a preincubation step (see el-Kest and Marth, 1992).

It is suggested that the controlled freezing of cellular material (maintaining the material at a 'holding temperature' for a certain period of time to optimise dehydration can reduce subsequent intracellular thaw damage by expanding ice crystals (el-Kest and Marth, 1992), however Kisidayova (1996) found no benefit to using a 2 step freezing technique on percentage cell recovery, indicated by cell motility. The mean recovery varied from 43 –80 %, but it was concluded that all preservation parameters should be specified separately for each protozoan species.

In a series of experiments Luchini *et al.* (1996) examined the effect of preservation method (lyophilisation or freezing, with or without glycerol) on the proteolytic activity of mixed rumen fluid digesting different feed sources *in vitro*. Other parameters examined were the microbial fraction used, centrifugation speed and dialysis (to reduce t=0 readings in blanks). Proteolytic activity was assessed by the release of total amino acids (**TAA**) from feeds and ammonia concentration in the supernatant at predefined times (up to 6 h). Preservation method altered total proteolytic activity but did not affect the overall ranking of feed products. Freezing was suggested as the optimum preservation method due to higher TAA in the blanks of lyophilised cultures at t=0, which suggested greater cell lysis during preservation. Glycerol addition significantly reduced the levels of NH_3 and TAA in the blanks at t=0, but did not affect the net release of these fractions after 6 h incubation. The preincubation of the frozen inoculum in a nutrient medium for 6 h, after thawing and before inoculation significantly improved the rate and extent of protein degradation. The implications of inoculum preservation on the celluloytic activity of mixed rumen fluid has not been assessed.

1.4.4.4 Culturing environment

When culturing rumen microorganisms *in vitro* the fermentative activity of the inoculum should be optimised by controlling the appropriate environmental condition (temperature, osmolarity and anaerobisis) providing growth factors, minerals and nitrogen sources and preventing endproduct inhibition.

Grant and Mertens (1992) examined the necessity for anaerobic conditions, tryptone, micromineral solution and reducing agent addition to culture medium for optimum NDF digestion. Bubbling media with CO_2 until saturated (indicated by a resazurin indicator) gave similar lag time, lower rates and higher extents of NDF digestion than continuously gassing with CO_2. There was no significant change in pH between either treatment but this study was confounded by vessel type. The authors recommend the use of reducing solution, micro minerals and nutritional supplements, particularly with substrates low in CP, to maximise digestion. The use of tryptone and microminerals for optimum cellulose digestion was supported by Cheng *et al.* (1955) and Martinez and Church (1970). The mineral requirements of ruminal microbial species was reviewed by Mackie and Therion (1984) and Komisarczuk-Bony and Durand (1992).

Grant and Mertens (1992) examined 3 buffers, Good buffer (Good *et al.,* 1966), McIlvaine buffer (Elving *et al.,* 1956) and Goering and Van Soest buffer (1970) and suggested that the Goering and Van Soest phosphate-bicarbonate buffer was most suitable to maintain *in vitro* pH 6.8 independent of the substrate and its fermentation. Grant and Mertens (1992) found no difference in NDF digestion with either the Van Soest buffer or McDougalls buffer. The buffer systems used in continuous fermenters are McDougalls buffer (1947), which is based on the cation and anion composition of sheep saliva, or the Weller and Pilgrim buffer. When used neat these buffers are pH 8.0 but to control osmolarity and reduce its negative impact on fibre digestion (Hoover *et al.*, 1984, Shriver *et al.*, 1986) buffers can be diluted. If CP of the diet is below 15 % it is recommended to include a urea supplementation of 0.5 g/l to compensate for indigenous recycling of nitrogen (Stern and Hoover, unpublished).

Branched-chain fatty acids, B-vitamins, and biotin are among some of the growth factors required for cellulolytic organisms (Dore and Gouet, 1991) and should be included in culturing media for purified inocula (Hidayat *et al.*, 1993). Significant improvements in total cell wall digestion are seen with low concentrations of BVFA (15.8 % and 24.8 % digestion in 24 h for 0.00 and 2.50 mM BCFA respectively), with no synergistic effects between acids (Gorosito *et al.*, 1985). An optimum level of 1.76 mM was suggested by regression equations. In the absence of BVFA, *Bacteroides amylophilius*

synthesise branch-chain AA from starch, CO_2 and ammonia. On death and lysis, the released AA are deaminated by *M. esldennii,* producing branched VFA which can support the cellulolytic organisms (Church, 1988).

Ammonia-N is required by cellulolytic microbes (Baldwin and Allison, 1983, Hespell 1984, Hoover *et al.,* 1998) and concentrations < 1.4 mM (dietary CP 12-13 % approx., Wallace, 1997) in rumen fluid may limit *in vitro* digestibility (Braver and Eriksson, 1967, Satter and Slyter, 1974). Satter and Slyter (1974) suggest that ammonia concentrations should not fall below 3.6 mM for optimum microbial activity, while Ricke and Schaefer (1996) cite a range of studies where optimum ammonia concentrations were found to range from 1 to 19.7 mM. Concentrations of 57 mM are reported to be toxic *in vivo* (National Academy Science, cited by Ricke *et al.,* 1996) but levels up to 92 mM have been reported in sheep (Hungate, 1966). Starch-digesting bacteria have been shown to obtain 66 % of their nitrogen from amino acids and peptides and only 34 % from ammonia (Chamberlain and Choung, 1995). Both sources of nitrogen are therefore used *in vitro*. However the use of casein to simulate ruminal soluble nitrogen and digestion kinetics is criticised by Cotta and Hespell (1986). Casein is highly soluble and readily hydrolysed *in vivo* unlike many soluble proteins which appear to be rate limited at the hydrolytic stage.

Osmolarity (moles of solute / l solution) is a controlling factor on microbial growth. It can be a function of diet, intake, microbial activity and water intake and influenced by ammonia, minerals and VFA concentration and methane production (Carter and Grovum, 1990). *In vivo* roughage and concentrate based diets have osmolarity levels in the range of 350 - 400 and 360 - 420 mOsmol/kg, respectively (Carter and Grovum, 1990). Pre-feeding values are approximately 250 mOsmol/kg (Engelhardt and Hauffe, 1975). Cellulose digestion *in vitro* was inhibited at 400 mOsmol/kg (Bergen, 1972) but inhibition is related to the compound used and under certain conditions no adverse affects or reduced fermentation for levels increasing to 500 mOsm/kg were seen (Okeke, 1978, Peter *et al.*, 1989). Protozoa are more sensitive than bacteria, and Gram negative bacteria are more sensitive than gram positive (Mackie and Therion, 1984). Microorganisms may be more insensitive to high osmolality solutions with sugar than salts (Mackie and Therion, 1984). It is suggested that ruminal microbes are resilient to the normal short term changes in osmolarity of ruminal fluid during a feeding cycle. Osmolarity is a consideration at extended incubation times due to endproduct buildup or in highly buffered continuous fermentation systems (Hoover *et al.*, 1976).

Johnson *et al.* (1958) found that the addition of 93.9 mM VFA (A:P:butyrate (B) was 50:40.5:3.4) decreased cellulose digestion. The addition of 62.6 mM VFA did not affect digestion though the

approximate concentration after 30 h would have been 198 mM (Piwonka and Firkins, 1996). Acetate and propionate concentrations of 50 and 40 mM at t=0, respectively had no effect on cellulose digestion (Johnson *et al.,* 1958). Acetate can be metabolized to butyrate by ruminal anaerobes such as *Butyrivibrio* and *Eubacterium* (see Gottschalk, 1986) and therefore could result in an elevated increase in butyrate production due to artificially elevated acetate levels *in vitro*.

1.4.4.5 Particle size

Sample preparation should optimise homogeneity of the sample, minimise physical losses, chemical losses, and chemical alterations during preparation (Mertens, 1993). Particle size for *in vitro* and *in sacco* experimental studies can vary with fresh or dried forages. Smaller particle sizes are preferred due to increased sample homogeneity, though long and chopped particles sizes are more favorable *in vivo* (Tafja *et al.,* 1999, Heinrichs *et al.,* 1999). Akins *et al.* (1974) have shown that the potential degradability of constituent plant parts is different, therefore kinetic studies of a ground sample is a weighted average of individual rates of several digestible fractions (Mertens and Ely, 1982). However this weighted average may be indirectly influenced by the botanical composition or stage of maturity of a herbage as the breaking or shattering of different constituent plant parts can differ (Emanuele and Staples, 1988).

A reduction in particle size can influence digestion kinetics (Dehority and Johnson, 1961, Menke *et al.,* 1979, Gerson *et al.,* 1988, Bowman and Firkins, 1993) and effects may be more prominent at shorter incubation times (Huntington and Givens, 1995). Akin (1976) showed that in 5 mm long sections of fresh grass leaf, very little of the tissue had been degraded from within after 6 h incubation, which would suggest that the initial rate of fermentation is dependent on the external microbial attack, which in turn is dependent on microbial population of the surface (Gerson *et al.,* 1988). Akin (1993) suggests that the *in vitro* use of particle sizes from 5 – 10 mm incorporate the ability of microbes to penetrate intact tissue. Uden (1992) comparing chopped and ground particles concluded that particle size has more influence on the lag than the rate of fermentation. A reduced particle size can also decrease the variations in DM degradation between different forage samples (Nocek and Kohn, 1988), though Michalet-Doreau and Cerneau (1991) found that the screen size of a mill can significantly affect the mean particle size of a sample, with a significant interaction between milling screen size and forage used. Drying of feeds may negatively affect the *in vitro* fermentation of samples, as the lag time of fermentation is extended as the feed hydrates (Miller and Hobbs, 1994). Hydration of samples prior to incubation did not benefit fermentation characteristics *in situ* (Corley *et al.,* 1998). High temperatures during particle size reduction can cause gelatinization of starch in concentrate feeds which can alter *in vitro* fermentation characteristics of a feed (Mansfield *et al.*, 1995).

1.4.4.6 Sample preparation

The impact of preparation techniques on subsequent *in vitro* DMD and *in sacco* digestion kinetics has been investigated (Vik-Mo, 1989, Hristov and Broderick, 1992, Lopez *et al.,* 1995) and where preparation techniques may not significantly affect the chemical composition of the forage the digestion kinetics can be compromised (van Soest and Mason, 1991, Cone *et al.,* 1995, Kostyukovsky and Marounek, 1995).

Effects of any preparation treatment were most notable in the early stages of incubation (6-24 h) (Vik-Mo, 1989, Lopez *et al.,* 1995). Vik-Mo (1989) compared the effect of oven drying (70 ^{0}C for 72 h) and freeze drying on the *in sacco* degradability of herbage and silage and estimates of DMD, OMD and nitrogen disappearance had method x feed interactions. Oven drying decreased the immediate soluble fraction of DM, OM and nitrogen, the respective rates of degradation for the silage fractions and the effective protein degradability for both forages. There was no comparison with the untreated fresh herbage for either forage in this study.

Drying was found to have the greatest effect on the WSC, DMD and acid detergent insoluble nitrogen (**ADIN**) content of forages, with the effects more severe with increasing temperature (Deinum and Maassen, 1994). Cone *et al.* (1995) suggest that maillard reactions and the binding of free phenolic acids to lignin, protein or hemicellulose may alter digestion kinetics. Lopez *et al.* (1995) examined the effect of preparation techniques on the *in sacco* degradability of fresh grass and an independent silage. The DM solubility, potentially and effective (outflow rate =0.033 /h) degradable fractions and lag times increased with drying. However a confound of particle size might suggest that the dried materials had a greater initial solubility due to particle loss. The degradation rate of CP but not DM was affected by preparation technique, while the solubility and degradability of nitrogen was higher for freeze dried than fresh and greater for frozen than fresh material.

Hristov and Broderick (1992) and Huntington and Givens (1995) omitted a fresh herbage treatment when looking at the effect of sample pre-treatment on *in sacco* degradability of silage and grass respectively. Hristov (1992) examined the effect of oven drying (60 ^{0}C) and freezing on silage DM and protein degradability but the experiment had a particle size confound. Huntington and Givens (1995) found that freeze-drying (**FD**) had the highest DM losses (56.7 vs. 53.7, for FD and average of other treatments). Freezing prior to oven drying (60 and 100 ^{0}C) or microwaving increased the DM degradability but this effect decreased as the heating temperature increased.

Oven drying will have a greater effect on the DM disappearance of silages and the formation of Maillard products in grass due to the higher concentration of VFA and protein/total N ratio of fresh silage and herbage, respectively. Lopez *et al.* (1995) did not quantify the concentration of VFA and could therefore not estimate the extent of loss or retention during preparation. It has been suggested that freeze-drying is the optimum preparation technique for *in sacco* studies (Vik-Mo, 1989, Lopez *et al.*, 1995, Huntington and Givens, 1995). However the milling of dried samples can introduce a confound of particle size into comparative work (Vik-Mo 1989, Lopez *et al.*, 1995) which can influence the immediately soluble fraction estimation and the estimation of rate of fermentation (Lopez *et al.*, 1995). There is no evidence of any extensive work of this type for the *in vitro* batch fermentation system. Many *in vitro* studies use the neutral detergent extraction procedure (Van Soest, 1972) in sample preparation which involves subjecting the material to high temperatures (100 ^{0}C) for up to 1 h in the presence of a detergent (Goering and Van Soest, 1970).

1.4.4.7 Substrate to inoculum ratio

The importance of microbial activity in the inoculum was discussed by Jessop and Herrero (1998) who, using a modelling technique for *in vitro* gas production, deduced that insufficient inoculum would give a reduced rate thus undermining a basic assumption i.e. the rate of fermentation was limited by the feed only. The substrate to inoculum and buffer ratio is normally 1 % w/v for gas and modified Tilley and Terry systems (Goering and Van Soest, 1970, Pell and Schofield, 1993, Theodorou *et al.*, 1994). The limiting factor when scaling down substrate inputs is the contribution of residual feed from the inoculum to the fermentation. This is characterised using a blank (Pell and Schofield, 1993). However Theodorou *et al.* (1994) found a linear relationship of gas pool size to substrate weight within the range of 0.2-2 g of substrate per bottle.

A suitable inoculum to buffer ratio is necessary to control pH and dilute end products of fermentation sufficiently but the contribution of blanks to the fermentation is related to inoculum volume. Therefore, a small inoculum is advantageous (Pell and Schofield, 1993). The gas systems of Beuvink, Menke and Cone use relatively large inoculums (33 %). Pell and Schofield (1993) found an inoculum size of 20 % sufficient to ensure maximum rate of gas production, whereas lower values were not. The smaller inocula were found to have greater lags than larger inoculum but the total volume of gas production was the same. This ratio is also used in the modified Tilley and Terry system.

Hidaya *et al.* (1993) looked at the effect of increasing the bacterial concentration in the inoculum on the digestion of hay and barley straw. Inoculum: buffer ratio was 33%. Bacterial pellets were resuspended 1.0, 0.2, 0.1 or 0.067 of the original volume using bacteria free rumen fluid and a salt

solution. Total VFA produced, rate of fermentation in the first 24 h and net gas production for barley straw increased with increasing bacterial density and proportions of VFA did not differ between treatments for either substrate. Net gas production for hay was lower for 0.067 treatment than 0.1 or 0.2. This might suggest a shift in MP production.

Fakhri *et al.* (1998) compared four systems of gas production that varied in quantity of buffer, % rumen fluid included, amount of digesta prepared and substrate pre-soaking. There were significant differences in VFA production (mM), and pH decreased to 6.04 in systems of high rumen fluid to buffer ratio (30 %) but not lower ratios of 20 and 10 %. Schofield and Pell (1995) found that gas production was not affected by the volume of the fermentation vessel tested. This was supported for DM and NDF digestion using the modified Tilley and Terry system (Sayre and Van Soest, 1972).

1.5 IMPACT OF MATURITY AND ENSILING ON RUMINAL MICROBIAL DIGESTION OF PERENNIAL RYEGRASS

1.5.1 Influence of maturity

In vitro studies have shown that in isolated form all hemicellulose and cellulose polysaccharides are fully digestible (Wilson, 1994), that an inverse relationship exists between forage NDF content and rumen degradation rate of OM (Cone 1996), and that lignification of the cell wall can have a linear or curvilinear effect on digestibility (Jung and Vogel, 1986). As herbage growth advanced, the *in vivo* extent of NDF digestion decreased (Bosch *et al.,* 1992, Huhtanean and Jaakola, 1994). This decrease can be associated with a longer lag and variable effects on the rate of fermentation (Bowman *et al.,* 1991). Huhtanean and Jaakola (1994) found a decrease in the rate and extent of DM and NDF digestion but no effect on the lag as forages matured. An increase in forage maturity greater than 35 days was found to decrease forage digestibility by 2.5 to 3 units/week (Keady *et al.,* 1995).

Maturity decreases the total nitrogen and the soluble protein fraction of herbage (Sanderson and Wedin, 1989b). Crude protein digestibility of fresh grass can vary from 47 to 87 % (Van Vuuren *et al*, 1991) and decreased with maturity (Amrane and Michalet –Doreau, 1993, van Vuuren *et al.,* 1990). Maturity does not greatly affect the AA composition of proteins (Hatfield, 1989) but will differentially affect the rate of protein digestibility between cellular fractions (Thomson, 1982).

The rumen fill value is suggested to increase with maturity as particle retention increases (Bowman *et al.*, 1991, Bosch and Bruining, 1995). However Bosch *et al.* (1992b) and Rinne *et al.* (1997) found that the passage rate increased with NDF content of the forage due to the higher functional specific gravity of the indigestible particles. Oba and Allen (1999) concluded from review, that a one unit decrease in NDF digestibility was associated with a 0.17 kg decrease in DMI, while 0.61 of reduced DMI with ensiled diets can be attributed to maturity alone.

Bosch *et al.* (1992) and Bosch *et al.* (1994) examined the effect of forage maturity on ruminal digestion. Rumen pH was <6.2 for 5, 3, 1 and 0 hours for four successive harvest differing in maturity. Protozoa numbers decreased with increasing maturity of the forage. Though OM and nitrogen digestions in the rumen were significantly higher with the early cut forages there was no improvement in efficiency of MP production and no change in LDR. This may be due to higher rumen recycling in the early stages due to higher protozoa numbers or a lack of synchrony in the earlier forages as the peak ammonia levels were 30.7 and 17.3 mmol/l for earliest and latest harvests. The minimum NH_3 levels never went lower than the optimum suggested by Satter and Slyter (1974) (3.6 mM) and faeces N

content was the same for all diets.

Bosch *et al.* (1994) found that the molar proportion of acetate increased in the rumen and butyrate decreased with increasing maturity of the ensiled forage, which were contrary to the findings of Beever *et al.* (1986). Propionate was not affected. Bowman *et al.* (1991) and de Visser *et al.* (1998) found that an A:P ratio of 3.2 was not greatly affected by maturity. Jung (1989) suggested that the change in VFA proportions might be due to an alteration in the microbial population and the toxic effects of free phenolic acids.

Beever *et al.* (1988) found that increasing maturity of an ensiled forage decreased gross energy and protein content, decreased rumen digestion of the NDF component and decreased non-ammonia nitrogen (NAN) flow to the small intestine. Tamminga *et al.* (1991) also found a negative effect of maturity on the degradation rate of grass silage DM, NDF and CP. Rinne *et al.* (1997) found that increasing maturity of the ensiled forage decreased DOMD (0.82, 0.82, 0.76 and 0.75 for increasing harvest date respectively) thus increasing the OM loss in faeces. Steen (1992) found a significant decrease in liveweight gain and carcass gain of finishing steers as the maturity of ensiled forages increased. These studies did not compare the ensiled forage with the fresh herbage but suggest that the negative impact of maturity pre-ensiling will hold for the digestion of the forage post-ensiling.

Some authors examined the effect of ensiling and forage maturity within different growth seasons on subsequent rumen digestion. Ensiling decreased the potentially digestible fraction though effects on rate seemed to be related to season, with the rates for all fractions higher in September than June, with little effect of ensiling (Lopez *et al.*, 1991). It is suggested that the cellulose:hemicellulose ratio may differentially influence rates of rumen fermentation. The cellulose:hemicellulose ratio is dependent on forage type, growth stage and growth season of the forage (Butler and Bailey, 1973). Regrowth grasses are not influenced by lignification to the same extent as first growths (see Bosch *et al.*, 1992, Givens *et al.*, 1993). Bosch *et al.* (1994) found no significant relationship between NDF content and *in sacco* degradation rate of CP with silages differing in maturity and harvesting season. The effect of season on rumen OM fermentation was not found to be significant by Ulyatt *et al.* (1988) and Beever *et al.* (1986) though the botanical composition changed. Harrison *et al.* (1994) concluded that the decline in first growth forage in spring was greater than the subsequent decline in regrowths (0.68 and 0.13 %/d for *in vitro* DMD respectively).

1.5.2 Influence of ensiling

Alterations in the biochemical composition of the herbage due to ensiling may affect the subsequent rumen digestion of the forage. Cushnahan and Gordon (1995) examined the effect of preservation duration on the ruminal digestion of perennial ryegrass. Increasing the duration of ensiling decreased the potential digestible fraction, increased the extent of DMD with variable effects on the rate of digestion when compared with the fresh herbage. Dry matter intake decreased with storage duration and was attributed to increases in ammonia-N and butyric acid concentrations. Lopez *et al.* (1991) also found no consistent effect of ensiling on the rate of degradation of the insoluble DM fraction.

When compared with the fresh forage, Petit and Tremblay (1992) found that ensiling, post wilting, increased the immediate DM and CP soluble fractions (17.9, 57.8, 27.3 and 78.5 % DM respectively) and decreased the extent of DM and CP digestion in the rumen (65.7, 45.4, 67 and 16.15 % respectively). Ensiling increased the extent of the grass DM and CP digestibility by 11.2-20.4 and 11.7-28.3 %, depending on assumed outflow rate. The lack of a significant effect on the rate of fermentation was attributed to the large variation in the disappearance rate among silages.

Cushnahan *et al.* (1995) examined the effect of restrictive and extensive preservation on the ruminal digestion of a perennial ryegrass sward. Preservation method did not affect the rate of DM, protein or ADF digestion, though the immediately soluble nitrogen fraction increased for the ensiled forages. There was no effect of ensiling on the lag or extent of fraction digestion, on ammonia concentration, on DMI or milk yield, though milk composition was altered with a reduction in fat and protein content of the extensively preserved forage. Total VFA concentration in the rumen was unaffected, though the NGR was lower for the extensively preserved forage.

O'Kiely and Flynn (1982) found no effect of ensiling on carcass production when animals were fed grass and well preserved forage. Keady *et al.* (1995) examined the effect of ensiling on the nutrient value of perennial ryegrass under restricted and untreated preservation conditions. There was a decrease in DMI for latter, and a decrease in milk yield and alteration of milk composition for both preservations. Preservation decreased the WSC content from 116 to 10 and 26 g/kg DM for untreated and restricted, respectively and also decreased the NDF content for both. The TVFA concentration was higher for the untreated, when compared with the restricted preservation and fresh herbage, the NGR ratio was lower for the restricted preservation when compared with the fresh herbage. There was no effect of ensiling on nitrogen retention, though urinary nitrogen excretion was higher for the fresh herbage.

In a review of the effect of ensiling on DMI and animal production, Keady and Murphy (1993)

concluded that ensiling can decrease the DMI by 3 % , decrease daily liveweight gain by 25 % and carcass gain by 8 %. In the absence of any effect on dry matter intake, the negative effect on performance may be associated with the loss of WSC fraction, lower microbial nitrogen flow and a lower efficiency of utilisation of metabolisable energy for animal production (see Keady *et al.,* 1995).

1.5.2.1 Nutrient synchrony

The increase in soluble nitrogen with the concomitant decrease in readily available carbohydrates (WSC and fermentable NDF) due to ensiling (and maturity) is suggested to develop a nutrient asynchrony for the ruminal microbial population. Several authors have reported inferior MP production by ruminants fed ensiled forages. For ensiled forages and herbages Harrison *et al.* (1994) reported that the efficiency of MP synthesis was 26.8 and 49.2 MN/kg OMADR respectively, Siddons *et al.* (1985) reported that the efficiency of MP synthesis was 21 and 26 g MN/kg OMADR respectively, while Gill *et al.* (1989) found the differences more extreme at 13-28 g and 33-58 g MN/kg OMADR respectively.

This would suggest that independent of or in association with the reduced energy potential of the carbohydrate fraction, ensiling results in the inefficient utilisation of ruminal ammonia-N by the microbial population. This may be attributed to its rapid removal from the rumen environment, through absorption or flow dynamics, or that the nitrogen content of the ensiled forage CP may limit optimum ruminal microbial growth.

Chamberlain and Choung (1995) have highlighted the difficulties of addressing nutrient asynchrony in the basal forage diet with *in vivo* studies. If the rate of supplemented readily fermentable carbohydrate is different there can be pronounced effects on ruminal pH and VFA patterns which can influence microbial growth or if synchronous or asynchronous diets rely on altering dietary components/composition there will be a confound of diet. Chamberlain and Choung (1995) suggest that altering the feeding rate of the protein supplement will offer the clearest interpretation of experimental results addressing the issue of nutrient synchronisation.

Shabi *et al.* (1998) reduced the ammonia concentration in the rumen by increasing the frequency of feeding from twice to four times daily. However the microbial DM and CP flow to the abomasum was higher on the latter and the authors concluded that available energy was the most limiting factor for microbial N utilisation. Kolver *et al.* (1998) decreased the ruminal ammonia peak of pasture grazing cows by 33 % by feeding a synchronous energy source. However though supplementation appeared to improve the capture of ruminal nitrogen, it did not affect the nitrogen status or performance of the animals.

Ammonia absorption from the rumen is extensive only when ammonia is unionised and ruminal pH is high (>6.8, Smith 1975) which are thought to be conditions seldom seen with silage based diets. It is noted that rumen ammonia concentrations will vary *in vivo* due to rumen outflow rate, rumen volume, and N recycling. Losses due to absorption of ammonia from the stomach were approximately 0.21 of intake (Chamberlain *et al.,* 1986) but ruminants have an ability to conserve N lost from the rumen by recycling plasma urea (Egan *et al.*, 1986). Therefore the capture of ammonia nitrogen may not be the most important influence on forage nutritive value post ensiling.

Lactic and acetic acid are the main end products of hexose metabolism during ensiling. In the rumen, the ATP benefit of hexose, lactic acid and acetic acid for rumen microbes is 4, 0.5 and 0 mol ATP/ mol carbohydrate unit respectively. Lactic acid concentration in ensiled forages can be as great as 15 % DM (McDonald *et al.*, 1991). Rumen microflora metabolism can adapt to high concentrations of lactic acid (Newbold *et al.,* 1987) though concentrations greater than 200 g/kg DM may exceed the fermentation capacity (Chamberlain, 1987). It can have a short half life in the rumen (25 min) with no selective utilisation of d- or l-lactate by rumen micro organisms (Chamberlain *et al.*, 1983). Ruminal *in vivo* concentrations on a grass diet varied from 2 mmol/l to 6 mmol/l (Dillion *et al.*, 1989).

Gill *et al.,* (1986) found that when sheep were offered perennial ryegrass at hourly intervals (139 g lacate/kg DM) the concentration of lactate in the rumen was low at all times (0.208 mmol/l) and 0.9 of lactate was metabolised in the rumen with the respective acetate, propionate and butyrate proportions of 0.6 : 0.35 : 0.05. Rinne *et al.* (1997) found an increase in lactate production during ensiling as the maturity of the forages decreased which did not support a subsequent response in rumen propionate production. The levels of lactate in the silages however were low (75, 76, 60, 47 g lactic /kg DM).

Jaakola and Huhtanen (1992) infused lactic acid continuously into the rumen of silage-fed bulls at a rate of 0, 40, 80 and 120 g/kg basal diet DM. They found that lactate was metabolised on a molar basis to 0.21 acetate, 0.52 propionate and 0.27 butyrate. Chamberlain *et al.* (1983) and Newbold *et al.* (1987) also found that propionate was the main endproduct of lactate digestion. However Counette (1981) has shown that the relative proportions of acetate and propionate produced from lactate are dependent on rumen pH, outflow rate and lactate concentration in the rumen. At pH 6.8, with a dilution rate of 0.25 /h, the acetate, propionate and butyrate proportions were 0.64, 0.33 and 0.03 respectively, with propionate increasing with decreasing dilution rate and butyrate increasing with decreasing pH (as cited by Gill *et al.*, 1986).

Jaakkola and Huhtanean (1992) also found with increasing infusions of lactic acid, a linear decrease in the number of rumen protozoa, a decrease in the efficiency of MP synthesis (20.4 and 13.4 g/kg OMADR for

control and 120 g/kg lactic acid respectively) and a linear relationship between lactic acid concentration and molar proportions of VFA. There were no effects on the flow dynamics of the rumen or pH (pH 6.2, 6.6, 6.4 and 5.9 for 0, 40, 80 and 120 respectively).

The pulse feeding of carbohydrate sources can increase lactic acid production and decreased pH immediately after feeding (Henning *et al.*, 1991), which would suggest a lower supply of ATP (Chamberlain, 1987) and possible energetic uncoupling of microbial growth (Russell and Dombrowski, 1980, Strobel and Russell, 1986). Pulse feeding may also influence the maintenance energy requirements of ruminal microbial populations. Maintenance energy requirements will affect bacterial Y_{ATP} and are thought to be generally higher for bacteria fermenting NSC than those fermenting SC (0.3 and 0.1 mg CHO/mg protein/h, Russell *et al.*, 1992). Henning *et al.* (1991) and Newbold and Rust (1992) concluded that the maintenance energy demands of bacteria in batch systems between synchronous and asynchronous situations are not greatly different. However, van Kessell and Russell (1996) using *in vitro* continuous culture techniques concluded that the maintenance energy requirements of mixed rumen bacteria cultured at 0.07 /h in energy-limiting ammonia-excess or energy-excess ammonia-limiting conditions were 0.09 vs. 0.96 mg of hexose equivalent/ mg protein/h respectively. Energy spilling is a term to define futile cycles of potassium, ammonium or protons through the cell membrane (van Kessell and Russell, 1996) and it can consume 50 % of total ATP generated by *S. bovis* thus increasing the maintenance energy requirements. Maintenance energy will be influenced by *in vivo* rumen function variability, rumen environment, substrate preference and/or species dominance and may partially explain the variable *in vivo* response of MP production to carbohydrate source.

1.5.2.2 Nutrient replacement

Inferior efficiencies of MP production may be due to an inefficient supply of other nutrients such as AA and peptides. Protozoa have no urease enzymes and can therefore not use urea or ammonia in the synthesis of AA while the three main bacterial cellulolytic species are non-proteolytic with a limited ability to incorporate AA (Weimer, 1992).

Using a diet of corn grain and oat straw (approx. 50:50) as the energy source Griswold *et al.* (1995) found peptides and AA increased ADF digestion when compared with urea but that nitrogen source had no effect on NDF digestion. Merry *et al.* (1990) reported an increase in cellulose digestion for fishmeal supplemented diets when compared with urea supplementation. Benefits of peptide supplementation to urea-N based diets are seen when the diets contain a large fraction of rapidly degraded carbohydrate (Maeng and Baldwin, 1975, Argle and Baldwin, 1989) suggesting the improved growth of amylotyic bacteria.

There appears to be a greater response to ammonia nitrogen supplementation when the basal diet is composed of slowly degradable structural carbohydrates. Crutz Soho *et al.* (1994) found no benefit in nitrogen source (ammonia, AA or peptide) infusion to the rumen of hay fed sheep, and *in vitro* AA and peptide, unlike urea supplementation did not stimulate the growth of cellulolytic microorganisms on cellulose substrate. Kernick *et al.* (1991, as cited by Griswold *et al.*, 1995) found that the *in vitro* digestibility of maize straw and alkaline treated wheat straw were not affected by peptide replacement of urea. Satter and Slyter (1974) suggest that cellulose digestion will be limited at ammonia concentrations less than 50 mg/l. Jones *et al.* (1998) found a linear decrease in *in vitro* fibre digestion as peptide nitrogen replaced urea as the nitrogen source. This decrease in cellulose digestion was associated with a decrease in ammonia concentration. This was supported by Bach *et al.* (1999) who found higher fibre digestion when pasture was supplemented with soybean hulls rather than corn or beetpulp, relating the latter two to decreases in ammonia concentration.

Chamberlain *et al.* (1982) found that different total nitrogen: non-protein nitrogen ratios in ensiled perennial ryegrass, with CP ranging from 133 to 148 g/kg DM did not affect rumen ammonia concentration (211 – 221 mg/l), OM digestion (0.78 to 0.82) or microbial flow to the duodenum (mean 23 g N/kg OMD). In this study the ratio of protein :AA: ammonia nitrogen was 5:5:1 to 10:5:1 for ensiled forages, which would suggest that protein nitrogen was not influential on rumen degradation. Rooke *et al.* (1985) found that the MP synthesis on silage based diets was improved with soyabean supplementation, when the ruminal ammonia concentration of unsupplemented diets was 100 mg/l. Keady and Murphy (1998) who examined the effect of sucrose or sucrose and fishmeal supplementation of the basal silage diet, concluded that the nutritive value of the ensiled forage was limited by the protein and/or AA content of the ensiled forage rather than by the energy content. There was no benefit of supplementation on the cell wall digestibility in this study.

Petit and Veira (1994) found no beneficial effect of protein supplementation on silage DM or NDF digestibility with a silage diet that had 14.4 % CP and maintained ammonia concentration at 10.23 mg NH_3/l. Rooke and Armstrong (1989) found no effect of continuous nitrogen supplementation (casein or urea) and sucrose supplementation on the rumen fermentation characteristics of silage based diet (127 g CP/kg DM). An inferior response to nitrogen supplementation of the basal diet when compared with previous work of Rooke *et al.* (1987) (0.7 vs. 1.9 g microbial N/g caesin-N infused, respectively) was attributed to the chemical composition of the basal diet and its potential to meet minimum ruminal requirements for NH_3 and peptide concentrations.

Supplementation of the basal silage diet with external energy sources can increase MP production which is attributed to the benefits of nutrient synchronisation (Chamberlain *et al.*, 1993, Henning *et al.* 1993, Sinclair *et al.,* 1993, Sinclair *et al.*, 1995, Van Vuuren *et al,* 1999). However supplementation of the basal diet with carbohydrate sources can negatively affect the *in vivo* NDF digestion of the basal diet (Rooke *et al.*, 1987, Rooke and Armstrong, 1989, Pwinoka *et al.,* 1994, van Vuuren *et al.,* 1999). Noziere *et al.* (1996) identified a negative effect on NDF digestion above 30 % supplementation of basal diet. Responses in MP synthesis can also vary between and within carbohydrate source ranging from 7 to 33 g MN/kg carbohydrate supplemented (cited by Chamberlain and Choung, 1995) and can be influenced by the composition of the basal diet (de Visser *et al.,* 1998, van Vuuren *et al.,* 1999).

The benefits of nitrogen supplementation may therefore be influenced by the protein content and concentration of the basal diet, the dependent microflora population and the carbohydrate content of the basal diet which will influence the microbial enzymatic activities and nutrient requirements. The benefits of carbohydrate supplementation may be influenced by an interactive effect with the NDF digestibility of the basal diet and the metabolic pathways used, maintenance energy of, and the substrate preferences of the microbial population.

1.6 Summary of research objectives

As outlined previously, forage preservation by ensiling is an important component of ruminant production in Ireland. The adverse effects on forage nutritive value have been attributed to a multitude of interactive processes (Steen *et al.,* 1998) with debate as to the relative importance of each (Chamberlain and Choung, 1995, van Os *et al.,* 1995, Steen *et al.,* 1998). *In vitro* studies may be used to explain some of the individual mechanisms underlying these interactive processes.

The aim of this thesis was to primarily examine the effect of ensiling on the *in vitro* ruminal fibre digestion of perennial ryegrass, which was harvested during late season (Chapter 3) or at different maturities (Chapter 4). To this end methodological issues, not previously or completely addressed in available literature, for batch *in vitro* fermentations were examined in Chapter 2 and Chapter 6, Section 6.1 to define the optimal *in vitro* experimental conditions to be used.

1.6.1 Methodological studies

The conventional vertical agitation of fermentation tubes may influence the dry matter digestion profile of forages by contributing to insufficient mixing and bridging of forage substrates during incubation. The objective of Section 2.1 was to examine the effect of vertical or horizontal agitation of culture

tubes during *in vitro* incubation on the variance within experimental treatments at any time point on the description of the NDF digestion profile

The separation of feeds into soluble and insoluble nutrient fractions is necessary to develop our understanding of the relationship between feed biochemical composition and ruminal *in vitro* digestion kinetics. Procedures for forage fractionation should be such that the biochemical structure or *in vitro* digestibility of the isolated fraction is not altered. The objective of Section 2.2 was to

- examine the effect of aqueous extraction temperature on the *in vitro* cell wall digestion kinetics of perennial ryegrass and silages differing in maturity
- examine the effect of extraction medium (water and neutral detergent solution) on the *in vitro* cell wall digestion kinetics of perennial ryegrass silages
- compare the *in vitro* digestion kinetics of the aqueous extracted CW material of perennial ryegrass silage with those estimated by the NDF content of the residues.

Potential variation within inoculum source for *in vitro* studies has been identified (Mauricio *et al.,* 1998, Weimer *et al.,* 1999), with little exploratory work reported which assesses the potential of inoculum preservation for use in ruminal *in vitro* studies (Luchini *et al.,* 1996). The objective in Section 2.3 was to identify an optimum method of inocula preservation for *in vitro* studies of forage apparent DM digestion.

Post-ensiling the water-soluble fraction is characterised by an increase in VFA concentration and a reduction in pH (McDonald *et al.,* 1991), both of which influence *in vitro* microbial activity (Peters *et al.,* 1989, Grant and Mertens, 1992). The objective of Section 6.1 was to develop a system of substrate neutralisation, which would stabilise the *in vitro* fermentation of a simulated silage WSC pre-inoculation and also to determine if substrate neutralisation altered the subsequent *in vitro* fermentation pattern of the residual WSC fraction post-ensiling.

Continuous fermentation systems are designed to incorporate the influence of flow dynamics on measures of *in vitro* digestion. Though fresh forages can be used in the Rusitec system, control of pH, LDR and SDR are important when examining the effect of forage maturity and ensiling on *in vitro* digestion and may be facilitated using a dual flow system. The objective of Chapter 5 was to establish and validate a semi-continuous fermenter

1.6.2 Effect of ensiling and maturity on cell wall digestion *in vitro*

The common objective of Chapter 3 and Chapter 4 was to examine the effect of alterations in the

soluble fraction of perennial ryegrass during preservation, on the subsequent digestion kinetics of the cell wall fraction. Therefore the digestion kinetics of the cell wall fraction of fresh and ensiled forages were described in two situations in both chapters. In the first situation the substrate was defined as the chopped fresh material of fresh and ensiled forages and the NDF digestion kinetics were evaluated. In the second situation the substrate was the isolated cell wall fraction of fresh and ensiled forages and the cell wall digestion kinetics were evaluated. As preservation method can influence the water-soluble fraction and structural fraction of perennial ryegrass, restrictive and extensive preservation conditions, using formic acid and sucrose supplementation respectively were imposed during perennial ryegrass preservation in Chapter 3 and Chapter 4.

An additional objective in Chapter 3 was to determine the effect of re-supplementing the water-soluble fraction pre- and post-ensiling on the apparent digestion of the isolated cell wall fraction of perennial ryegrass pre- and post-ensiling. An additional objective of Chapter 4 was to examine the effect of maturity from 7 to 16 weeks regrowth on the *in vitro* digestion of fresh and ensiled perennial ryegrass.

In Chapter 6, the nutritive potential of the water-soluble fraction pre- and post-ensiling was addressed. The objective of Section 6.2 was to examine the effect of ensiling *per se* on the nutritive potential of the soluble fraction using batch culture. The objective of section 6.3 was to examine the effect of the soluble fraction pre- and post-ensiling on the *in vitro* digestion of the structural fraction pre- and post-ensiling using semi-continuous culture.

CHAPTER 2

EXPERIMENTAL METHODOLOGY – BATCH STUDIES

2.1 THE EFFECT OF CULTURE TUBE ORIENTATION ON THE *IN VITRO* DIGESTION OF PERENNIAL RYEGRASS SILAGE.

Introduction

To improve the incubation capacity of any *in vitro* procedural run, test tubes are the preferred culture vessel. However, when culture tubes were incubated in an upright position in preliminary studies, the release of fermentation gases caused random 'bridging' where the dry matter was raised above the inoculum. Bridging occurred with milled (particle size 2 mm) and chopped (particle size 1 cm) samples.

Culture tubes were manually mixed in the former studies to re-suspend the substrate. The suspension of substrate particles above the incubation medium may increase the variation between replicates at any specified sampling time. The vertical orientation of cultures during incubation also results in passive mixing which may be inadequate for optimal mixing when large substrate particles are incubated. Grant and Mertens (1992) concluded that fermentation vessel (125 ml Erlenmeyer flask or 50 ml polypropylene tube) had no effect on the *in vitro* neutral detergent fibre digestion of an incubated milled substrate. However the effect of orientation may influence the digestion profile of the substrate.

Objective

The objective of this study was to examine the effect of vertical or horizontal agitation of culture tubes during *in vitro* incubation

- on the variance within experimental treatments at any time point
- on the description of the NDF digestion profile

Materials and methods

Experimental treatments

Culture tubes used for the vertical agitation (**V**) were glass tubes, 245 mm length, 28 mm I.D. and 150 ml volume, with a bunsen valve (**Figure** 2.1.1a). These culture tubes were modified to allow for horizontal incubation with the addition of a glass side arm for gas release (7 mm I.D. and 20 mm in length) and a screw cap lid (**H, Figure** 2.1.1b). The *in vitro* substrate was perennial ryegrass silage,

which was chopped to 1cm length using a paper guillotine (**PS1**) and frozen, or dried at 45 ^{0}C for 48 h and milled to 2 mm particle sizes (**PS2**) (**Table** 2.1.1).

Figure 2.1.1a Culture tube for vertical agitation

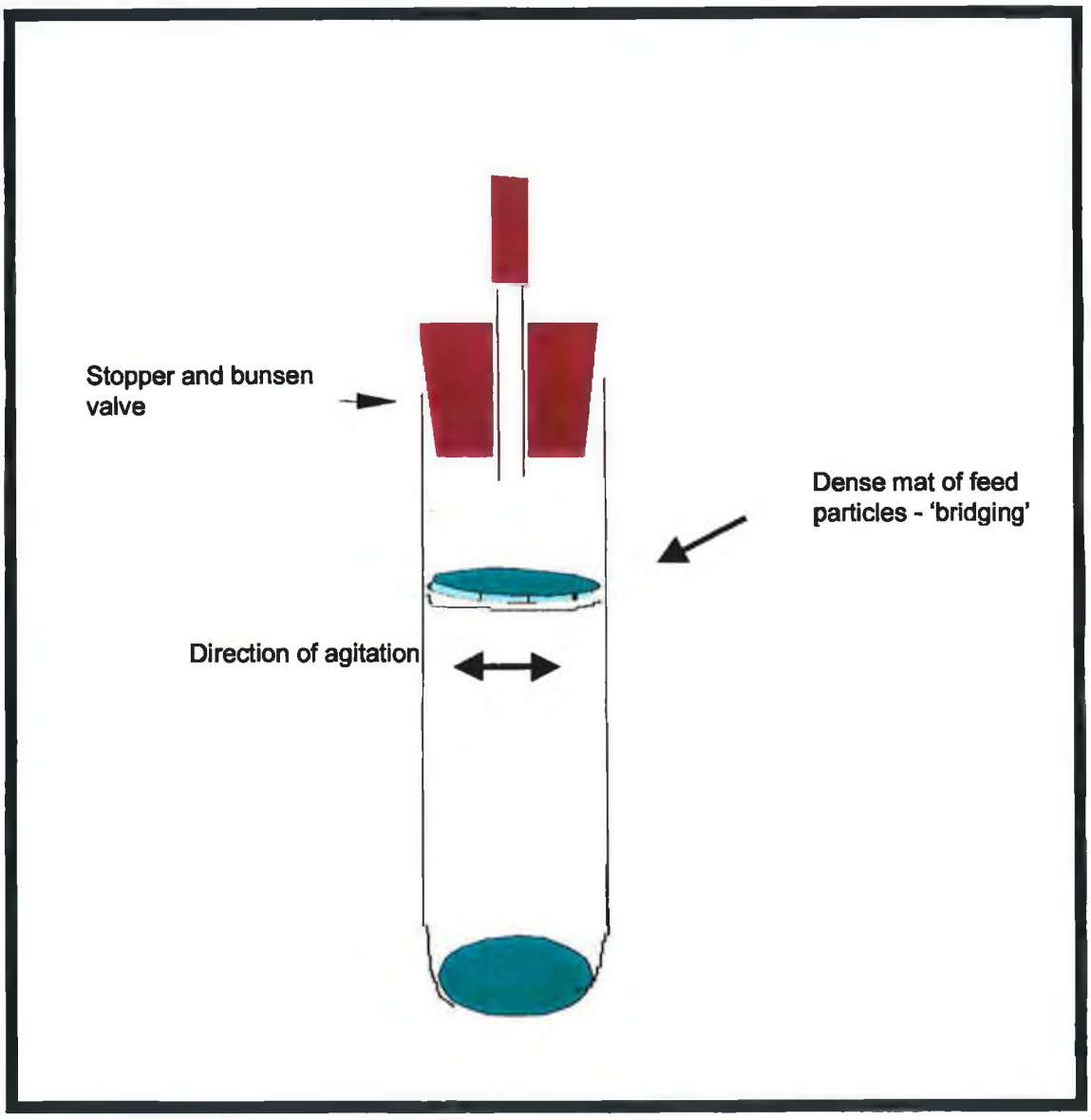


Figure 2.1.1b Culture tube for horizontal agitation

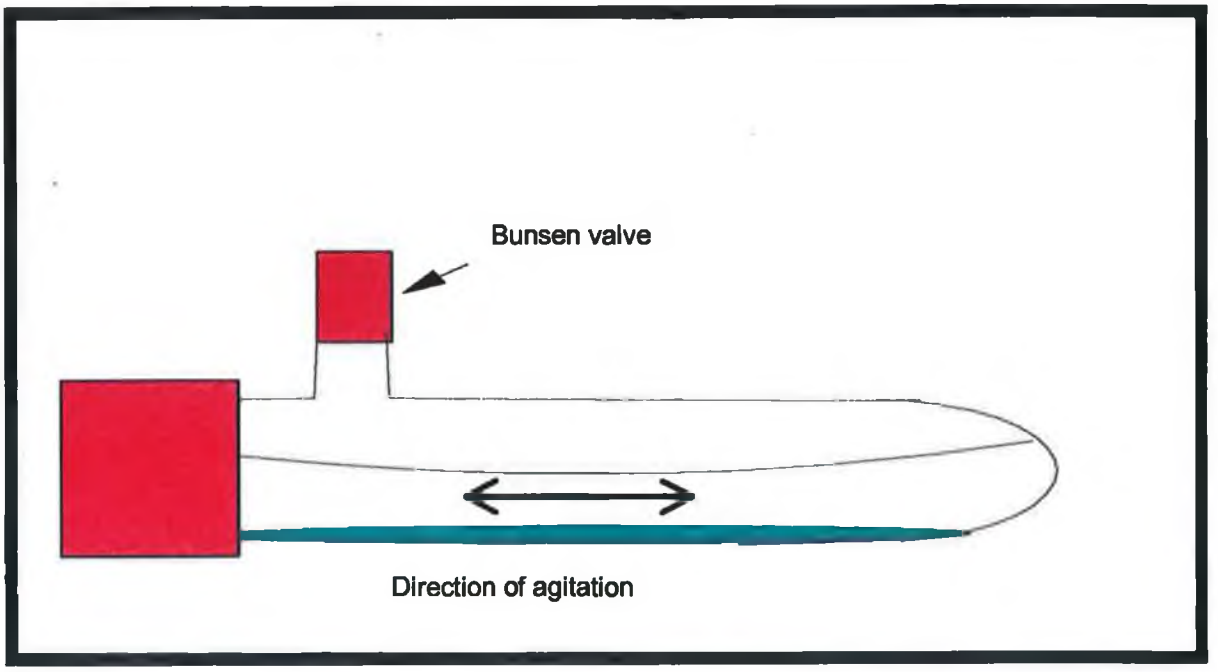


Table 2.1.1 Chemical composition of control silage (g/kg dry matter (DM) (sd.))

	Substrate
DM digestibility	667.0 (10.61)
Digestible organic matter	639.3 (10.87)
Crude protein	162.7 (3.30)
Ash	102.0 (1.63)
Neutral detergent fibre	554.0 (1.63)
Acid detergent fibre	344.8 (9.53)

In vitro technique

Modified Tilley and Terry technique (Section 1.4.2.1)

Inoculum preparation

A representative sample of rumen fluid was collected pre-feeding from 3 steers fed grass silage *ad libitum*. On the morning of inoculation, rumen fluid was removed through the ruminal fistula using a 200 ml plastic container and stored in a preheated CO_2 flushed thermos flask. Solid digesta was sampled from every animal and stored at 39 ^{0}C in sealed bags previously flushed with CO_2. In the laboratory, the rumen fluid was filtered through 100 μm mesh under a continuous flow of CO_2 at 39 ^{0}C and the filtered contents continuously mixed with a magnetic stirrer. The rumen is estimated to contain 10-12 % DM (Church, 1988). Therefore for every litre of fluid collected, 100 g of solid digesta from the animal was washed to remove solid-associated microbes. The washing procedure used was described by mixing 50 g of digesta with 100 ml of rumen fluid in a CO_2 flushed bag. The contents were then stomached using a stomacher (Lab blender 400) for 5 min., after which the contents were pooled with the rumen fluid filtrate by filtering through 100 μm mesh (based on Merry *et al.,* 1983). The inoculum was continuously stirred under a stream of CO_2.

In vitro method

For dried and milled substrates, 1 g DM was weighed into each culture tube and 80 ml buffer and 4 ml reducing solution (**Table** 2.1.2) were then added under anaerobic conditions. All cultures were incubated at 39 ^{0}C, 18 h prior to inoculation. For wet frozen forages, the silages were thawed at 4 °C and 1 g of DM equivalent weighed into each culture tubes on the morning of inoculation. At inoculation, 20 ml of prepared ruminal fluid was added to culture tubes within 1 h of sampling, under anaerobic conditions using a previously calibrated hand-held dispenser. Cultures were incubated either horizontally or vertically, in a temperature controlled Brunswick incubator set at 39 ^{0}C, with agitation of the tubes maintained at 80 revs/min. Cultures were removed in triplicate 9 times over 96 h. The pH of each was checked when removed from the incubator and recorded if greater than pH 6.8. Residues were recovered by vacuum filtration through 100 μm mesh and washed 3 times with 10 ml hot water. Residues were then dried at 40 ^{0}C for 48 h and weighed. All silage preparations were incubated in each of two consecutive *in vitro* runs.

Statistical analysis

Data pertaining to within treatment variation for DM disappearance were analysed for each time point using the Chi Squared (or Bartletts) Test (Steel and Torrie, 1960). A model appropriate to a factorial design was used for apparent DM disappearance where orientation and particle size were the main factors and each run was treated as an experimental block.

Table 2.1.2 Components of Goering and Van Soest buffer and reducing solution

Component		in H_20	/ litre * final
H_2O		(g/l)	500
Buffer	NH_4HCO_3	4.0	250
	$NaHCO_3$	35.0	
		(g/l)	
Macro mineral	Na_2HPO_4	5.7	250
	KH_2PO_4	6.2	
	$MgSO_47H_2O$	0.6	
		(g/100 ml)	
Micro mineral	$CaCl_22H_2O$	13.2	0.25
	$MnCL_2.4H_20$	10.	
	$CoCl.6H_2O$	1.0	
	$FeCl_2.6H_2O$	8.0	
Casein			5.0
Resazurin			2.5
Reducing solution (g/100 ml)			
Cysteine HCL		0.625	
H_20		95.0	
1M NaOH		4.0	
Sodium sulphide		0.625	

*Final buffer was gassed for 4 h with CO_2

Results and discussion

When vertically agitated, the available surface area ($2\pi rh$) to a working volume (πr^2 h) ratio: for a 100 ml volume in each culture was 0.7. When the modified culture tubes were horizontally incubated, the available surface area:volume ratio was 1.1. This increased ratio allowed for greater mixing of the cultures and presumably a greater diffusion of rumen fluid between the substrate particles.

For apparent DM disappearance there was no effect of orientation and PS on within treatment variation (0.0274, 0.0218, 0.0357 and 0.0367 g^2 DM for vertical PS1, PS2, horizontal PS1 and PS2, respectively where $\chi^2 = 4.71$, $p > 0.05$).

The within treatment variation was periodically influenced by experimental treatments with a significant effect of treatment at 7 h ($p<0.05$), 24 h ($p<0.01$) and 48 h ($p<0.05$) (**Table** 2.1.3). The Barletts test does not describe where the significant effect occurred between treatment means. However the within treatment variation was numerically greater for PS1 when vertically incubated for 7 and 24 h but not at 48 h where the horizontal agitation had greater variation. The variation for PS 2 was also less when horizontally agitated at 24 h but not at 48 h, with little difference at 7 h.

Table 2.1.3 Effect of orientation (O) and particle size (P) on within treatment variation at each time point for apparent dry matter disappearance.

O[a]	P	Time (h)								
		0	3	7	12	24	36	48	72	96
V	2mm	0.0047	0.0051	0.0070	0.0068	0.0037	0.0057	0.0005	0.0023	0.009
	1cm	0.0031	0.0022	0.0029	0.0022	0.0113	0.0004	0.0012	0.0043	0.0041
H	2mm	0.0060	0.0103	0.0077	0.0192	0.0001	0.0050	0.0069	0.0015	0.0023
	1cm	0.0016	0.0021	0.0003	0.0035	0.0047	0.0035	0.0039	0.0010	0.0023
χ^2_3		4.74	4.07	9.74	6.32	15.78	6.89	8.2	2.73	2.45
sig.		ns	ns	*	ns	**	ns	*	ns	ns

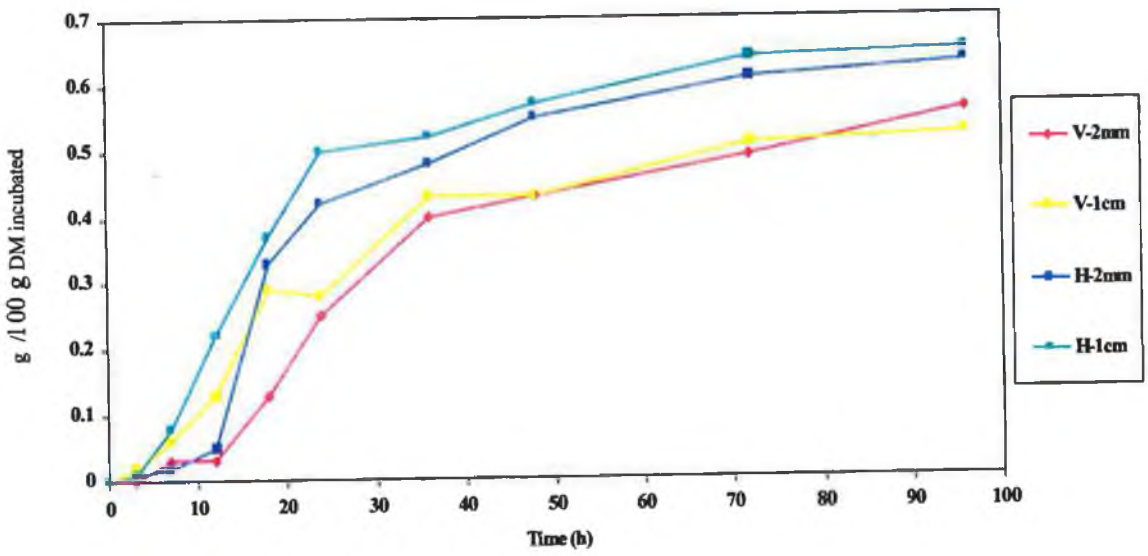
[a] V = vertical orientation ; H = horizontal orientation

Though there is evidence that the method of agitation can affect the *in vitro* fermentation profile in gas production systems (Rymer *et al.*, 1998, Getachew *et al.*,1998), similar information for the modified Tilley and Terry *in vitro* technique is scarce. Polypropylene tubes are normally the culture vessel of choice for use in kinetic studies based on gravimetric measurements but due to the dimensions of these tubes substrate bridging can occur (Miller and Hobbs, 1994). In this study though the horizontal orientation of culture tubes prevented bridging, it did not subsequently have a consistent influence on within treatment variation.

Analysis of variance requires that the homogeneity of treatment data sets are not different. Because of this the DM disappearance over time was not statistically analysed. However from **Figure** 2.1.2 the horizontal orientation of fermentation tubes gave a superior digestion profile at each particle size. This is supported by Stevenson *et al.* (1997) who concluded that the *in vitro* substrate fermentation profile

Analysis of variance requires that the homogeneity of treatment data sets are not different. Because of this the DM disappearance over time was not statistically analysed. However from **Figure** 2.1.2 the horizontal orientation of fermentation tubes gave a superior digestion profile at each particle size. This is supported by Stevenson *et al.* (1997) who concluded that the *in vitro* substrate fermentation profile (0 and 24 h measurements only) was improved by increasing the ASA:V ratio but there was no effect on the measured parameters (VFA and MP production) when tubes were shaken or stationary.

Figure 2.1.2 The effect of orientation (V = vertical ; H = horizontal) and particle size (2 mm or 1 cm) on apparent dry matter digestion *in vitro*



The kinetics of fibre digestion may be positively influenced by reducing substrate particle size (Bowman and Firkins, 1993, Huntington and Givens, 1995) as the opportunity for cellulolytic microbes to adhere to the fibrous surface increases (Akin, 1993, Gerson, 1988). The apparently superior fermentation profiles of the chopped substrates in this study may reflect differences in substrate preparation. Freezing can disrupt the structural fraction due to the freeze-thaw process making the substrate more susceptible to digestion (Huntington and Givens, 1995) and higher temperatures during oven drying and milling may adversely influence substrate digestibility (Deinum and Massen, 1994, Lopez *et al.*, 1995).

Conclusion

It is concluded that

- within treatment variation in the modified Tilley and Terry technique was not consistently influenced by orientation or particle size

Implications

Though the horizontal agitation of cultures did not consistently influence within treatment variation, its use was adapted for all further *in vitro* studies due to the enhanced *in vitro* fermentation profiles for perennial ryegrass apparent DM.

2.2 EXTRACTION OF NEUTRAL DETERGENT FIBRE FROM PERENNIAL RYEGRASS

Introduction

Early *in vitro* and *in vivo* research was concerned with the description of the fermentation kinetics of whole forages (Goering and Van Soest, 1970, Nocek, 1988, Michalet-Doreau and Ould-Bah, 1992). As the understanding of the relationship between feed biochemical composition and ruminal *in vitro* digestion kinetics develops, there is a greater need to recognise the existence of differential fermentation profiles of feed soluble and insoluble energy and protein pools in ruminal digestion (Russell *et al.*, 1992, France *et al.*, 1993, Pitt *et al.*, 1996). These pools differ in the kinetics (Doane *et al.*, 1997) and endproducts of fermentation (Murphy *et al.*, 1982, Friggens *et al.*, 1998). Therefore the separation of feeds into soluble and insoluble nutrient fractions is becoming a necessary step in *in vitro* nutritional and kinetic studies of ruminant feeds.

Kinetic data on fractions of individual feeds may be obtained from a single *in vitro* time point measurement or application of multiphasic kinetic models (Schofield and Pell, 1995a). When a fraction is difficult to isolate a method of curve subtraction may be employed. The digestion profile of the entire forage is characterised, as is the profile of a suitably isolated fraction such that when the two curves are subtracted data is generated which describes the remaining fraction. The reliability of this procedure will depend on the effectiveness of the original extraction procedure. To date little information has been published on the validation (Schofield and Pell, 1995a, Stefanoan *et al.*, 1996, Doane *et al.*, 1997b, Hall *et al.*, 1997) or application of the technique (Doane *et al.*, 1997a, Blummel and Bullderick, 1997).

Fraction isolation can be complicated by the biochemical composition of the feeds, which can contain variable amounts of soluble hexoses, storage and structural carbohydrates. Chemical isolation procedures for any feed fraction can be elaborate and complicated (Moore *et al.*, 1994). Extraction procedures currently employed, range from aqueous extraction (Smith, 1981) to refluxing in detergent solutions (Goering and Van Soest, 1970), and are obviously dictated by the fraction required (Moore *et al.*, 1994). However the increasing severity of extraction may adversely effect the subsequent *in vitro* digestion kinetics (Theurer, 1986, Kostyuovsky and Marounek, 1995, Haddad *et al.*, 1995).

Neutral detergent solution removes cytoplasmic proteins, and soluble and structural carbohydrates to variable degrees (Van Soest *et al.*, 1991). The NDF fraction which is generally considered to be the cell wall fraction is actually a subfraction of the cell wall (Van Soest, 1982). This is an accepted

generalization in studies dealing with fibre digestion and concepts of rumen fill. However when addressing issues of total nutrient availability to the cellulolytic microbial community and nutrient supply to the host, all fractions should be considered.

The soluble and structural fractions of perennial ryegrass and their alterations due to maturity and ensiling are important in ruminant nutrition. There is currently no published literature on either the validation or application of an isolation procedure for the cell wall fraction of perennial ryegrass or perennial ryegrass silage. Perennial ryegrass and silage has a biochemical structure amenable to a simplified procedure of component fractionation. The carbohydrate fraction is composed mainly of the structural carbohydrates and water soluble storage polysaccharide, fructan and sugars (McDonald *et al.,* 1991). In silage, the endproducts of fermentation are also water soluble (a heterogeneous mixture of organic acids, sugars, VFA and lactate in addition to nitrogenous compounds and lipid (McDonald *et al.,* 1991)). However the fibre component is complex and can vary in physical, chemical and nutritional properties as the plant matures.

The aim of this study was to validate a non-chemical isolation procedure for the structural component of perennial ryegrass forages. Three independent methodology studies are reported.

2.2.1 Objective

To examine the effect of aqueous extraction temperature on the *in vitro* CW digestion kinetics of perennial ryegrass and silages differing in maturity.

Materials and methods

Maturity and ensiling treatments

Perennial ryegrass plots (n=3) were harvested at 4 maturities, representative of early vegetative to full-head out growth stages. On any day of harvesting, the grass forage (**G**) was mixed, precision chopped and ensiled for 8 weeks in mini-silos (n=6, O'Kiely and Wilson, 1991). Restrictive (**R**, 5 ml formic acid / kg fresh weight, 85% formic acid) or extensive (**E**, 20 g sucrose/kg fresh weight) ensiling conditions were examined with the aim of influencing the microbial fermentation of structural components during preservation.

Sample preparation

For every harvest date, all three forages were dried at 40 ^{0}C, milled through a 2 mm screen (**Dr**) and 200 g of forage DM weighed into a nylon bag (aperture 100 µm). Using an automatic washing machine, forages were washed with cold water for 30 min and then submersed in 8 l of water. With

continuous agitation the temperature of the water was raised and maintained at 70 ^{0}C for 1 h. The cold wash was repeated and the residue dried at 40 ^{0}C for 48 h (**F70**). This procedure was repeated with 200 g forage DM but the temperature during agitation was maintained at 20 ^{0}C (**F20**). For every forage, each fraction was prepared three times and pooled for *in vitro* incubations.

In vitro technique

Gas pressure transducer (Section 1.4.2.2)

Inoculum preparation

As described in Section 2.1

In vitro method

The isolated fractions (F20 and F70) of all forages, from harvest 1 to 4, were incubated (n=2) in each *in vitro* run which were repeated within 7 days. Serum bottles of nominal volume 100 ml, contained 1g substrate, 10 ml inoculum, 85 ml buffer and 4 ml reducing solution (**Table** 2.1.2) and were prepared under anaerobic conditions (Theordorou *et al.*, 1994). Sealed bottles, with all components added except for the inoculum, were incubated at 39 ^{0}C for 18 h prior to inoculation. Blanks were included (n=3) to correct for gas production from residual feed fermentation in the inoculum. On the morning of inoculation, 10 ml of rumen fluid was added to each serum bottle, within 1 h of sampling, using a 20 ml syringe. All cultures were vented 10 min after inoculation and the time noted as t=0. Gas volume and pressure readings were taken at intervals so as not to allow the headspace pressure to increase above 7 psi (Theodorou *et al.,* 1994). Cultures were incubated in a 39 ^{0}C waterbath, without agitation, other than inversion after sampling. At the end of the incubation period (96 h) the pH of all cultures was measured and a 2 ml sample removed and acidified with 200 µl of 5M H_2 SO_4 before freezing for subsequent VFA analysis. The residue of each serum bottle was recovered, dried at 40 ^{0}C for 48 h and weighed.

Table 2.2.1 Neutral detergent solution

Component	Quantity
Distilled water (l)	1
Sodium lauryl sulfate (g)	30
EDTA disodium salt (g)	18.61
Sodium borate decahydrate (g)	6.81
Disodium hydrogen phosphate (g) anhydrous	4.56
2-ethoxyethanol (ml)	10

Chemical analysis

The following biochemical components of all herbage fractions isolated and incubated were defined: dry matter digestibility (DMD, Tilley and Terry, 1963), NDF/ADF (Van Soest, 1963), crude protein (CP, Association of analytical chemists (AOAC) method 990-03 Instrument Leco FP-428), acid detergent insoluble nitrogen (ADIN) (Instrument Leco FP-428), digestible organic matter (DOMD, Alexander and McGowan , 1961) and crude ash (Ash, SI 200 of 1984 6. Mineral Substances 6.1).

Statistical analysis

Data were analysed using the statistical package Genstat 5 (Lawes Agricultural Trust, 1990) and the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985). Data were analysed using a model appropriate for a split-plot design, where harvest was in the main plot, and forage and component in the sub-plots.

The pattern of NDF disappearance over time for each treatment was characterised by the unmodified Gompertz model (Bidlack and Buxton, 1992) described by

$$y = (a+c) \exp[-\exp\{-b(x-m)\}]$$

where a+c = upper asymptote, a = lower asymptote, m = x value at the maximum slope of the curve, m-1/b = lag, b = fractional degradation rate governed by the constant (x-m).

Results and discussion

Chemical composition

The chemical composition of the fresh herbages and respective silages are detailed and discussed in Chapter 4 (**Table** 4.3). Briefly, advancing maturity of the fresh herbage was evident from the linear increase in forage NDF ($p<0.001$) and ADF ($p<0.001$) structural components from maturity stage (**M**) 1 to M4. Lignin concentration in the cell wall material increased linearly with maturity. Increases in lignin concentration have previously been associated with reductions in forage digestibility (Jung and Allen, 1995). There was a linear decrease in forage DM digestibility in this study as the herbage matured. The CP content of the DM fraction linearly decreased ($p<0.001$) as the perennial ryegrass matured. Ensiling decreased the NDF content of herbages in M1 and M2 and increased the ADF content in M3 and M4 which reflects the biochemical changes in herbage due to maturity and the subsequent increase in the resistance of the cell wall structure to acid and enzymatic hydrolytic effects. The effect of ensiling on the CP fraction was variable as ensiling can alter the proportional

representation of various nitrogen fractions without affecting or slightly increasing total CP concentration (McDonald *et al.,* 1991). The ADIN content of the DM did not change with maturity.

Moore *et al.* (1994) suggested that the effectiveness of extraction procedures for fractional isolation of forage components may be differentially influenced by any biochemical alterations in the forage matrix. The CP content of herbages changes with maturity (Sanderson and Weidin, 1989a) and ensiling (McDonald *et al.,* 1991) which may differentially affect the formation of Maillard products during aqueous extraction. Thus forages differing in stages of maturity and influenced by restricted and extensive ensiling conditions were used to examine the effectiveness of aqueous extraction at 70 ^{0}C for CW isolation of perennial ryegrass. The extraction temperature was chosen from preliminary work which suggested that no Maillard reaction products were formed at 70 ^{0}C. The use of the F20 fraction for the subsequent *in vitro* digestion assumes that aqueous extraction at 20 ^{0}C does not interfere with the biochemical nature of the insoluble forage fraction.

There was a significant maturity x forge x component interaction for the NDF ($p< 0.01$), ADF ($p<0.01$) and CP ($p< 0.001$) content of the forages (**Table** 2.2.2). The biochemical alterations due to maturity and ensiling therefore have an interactive effect on component solubilities, as suggested by Moore *et al.* (1994).

The Dr fraction represented the experimental control with all biochemical components present in their true proportions. The true WSC sugars (glucose, fructose and sucrose) are cold water soluble and the structural carbohydrates (pectins, galactans, β-glucans, arabans etc, Butler and Bailey, 1973) are hot water soluble to varying degrees. Therefore the F20 and F70 fractions were without all WSC and protein components such as the cytoplasmic proteins. A significant effect of component on the NDF and ADF concentrations was expected as the extraction procedure concentrated the NDF fraction. Removal of aqueous soluble proteins and the concurrent increase in NDF concentration reduced the CP content of the fractions.

Table 2.2.2 The chemical composition (g/kg DM) of isolated fraction (C)[a] as influenced by maturity and forage type

Maturity[b]	Forage [c]	NDF			ADF			CP		
		Dr	F20	F70	Dr	F20	F70	Dr	F20	F70
1	Grass	521.5	798.0	844.0	301.5	480.5	504.5	204.5	127.5	114.0
	Restrictive	472.0	760.5	822.0	294.0	473.5	494.5	199.0	138.0	129.5
	Extensive	470.0	759.0	788.5	297.5	474.0	489.0	194.0	131.0	119.5
2	Grass	535.0	827.5	863.0	310.5	492.5	500.5	171.0	113.5	113.0
	Restrictive	512.0	794.5	859.0	320.0	490.5	527.0	175.0	123.5	102.5
	Extensive	503.0	789.5	859.5	320.0	502.5	542.5	171.0	104.0	90.3
3	Grass	592.5	842.0	866.5	348.0	518.0	536.5	113.0	95.4	95.1
	Restrictive	612.0	841.5	883.5	376.5	542.5	530.0	127.5	99.0	84.9
	Extensive	587.5	839.5	869.5	367.0	506.0	530.0	118.0	84.3	79.1
4	Grass	615.0	874.5	931.5	369.0	513.5	559.5	97.5	80.95	72.5
	Restrictive	619.5	885.5	909.0	379.5	527.5	545.5	113.0	77.05	70.6
	Extensive	602.0	876.0	917.0	363.0	533.5	555.5	102.5	66.95	63.1
		sig.	**s.e.d.**		**sig.**	**s.e.d.**		**sig.**	**s.e.d.**	
	M	***	1.51		***	2.58		***	0.43	
	F	***	2.14		ns	2.84		***	0.86	
	C	***	2.06		***	2.34		***	0.71	
	MxF	***	3.80		ns	5.31		ns	1.46	
	MxC	***	2.68		***	4.61		***	1.23	
	FxC	ns	3.61		ns	4.36		***	1.32	
	MxFxC	**	6.95		**	8.48		***	2.48	

[a] Forage cell wall fractions were described by drying (Dr), washing Dr at 20 ^{0}C for 1 h and drying (F 20) or washing Dr at 70 ^{0}C for 1 h and drying (F 70) where drying was described as 40 °C for 48 h.

[b] Grass was harvested at 7, 10, 12 and 16 weeks regrowth, referred to as 1, 2, 3 and 4 stages of maturity (M) respectively.

[c] Forages (F) were ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

The F70 had consistently higher NDF and ADF or lower CP content, either numerically and/or statistically when compared with the F20 fraction for all forages and maturities. When compared with the control the F70 fraction had higher and lower carbohydrate and protein fractions respectively by

56.8, 56 and 36.5 % for NDF, ADF and CP respectively but a large proportion was removed by the 20 ^{0}C wash (48.8, 49.6 and 30.5 % respectively).

A comparison of the maturity x component means suggested that the greatest water soluble fraction (average of F20 and F70) was present in the early stages of maturity (36.4, 63.1, 63.3 and 30.9, 46.8, 45.5 % for CP, NDF, ADF of M1 and M4 respectively). There was little difference between F20 and F70 for all components as the forages matured.

For NDF, the significant harvest x forage interaction ($p< 0.001$) described the susceptibility of the unlignified NDF structure to hydrolysis during ensiling in the earlier stages of maturity. However for M3 and M4 the NDF concentration did not differ between forages. The NDF content of the F20 and F70 was increased by 59, 68, 44 and 50 % when compared with the control for M1 and M4 respectively, while the CP content was decreased by 54, 58, 28 and 34% respectively. This suggested that F70 removed more of the soluble component than F20, but as the forage matured there was little change in the fraction soluble in hot water.

Kinetics of in vitro fermentation

When assessing and comparing *in vitro* gas production profiles, it is important to refer to the VFA concentration and proportions as these parameters can influence the direct and indirect gas production profiles of a system as discussed in Section 1.4.2.2. Other factors may also be involved and are discussed in detail in Chapter 3.

There was a significant effect of forage component on all VFA measured ($p<0.001$, **Table** 2.2.3). Total VFA production decreased with maturity, with a significant increase due to ensiling in M2 ($p<0.05$). Total VFA production was greater for F70 ($p<0.001$) which may reflect the fermentation of structural carbohydrate to VFA in the absence of fermentable nitrogen, as the CP content of F20 was greater (**Table** 2.2.2).

There was a significant three-way interaction for the NGR ratio ($p<0.05$) which reflected a consistently higher NGR for F70 for each forage type, except for E at M4. This was supported mainly by an increase in propionic acid for F20, rather than acetic or butyric acid which may reflect the more fermentable nature of the residue extracted at 20 ^{0}C.

The F70 fraction had a greater proportion of Tiso post F70 fermentation ($p<0.001$) indicating greater protein metabolism during *in vitro* incubation, though the CP content was lower than F20. As the

VFA concentrations are endpoint measurements only, it is difficult to speculate if this protein originates from the substrate fraction incubated, the included protein supplement in the buffer or from cell lysis due to substrate depletion. However inherent variations in the acetate : propionate ratio will result in differences in the proportion of indirect gas produced for F20 and F70 fractions.

The rate of *in vitro* fermentation was not affected by the isolated fraction (**Table** 2.2.4) and the main effects are attributed to the expected alterations in digestion due to maturity and ensiling. The rate can be decreased by lignification (Jung and Deetz, 1993). The rate may also be decreased by the formation of Maillard products (Moore *et al.*, 1994). It can therefore be stated that heating of a prewashed forage to 70 ^{0}C for 1 h to remove soluble proteins, did not cause sufficient alterations in the biochemical composition to alter the rate of structural carbohydrate digestion.

There was a significant three-way interaction for the lag of substrate digestion ($p < 0.05$) which may be attributed to the decrease in gas production for the F20 fraction of E at M3, however the differences between treatments were small. Stefanon *et al.* (1996) also found very small but significant variations in lag time with the *in vitro* gas system and concluded that there was no biological relevance in such small numerical differences.

The extent of fraction degradation is quoted as ml gas/g isolated fraction (estimated extent) or g/g isolated fraction (real extent). The significant three-way interaction of the EE ($p<0.05$) was attributed to the lower extent for F20 in M1 and M2 and the higher extent in M3 and M4 when compared with F70. This effect was not evident in the RE value and may be attributed to the differences observed in VFA proportions, as discussed earlier.

For the real extent, there was a significant M x F interaction ($p<0.01$) which described a greater extent of forage digestion for ensiled forages at M1 and M2 ($p<0.05$), with no difference in extent at M3 and M4. This may reflect the weakening of chemical interactions within the structural fraction during ensiling. The potential hydrolytic and proteolytic benefits on fibre digestion post-ensiling are not seen when the ensiled forage becomes increasingly lignified. The significant M x C interaction ($p<0.001$) reflects a greater extent of digestion for F20 at M1 and M2 ($p<0.05$) but not at M3 and M4. This again may be attributed to the lignification of the structural component due to maturation, with the concurrent reduction in the immediately soluble fraction and structural fraction soluble at 20 ^{0}C.

Table 2.2.3 Volatile fatty acid production for the forage fractions[a] as influenced by maturity and forage type *in vitro*

Maturity [b] (M)	Forage [b] (F)	Component (C)	Total VFA	NGR [c]	% Acetate	% Propionate	% Butyrate	%Total Iso-acids [d]
	Grass	F20	56.7	3.6	68.4	22.7	6.4	1.5
		F70	78.7	4.2	66.7	19.3	6.9	4.3
1	Restrictive	F20	58.2	3.7	68.3	22.5	6.8	1.4
		F70	73.6	4.7	68.8	17.7	7.0	3.9
	Extensive	F20	51.3	3.7	68.7	22.1	6.8	1.5
		F70	70.2	4.2	66.9	19.1	6.7	4.5
	Grass	F20	50.4	3.7	68.6	22.5	6.9	1.3
		F70	66.4	4.2	66.4	19.4	7.0	4.3
2	Restrictive	F20	53.6	3.7	67.8	22.4	6.9	1.7
		F70	79.2	4.1	66.7	19.8	69	3.9
	Extensive	F20	65.0	3.6	69.4	22.4	5.3	1.8
		F70	85.9	4.2	66.7	19.2	7.1	4.2
	Grass	F20	42.5	3.0	66.3	25.8	6.2	1.2
		F70	67.3	3.9	64.8	20.3	7.1	4.7
3	Restrictive	F20	47.8	3.0	66.8	26.0	5.7	1.1
		F70	63.8	3.9	65.0	20.1	6.7	4.9
	Extensive	F20	46.4	2.9	65.9	26.6	5.8	1.2
		F70	65.8	3.8	65.6	20.9	6.5	4.5
	Grass	F20	41.8	3.4	68.5	23.8	5.8	1.5
		F70	62.3	3.8	64.6	20.9	7.0	4.3
4	Restrictive	F20	46.8	3.1	66.6	25.7	5.9	1.6
		F70	67.1	4.2	66.4	19.3	6.9	4.6
	Extensive	F20	43.0	3.6	68.2	22.6	6.8	1.7
		F70	62.7	3.4	64.1	22.1	5.5	4.7

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
M	***	1.64	***	0.08	***	0.26	***	0.26	*	0.16	ns	0.14
F	ns	1.42	ns	0.07	ns	0.23	ns	0.23	ns	0.14	ns	0.13
C	***	1.73	***	0.05	***	0.20	***	0.21	***	0.10	***	0.10
MxF	**	2.84	ns	0.14	ns	0.45	ns	0.45	ns	0.27	ns	0.25
MxC	ns	2.95	*	0.11	*	0.38	**	0.40	ns	0.21	*	0.20
FxC	ns	2.55	*	0.09	**	0.33	*	0.34	ns	0.18	ns	0.18
MxFxC	ns	5.11	*	0.19	ns	0.67	*	0.69	***	0.37	ns	0.35

Forage cell wall fractions (C) were described by drying (Dr), washing Dr at 20 °C for 1 h and drying (F 20) or washing Dr at 70 °C or 1 h and drying (F 70) where drying was described as 40 °C for 48 h.

Grass was harvested at 7, 10 12 and 16 weeks regrowth, referred to as 1, 2, 3 and 4 stages of maturity (M) respectively. Forages (F) were ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

The non-glucogenic ratio (NGR) was calculated from VFA concentrations such that NGR=[(Acetate + 2 x Butyrate) / Propionate)]

Total iso-acids refers to the sum of the branched VFA = (isobutyric + iso valeric)

Table 2.2.4 The kinetic parameters of *in vitro* digestion of isolated fractions[a] as influenced by maturity and forage type

Maturity [b] (M)	Forage (F) [b]	Component (C)	Rate (/h)	Lag (h)	Extent (ml gas/g C)	Extent (g / g C)
	Grass	F20	0.11	2.4	268	0.75
		F70	0.10	2.0	278	0.74
1	Restrictive	F20	0.12	2.2	280	0.83
		F70	0.12	3.1	287	0.78
	Extensive	F20	0.13	2.7	279	0.84
		F70	0.13	2.9	277	0.79
	Grass	F20	0.09	1.2	269	0.70
		F70	0.09	1.2	271	0.65
2	Restrictive	F20	0.10	2.0	268	0.77
		F70	0.10	1.7	281	0.71
	Extensive	F20	0.09	0.7	269	0.75
		F70	0.09	1.2	280	0.72
	Grass	F20	0.09	0.8	237	0.59
		F70	0.09	1.5	239	0.61
3	Restrictive	F20	0.10	1.5	253	0.63
		F70	0.09	1.5	258	0.63
	Extensive	F20	0.08	1.7	241	0.61
		F70	0.08	1.1	247	0.60
	Grass	F20	0.07	0.3	204	0.50
		F70	0.07	0.2	213	0.54
4	Restrictive	F20	0.07	0.7	221	0.53
		F70	0.07	0.7	225	0.55
	Extensive	F20	0.07	0.8	221	0.54
		F70	0.07	1.2	228	0.53

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
M	**	0.007	ns	0.54	**	8.1	***	0.010
F	**	0.002	ns	0.27	**	2.2	***	0.005
C	ns	0.001	ns	0.10	***	0.8	ns	0.005
MxF	**	0.007	ns	0.70	ns	8.8	**	0.013
MxC	ns	0.007	ns	0.56	ns	8.1	***	0.012
FxC	ns	0.002	ns	0.30	ns	2.4	ns	0.008
MxFxC	ns	0.008	*	0.74	*	9.0	ns	0.017

[a] Forage cell wall fractions (C) were described by drying (Dr), washing Dr at 20 ^{0}C for 1 h and drying (F 20) or washing Dr at 70 ^{0}C for 1 h and drying (F 70) where drying was described as 40 °C for 48 h.

[b] Grass was harvested at 7, 10 12 and 16 weeks regrowth, referred to as 1, 2, 3 and 4 stages of maturity (M) respectively. Forages (F) were ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

2.2.2 Objective

To examine the effect of extraction medium (water and neutral detergent solution) on the *in vitro* cell wall digestion kinetics of perennial ryegrass silages

Materials and methods

Experimental treatments

A perennial ryegrass silage was dried at 40 ^{0}C for 48 h. The dried material was subdivided into three equal parts. From each, the forage fractions Dr, F70 and NDF were prepared as described where the material was chopped to 1cm length (**Dr**). The F70 fraction was prepared as described previously. The NDF of the DM fractions was extracted based on the procedure of Schofield and Pell (1995) where 150 g DM was autoclaved for 1 h at 100 ^{0}C with 6250 ml neutral detergent solution (**Table** 2.2.1). Post autoclaving, the NDF residue was filtered through a 45 μm mesh and washed with hot water. The residue was then washed with ethanol and acetone (1 litre of each) before soaking in 3 litres 1M $(NH_4)_2SO_4$ overnight at 39 ^{0}C to remove trace elements of ionically bound detergent. The filtration and wash was then repeated and the residue dried at 40 ^{0}C for 48 h (**NDF**).

In vitro technique

Modified Tilley and Terry (Section 1.4.2.1)

Inoculum preparation

As described in Section 2.1.

In vitro procedure

As described for dried substrates (Section 2.1). Culture tubes were horizontally incubated. The DM, F70 and NDF fractions of each forage were incubated. Cultures from each treatment were sampled in triplicate (one from each substrate) 11 times over 96 h.

Chemical composition

As described in Section 2.2.1

Statistical analysis

Data were analysed using the statistical package Genstat 5 (Lawes Agricultural Trust, 1990) and the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985). Data pertaining to forage chemical composition was analysed using a single factor completely randomised analysis of variance. The effect of time on NDF disappearance was analysed using a model appropriate to a split-

plot where component was in the main plot and time in the sub-plot. The kinetic data of the Gompertz equation were analysed using a single factor completely randomised analysis of variance. Within significant interactions means were compared using the LSD test (Steel and Torrie, 1960).

Results and discussion

Chemical composition

The chemical compositions of the perennial ryegrass silage is shown in **Table** 2.2.5. Extraction of the NDF fraction with neutral detergent solution decreased the DMD when compared to the Dr and F70 ($p<0.001$). Morrison (1988) found that the NDF extraction increased the digestibility of barley straw in the first stage of the Tilley and Terry estimate when compared to a washed residue. The neutral detergent solution was thought to primarily attack acetic and phenolic acid residues increasing the digestibility of a substrate more highly lignified than perennial ryegrass, by removing chemical and stearic hindrances. The Tilley and Terry (1963) estimation of *in vitro* DMD relies on two stages, the first is an *in vitro* microbial digestion with rumen inoculum and the second is an acid/pepsin hydrolytic step.

Table 2.2.5 Chemical composition of forage fractions

	Component [a]				
	Dr	**F70**	**NDF**	**sig.**	**s.e.d.**
Composition of dry matter (g/kg DM)					
Dry matter digestibility	754.0	643.0	480.0	***	28.30
Crude protein	162.0	82.5	97.9	***	0.67
Neutral detergent fibre	504.5	864.0	885.0	***	3.02
Acid detergent fibre	298.0	505.7	514.3	***	4.44
Ash	82.1	33.7	37.2	**	10.21

[a] Forage cell wall fractions (C) were described by drying (Dr), washing Dr at 70 °C for 1 h and drying (F 70) or extracted with neutral detergent fibre solution (NDF), where drying was described as 40 °C for 48 h.

The low estimates of DMD for the NDF component are more likely due to the formation of insoluble Maillard reaction complexes during the isolation procedure (Kostyukovsky and Marounek, 1995) rather than to the incomplete removal of the detergent which can interfere with rumen microbial activity (see Pell and Schofield, 1995). The proportion of ethanol and acetone used to rinse the recovered detergent residues was less than that of Pell and Schofield (1995). However other authors (Blummel and Becker, 1997) have omitted ammonium sulphate and the ethanol/acetone steps, opting to rinse thoroughly with hot water, and reported no negative effects on digestion.

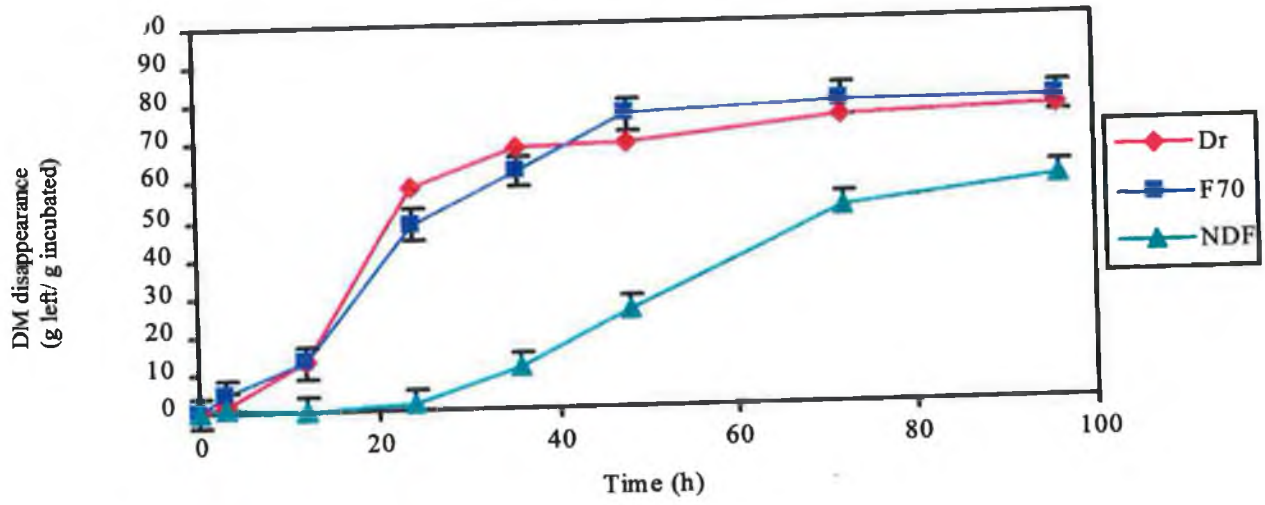
Detergent solution extraction increased the NDF content ($p<0.001$) when compared with F70 and Dr. The ADF content was also increased with detergent extraction ($p<0.001$). When compared with the Dr fraction, extraction method decreased the ash content of the residue ($p<0.01$) but there was no effect of extraction procedure on the ash content.

The NDF content of the detergent extract when estimated in routine laboratory analysis was less than 100 % . This may be an artefact of the procedure used. When forage fractions were isolated the particle size was 1 cm but in routine laboratory analysis the DM is milled to 2 mm, before analysis. It is possible therefore that extraction procedures can be influenced by sample preparation and reducing particle size will improve the efficiency of extraction.

In vitro digestion kinetics

The digestion profiles of the neutral detergent component determined using the procedure of Goering and Van Soest (1970) of all incubated fractions are shown in **Figure** 2.2.1. The digestion curves were parallel which Doane *et al.* (1997a, 1997b) suggested was representative of the non-interactive nature of isolation procedure and biochemical structure of the isolate. However differences between profile time points were significant.

Figure 2.2.1 Apparent dry matter disappearance over time, for cell wall fractions described by drying at 40 ^{0}C for 48 h (Dr), washing Dr at 70 ^{0}C for 1 h and drying (F 70) or extraction using neutral detergent fibre solution and drying (NDF).



The rate of degradation was not affected by any extraction procedure but the lag was increased ($p<0.001$) and the extent decreased ($p<0.001$) by NDF isolation (**Table** 2.2.7). This would suggest alterations in the structural component. The F70 fraction was not different from that of the original Dr description.

Table 2.2.6 Kinetic parameters for *in vitro* digestion of forage fractions

	Component [a]			
	Dr	**F70**	**NDF**	**s.e.d.**
Lag (h)	10.0[a]	10.3[a]	38.2[b]	2.52
Rate (/h)	0.10	0.06	0.07	0.018
Extent (g/100 g incubated)	74.2[a]	78.6[a]	59.2[b]	4.92

[a] Fractions (C) described by drying at 40 °C for 48 h (Dr), washing Dr at 70 °C for 1 h and drying (F 70) or fibre extraction using neutral detergent fibre solution and drying (NDF).
Note: Within rows means with a common subscript do not differ significantly ($p<0.05$)

The negative effect on the *in vitro* digestion of the NDF isolate may not be attributed to residual detergent residues as discussed earlier. Ensiled products due to plant and microbial proteolytic activities have a high residual concentration of soluble organic and inorganic nitrogen sources (McDonald *et al.,* 1991). The F70 isolation method, unlike the NDF isolation technique, removed all soluble protein sources before increasing the extraction temperature. The severe negative effect of NDF extraction on the subsequent *in vitro* digestion may reflect the formation of maillard products.

In vitro gas studies have found the specific rate of the fractionated NDF component to be higher than the unfractionated DM (Pell and Schofield, 1995, Kennedy *et al.,* 1999). Morrison (1988) found a greater *in vitro* digestibility for the NDF isolate, while Doane *et al.* (1997a, 1997b) found similar extents of digestion between fractions. Disparities between these findings and data presented here may be attributed to differences in the biochemical structure of the experimental materials. In some studies (Pell and Schofield, 1995, Kennedy *et al.,* 1999) forages were in a very late stage of maturity, with subsequent low digestibility. In these situations, as with the work of Morrison (1988), the chemical treatment may have improved the digestibility of the lignified complexes. Kennedy *et al.* (1999) stated that the beneficial effects of extraction on cell wall digestibility were not found for legume forages, whose digestibility is not severely restricted by lignin deposition.

2.2.3 Objective

To compare the *in vitro* digestion kinetics of the aqueous extracted CW material of perennial ryegrass silage with those estimated by the NDF content of the residues.

Materials and methods

Ensiling treatments

A perennial ryegrass sward (n=3) was harvested and fresh herbage precision chopped, pooled and ensiled for 8 weeks in mini-silos (n=6, O'Kiely and Wilson, 1991) using restrictive (5 ml formic acid /kg fresh weight, 85 % formic acid) or extensive (15 g sucrose/kg fresh weight) ensiling conditions. All herbages were sampled for chemical analysis.

Sample preparation

Forages were dried at 40 ^{0}C, chopped to 1 cm and the F70 component prepared as previously described (**F70**). Post *in vitro* incubation the residues were recovered, weighed and the NDF residue at each time point was measured.

In vitro technique

The modified Tilley and Terry (Section 1.4.2.1)

Inoculum preparation

As previously described in Section 2.1.

In vitro procedure

As described in Section 2.1 with the following modifications: *in vitro* cultures were horizontally incubated and sampled 11 times in triplicate over 96 h.

Statistical analysis

Data were analysed using the statistical package Genstat 5 (Lawes Agricultural Trust, 1990) and the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985). Data were analysed using a single factor completely randomised analysis of variance. Within significant interactions means were compared using the LSD test (Steel and Torrie, 1960).

Results and discussion

It is important to determine the differences in the predicted kinetics of substrate digestion when using the recovered F70 fraction or the neutral detergent soluble treated residue post-incubation. This would allow for more accurate comparisons of experimental results between studies utilising different procedures (as in Chapter 3).

When the digestion curves of the F70 residue and the NDF residue were described by the Gompertz model the rate and lag were unaffected by the fraction used (**Table** 2.2.7). The lag increased with ensiling ($p<0.01$). The extent of forage digestion was lower when expressed as an NDF residue ($p<0.001$). This may be attributed to the severity of the NDF extraction procedure, which could possible underestimate the *in vitro* digestion of the intact structural fraction represented by the F70 fraction.

Post-incubation, the difference in sample weight at any time point between the F70 residue and the recovered NDF fraction ranged between 7 – 21 %. An incomplete removal of the WSC by F70 extraction was unlikely. A 7 % variation in the latter stages of fermentation when it may be presumed that the residual substrate was composed of structural carbohydrates would suggest that the NDF extraction removes a fractional component insoluble to water at 70 ^{0}C. This is likely to be ash and/or ether extract, which can be 7-12 % and 9-11 % of forage DM respectively (McDonald *et al.*, 1991).

Table 2.2.7 The effect of forage type and residue component on *in vitro* digestion kinetics

Forage [a] **(F)**	**Residue**[b] **(C)**	**Rate (/h)**	**Lag (h)**	**Extent (g/g F70 or /g NDF)**
Grass	F70	0.11	9.30	0.66
	NDF	0.11	9.60	0.47
Restrictive	F70	0.10	10.90	0.68
	NDF	0.08	7.90	0.48
Extensive	F70	0.11	14.80	0.65
	NDF	0.07	12.20	0.43
	F	ns	**	ns
	C	ns	ns	***
	FxC	ns	ns	ns
	s.e.d.	0.018	1.70	0.031

[a] Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[b] Substrates were prepared by washing dried forages at 70 ^{0}C for 1 h and drying (F 70) or washing with neutral detergent fibre solution and drying (NDF).

Conclusion

Procedures for forage fractionation should be such that the biochemical structure or *in vitro* digestibility of the isolated fraction was not altered. It is concluded from these studies that

- the aqueous extraction of all soluble protein and carbohydrate fractions before heating forage residues to 70 ^{0}C for one hour did not cause any biologically significant alteration in the kinetics of fraction digestion.
- the NDF extraction but not F70 extraction negatively affected the *in vitro* digestion kinetics of perennial ryegrass silage
- the extent of digestion estimated from the incubation of F70 was greater than that estimated from the NDF content of the residues

Implications

As the NDF extraction procedure altered the inherent *in vitro* digestion characteristics of forages in these studies, the F70 fraction was deemed more representative of a the structural component ingested by silage fed ruminants.

2.3 EFFECT OF INOCULUM PRESERVATION ON *IN VITRO* FORAGE APPARENT DRY MATTER DIGESTION

Introduction

Inoculum variation can influence *in vitro* measurements and thus compromise the measurement of any intrinsic parameter. The aim of batch inoculum preservation is to ensure that a sub-sample of inoculum removed from storage does not vary between samplings or ideally from the original inoculum. Much of the exploratory work to assess problems or potentials of microbial preservation methods has been carried out with pure cultures (Lievense *et al.,* 1994, Castro *et al,* 1995, Castro *et al,* 1997, To and Etzel, 1997). Microbial survival during storage is dependent on the strain of the microorganism, growth conditions, age of the culture, nature of the suspending medium and processing conditions (el-Kest and Marth, 1992).

Frozen cultures can suffer cellular injury as the temperature declines due to disruption of the cellular membrane (Moss and Speck, 1963, el-Kest *et al.,* 1991, el- Kest and Marth, 1991), though Johnson and Etzel (1995) found no effect of a freeze storage duration up to 4 weeks when studying *Brevibacterium linens*. The freeze-thaw damage can be reduced or alleviated by controlled reductions in temperature and/or the use of cryoprotectants. To and Etzel (1997) however, found that the addition of glycerol did not improve the survival of *B. linens* after freezing and thawing. Metabolic disruptions of the cell can also be overcome by supplying the microbes with their nutritional requirements during fermentation or in a preincubation step (el-Kest and Marth, 1992).

In a series of experiments, Luchini *et al.* (1996) examined the effect of preservation method on the proteolytic activity of mixed rumen fluid *in vitro*. Freezing was suggested as the optimum preservation method while the pre-incubation of the frozen inoculum in a nutrient medium for 6 h after thawing and before inoculation significantly improved the rate and extent of protein degradation.

Objective

The objective of this study was

- to identify an optimum method of inocula preservation for *in vitro* studies of forage apparent DM digestion

Materials and methods

Inoculum collection

As detailed in Section 2.1

Experimental treatments

All treatments were prepared under anaerobic conditions.

Inocula was used to inoculate culture tubes immediately after preparation (**P1**).

Inocula was frozen in 3 x 200 ml volumes under CO_2 and stored at –20 ^{0}C (**P2**).

Inocula (3 x 200 ml) was centrifuged at 20,000 g (Sorvall RC-5B Superspeed) for 20 min. at 39 ^{0}C. The microbial pellet was reconstituted to 5 % of the original volume with a McDougalls buffer (**Table** 2.3.1). The solution was stirred for 20 min. in an ice bath under CO_2 and subsequently stored at –20 ^{0}C. On the day of inoculation, the suspension was thawed at room temperature and centrifuged. The recovered pellet was washed with 10 ml of preheated McDougalls buffer. After centrifugation the pellet was resuspended to its original volume with preheated McDougalls buffer (**P3**).

Microbial protein pellets were prepared as for P3, but frozen in 50:50 (v/v) solution of glycerol-McDougalls buffer (**Table** 2.3.1, **P4**).

The P3 preparations were thawed at 39 ^{0}C, centrifuged and the pellets reconstituted to the original volume using a defined medium (**Table** 2.3.2). The microbial pellets were pre-incubated under anaerobic conditions at 39 ^{0}C for 6 h after which the suspensions were centrifuged. Any pellet was reconstituted to the original volume with preheated McDougalls buffer. All preparations were pooled and used to inoculate the culture tubes (**P5**).

The P4 preparations were pre-incubated as in P5 (**P6**).

Table 2.3.1 McDougalls buffer (1947)

Chemical	g/l distilled H_20
Sodium hydrogen carbonate	9.8
Di-sodium hydrogen phosphate	9.3
Sodium chloride	0.47
Potassium chloride	0.57
Calcium chloride	0.052
Magnesium chloride	0.13
[b]L-cysteine hydrochloride monohydrate	0.25
[b]Micromineral solution	0.25

[b]These components were added in the stated amount per litre of diluted buffer.

In vitro procedure

As described in Section 2.1 with the following modifications: a dried milled silage (**Table** 2.3.3) was used as substrate. Blanks were prepared in triplicate for each treatment. Cultures for each treatment were sampled in triplicate 9 times over 72 h. All cultures and respective blanks were sampled under anaerobic conditions for VFA analysis at each time point (Ranfft, 1973).

In vitro procedure

As described in Section 2.1 with the following modifications: a dried milled silage (**Table** 2.3.3) was used as substrate. Blanks were prepared in triplicate for each treatment. Cultures for each treatment were sampled in triplicate 9 times over 72 h. All cultures and respective blanks were sampled under anaerobic conditions for VFA analysis at each time point (Ranfft, 1973).

Table 2.3.2 Components of the pre-incubation medium as described by Luchini *et al.* (1996)

Solutions	**(ml/l)**	**Prepared in BMS** [c]
Buffer[a] , macromineral and micromineral solution[b]	739	
Pectin [c]	100	
Soluble carbohydrate	50	g/50 ml
Maltose		0.675
Glucose		0.337
Sucrose		0.337
Starch		2.5
Vitamin	100	mg/l
Thiamine HCL		20
Ca-D-panthotenate		20
Nicotinamide		20
Riboflavin		20
Pyridoxine HCL		20
p-aminobenzoic acid		1
Biotin		0.5
Folic acid		0.125
Vitamin B-12		0.2
Tetrahydrofolic acid		0.125
Volatile fatty acid [d]	10	ml/100 ml
Acetic acid		17
Propionic acid		6
n-butyric acid		4
Iso-butyric acid		1
n-valeric acid		1
Iso-valeric acid		1
DL-α-methyl-butyric acid		1
Hemin [e]	1	
Mercaptoethanol [f]	0.16	

[a] Goering and Van Soest (1970) except that NH_4HCO_3 was replaced by an equimolar amount of $KHCO_3$

[b] As described in **Table** 2.1.2.

[c] Solution contained 2.65 g pectin diluted in 100 ml of heated (70 ^{0}C) buffer-mineral solution (**BMS**) and stirred vigorously for 1 h

[d] pH adjusted to 7 with NaOH

[e] 100 mg was dissolved in a solution of 50 ml of 50 %(v/v) ethanol and 50 ml of 0.05 M NaOH

[f] Added as a reducing agent

Table 2.3.3 Chemical composition of standard milled silage (g/kg dry matter (sd.))

	Standard	
Dry matter digestibility	776.0	(12.02)
Digestible organic matter	714.0	(14.25)
Crude protein	187.3	(0.94)
Ash	83.0	(4.50)
Neutral detergent fibre	450.5	(1.50)
Acid detergent fibre	259.0	(2.00)

Curve fitting

As described in Section 2.2

Statistical analysis

Data were analysed using the General Linear Model Procedure (Proc GLM) of Statistical Analysis Institute (1985) and the statistical package Genstat 5 (Lawes Agricultural Trust, 1990). Data pertaining to the kinetic parameters of the Gompertz equation were analysed using a model appropriate for a single factor randomised design. Data pertaining to VFA were analysed using a model appropriate to a split-plot with preparation method in the main plot and time in the sub-plot. Within significant interactions means were compared using the LSD test (Steel and Torrie, 1960).

Results and discussion

Methods of inoculum preservation, to eliminate variation that could occur with the repeated collection of rumen fluid from silage-fed donor animals, were examined. The main methods of microbial preservation are freeze drying (lyophilisation), spray drying or freezing. Freeze- and spray-dyers are expensive to build and operate and high temperatures with the latter can cause chemical and cellular alterations of the inoculum. In addition the viability of stored inoculum can be dependent on the humidity and storage atmosphere, with evidence that oxidation of the fatty acid content of membrane lipids can occur if these conditions are not optimum (Castro *et al.*, 1995).

There is also evidence to suggest that the controlled freezing of cellular material (maintaining the material at a 'holding temperature' for a certain period of time to optimise dehydration (el-Kest and Marth, 1992) can reduce subsequent intracellular thaw damage by expanding ice crystals. Kisidayova (1996) found no benefit to using a 2-step freezing technique on percentage cell recovery of entodiniomorphid protozoa, indicated by cell motility though it was concluded that all preservation parameters should be specified separately for each protozoan species.

Frozen cultures can suffer cellular injury due to the disruption of the chemical and functional nature of the cellular membrane and dehydration of the cell due to the formation of ice crystals. The cell is also susceptible to osmotic shock on thawing and disruption of protein structures and functions, which are often temperature sensitive (el-Kest and Marth, 1992). However, Luchini *et al* (1996) concluded that freezing, rather than freeze drying of mixed rumen inoculum in the presence of a cyroprotectant gave optimal protein degradation results. The effect of freezing directly **(P2),** freezing a bacterial pellet with and without the presence of a cryoprotectant (**P3** and **P4,** respectively) and the impact of an incubation step pre-inoculation on **P3** and **P4** (**P5** and **P6,** respectively) on the resultant cellulolytic activity of the inoculum were examined.

The kinetics of apparent DM digestion are summarised in **Table** 2.3.4. Method of preservation had no effect on the fractional rate constant. Luchini *et al.* (1996) found that rate of protein digestion post preservation was four to eight times lower than the control. The rate is a mathematical parameter describing the changing shape of the digestion profile and is therefore influenced by incubation duration. In contrast to the present study, the work of Luchini *et al.* (1986) was of short incubation duration (6 h).

Table 2.3.4 The kinetic parameters of apparent dry matter digestion (DM) for each preparation

Treatment of inocula prior to inoculation of culture tubes	**Lag (h)**	**Rate (/h)**	**Extent (g/g DM)**
Fresh	0.00^{a}	0.05	85.9^{a}
Frozen at –20 ^{0}C	2.90^{b}	0.04	82.6^{b}
Microbial pellet reconstituted to 5% volume with McDougalls buffer and frozen at –20 ^{0}C (**P3**)	9.30^{c}	0.07	67.8^{d}
Microbial pellet reconstituted to 5% volume with 50:50 (v/v) glycerol: McDougalls buffer and frozen at –20 ^{0}C (**P4**)	5.20^{b}	0.04	86.6^{a}
P3 was preincubated for 6 h prior to inoculation using a nutrient medium [a]	12.80^{d}	0.05	77.5^{c}
P4 was preincubated for 6 h prior to inoculation using a nutrient medium	4.10^{b}	0.04	87.1^{a}
s.e.d.	1.86	0.005	2.77
sig.	***	ns	***

Note: Within columns means with a common subscript do not differ significantly ($p<0.05$).

[a] Nutrient medium was defined by Luchini *et al.* (1996)

The negative impact of preservation method seen by the latter is obvious in the significant increase in the lag of fermentation in this study ($p<0.001$). All preservation techniques increased the lag of digestion ($p<0.05$). Freezing of the complete inoculum had a shorter lag than freezing in McDougalls buffer with or without a pre-incubation step ($p<0.05$). The lag of P2 was not different when compared with a microbial pellet frozen in the presence of a cryoprotectant, with or without a preincubation step.

Cryoprotectants are low molecular weight compounds that can protect the cells from damage incurred during freezing and/or storage, by decreasing the fraction of electrolytes both inside and outside of the cell. Larger compounds and a complex of undefined substances such as blood, extracts of malt or bacteria can also be used (el-Kest and Marth, 1992). To and Etzel (1997) found that the addition of glycerol did not improve the survival of *B. linens* after freezing and thawing which would suggest that glycerol may not be a universal protectant for mixed rumen microbial populations. The results suggest that rumen liqour may have a cryoprotectant effect.

Pre-incubation did not further reduce the lag of digestion for the inocula stored in the presence of a cryoprotectant. Metabolic disruptions of the cell can be overcome by supplying the microbes with their nutritional requirements during a pre-incubation step (see el-Kest and Marth, 1992) and the benefits of such a procedure have been reported previously (Luchini *et al.,* 1996). This would suggest that freezing in McDougalls buffer alone caused irreversible damage during preservation. Inoculum preserved by freezing was not pre-incubated before inoculation as the rumen liquor is an indigenous nutrient medium.

There was a significant preservation method x time interaction for all measured parameters of VFA production ($p<0.001$, **Table** 2.3.5). The long lag of P5 significantly delayed TVFA production ($p<0.05$) and the presence of a high initial TVFA value for the P2 preparation would suggest a residual fermentation during freezing or during thawing which may be associated with the fermentation of feed in the residual nutrients in the inoculum. Though the pre-incubation step did not improve the lag of apparent DM digestion for inocula preserved with a cryoprotectant there was a significant beneficial effect on TVFA production for P6.

At 96 h, inocula preserved by freezing alone or in the presence of a cryoprotectant, with pre-incubation had similar TVFA concentrations to that produced by enzymatic activity of the fresh inocula. However the high initial TVFA for P2 is noted and would suggest that the P6 fermentation

was most similar to the fresh inocula, assuming that no TVFA production resulted from the preliminary pre-incubation step.

Variations in the NGR appear to be most extreme when TVFA concentrations are low. However, as TVFA production increases over time, the NGR is more dependent on apparent DM digestion and at 72 h there is no difference between any treatment in the NGR.

The extent of digestion for the frozen inoculum was significantly lower than the control and inocula incubated in the presence of a cryoprotectant with or without pre-incubation ($p<0.05$), which may reflect microbial deterioration during storage or selective loss of microbial species. However, the inoculation of each fermenter tube with uncentrifuged inocula will contribute approximately 0.4 g DM/20 ml rumen fluid to the culture (experimental observation). In the absence of any negative effect on lag and rate, when compared with pre-incubated inoculum, this contaminant DM material may have elevated the final 96 h residue weight when compared with treatments incorporating inocula centrifugation and washing. As expected from the previous discussion, freezing of a microbial pellet in McDougalls buffer significantly reduced the extent when compared with all other treatments ($p<0.05$).

It should be noted that the benefits of cryoproptectant inclusion and pre-incubation may have been more evident had the storage period being longer as some authors have noted a significant effect of storage duration (Moss and Speck, 1963, el-Kest and Marth, 1992,) and storage temperature (el-Kest *et al.,* 1991) on subsequent inoculum viability. The storage duration in this study was 14 days.

Table 2.3.5 The effect of inoculum preservation method on total volatile fatty acid concentration (mmol/l) and non-glucogenic ratio during *in vitro* digestion of a milled silage.

Parameter	Preservation (P) [a]	Time (T)								Significance					
		0	9	12	18	24	36	48	72	P		T		PxT	
	C	3.2	38.7	27.8	65.4	65.8	71.1	82.3	82.3	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig
	P2	19.8	28.2	31.7	58.9	53.9	65.7	58.8	72.3						
Total VFA	P3	0.3	27.5	29.4	39.1	50.2	52.1	55.2	52.0						
	P4	0.4	6.8	9.0	18.9	35.3	51.1	64.2	64.3						
	P5	0.4	9.0	7.3	8.2	15.6	13.6	39.8	50.8						
	P6	1.4	23.0	31.0	46.5	59.8	65.6	65.6	80.0	2.84	***	2.76	***	7.01	***
	C	2.4	2.6	2.7	3.7	3.6	3.5	3.5	3.6						
	P2	4.1	2.2	3.7	4.0	4.7	3.0	2.8	2.4						
Non glucogenic ratio (NGR) [b]	P3	4.7	2.1	2.6	3.1	3.1	2.6	2.7	2.6						
	P4	2.2	4.0	4.0	2.5	3.2	2.4	2.6	3.0						
	P5	3.1	2.4	5.1	6.7	3.8	1.7	2.0	2.4						
	P6	4.7	1.7	2.2	2.7	2.7	2.4	2.7	3.0	0.18	*	0.30	***	0.71	***

[a] C= fresh inocula, P2= Inocula frozen at –20 ^{0}C, P3 = the microbial pellet reconstituted to 5 % volume with McDougalls buffer and frozen, P4 = microbial pellet reconstituted to 5 % volume with 50:50 (v/v) glycerol:McDougalls buffer and frozen, P5 = P3 preincubated in a nutrient medium (Luchini *et al.*, 1996) for 6 h prior to inoculation and P6 = P4 preincubated in a nutrient medium (Luchini *et al.*, 1996) for 6 h prior to inoculation.

[b] The non-glucogenic ration (**NGR**) is calculated from volatile fatty acid concentrations such that NGR = [(Acetate +2xButyrate)/Propionate)]

Conclusion

It is concluded that for short-term storage

- inocula preservation by freezing did not affect the rate of apparent DM digestion, imposed a lag on digestion and variably affected the extent of digestion *in vitro*
- the preservation of rumen inocula by freezing in the whole state or in the presence of a cyroprotectant had minimum negative effects on the *in vitro* apparent DM digestion kinetics of a dried milled perennial ryegrass when compared with fresh inocula
- inclusion of a cryoprotectant reduced the lag and increased the extent of *in vitro* apparent DM digestion when compared with inocula frozen in buffer alone
- pre-incubation of inocula did not improve the *in vitro* kinetics of cellulolytic activity for inocula preserved in the presence of a cryoprotectant but significantly improved the rate of TVFA production and final TVFA concentration. Pre-incubation improved the extent and not the lag of inocula preserved in the presence of a buffer.

Implications

Rumen fluid may be preserved by freezing at – 20 ^{0}C or in the presence of a cryoprotectant, with subsequent pre-incubation in a nutrient medium for periods of short duration.

2.4 APPLICATION OF THE *IN SACCO* TECHNIQUE TO *IN VITRO* INCUBATIONS

Introduction

Ruminant diets of perennial ryegrass silage are often supplemented to improve the nutritive value of the basal diet. The influence of non-structural carbohydrate supplementation on fibre digestion *in vitro* has been found to be pH dependent (Pwionka and Firkins, 1993, Pwionka and Firkins, 1996), while Grant and Mertens (1992) concluded that a substrate preference and/or a negative bi-phasic pH effect may inhibit NDF digestion. Supplementation of the basal diet with carbohydrate sources negatively affected the *in vivo* (Rooke *et al.*, 1987, Dawson *et al.*, 1988, Rooke and Armstrong, 1989, Pwionka *et al.*, 1994), and *in vitro* (el-Shazyl *et al.*, 1961, Mertens and Loften, 1980, Pwionka and Firkins 1993) NDF digestion.

As *in vitro* techniques maintain a constant pH, negative influences in these systems may be attributed to a substrate preference during microbial fermentation. Currently *in vitro* methodologies are restricted in that all substrates are pooled within the fermentation tube. Substrate digestion is therefore a composite of all component digestion profiles. Following this it would be advantageous to apply the standard *in sacco* technique (Nocek, 1988, Huntington and Givens, 1995) for use in the modified Tilley and Terry *in vitro* technique (Goering and Van Soest, 1970). This would facilitate the study of individual feed NDF digestion profiles when incubated within a common culture tube.

Objective

The objective of this study was to

- determine if the *in vitro* digestion profile of a milled perennial ryegrass silage was restricted when incubated within nylon bags *in vitro.*

Materials and methods

Experimental treatments

Polyester bags (Ankom Co., New York) of a nominal pore size of 50 ± 15 μm and 100 x 50 mm were used. The sample (mg):surface area (cm^2) ratio was kept constant at 20:1 which is within the suitable range quoted by Nocek (1985). The modified fermentation tubes described in section 2.1 were used. A dried and milled silage (**Table** 2.4.1) was used as the experimental substrate. The experimental treatments assigned were 1 g of substrate incubated in free suspension (**T1**), 1 g of substrate incubated *in sacco* (**T2**), 0.5 g of substrate *in sacco*, incubated in duplicate (**T3**) (post fermentation each bag was

randomly assigned to sub sample (**SS**) **A** or **SS B**, where **T3** = SSA+SSB) and 0.5 g of substrate *in sacco* (**SS C**) and 0.5 g of substrate in free suspension (**SS D**).

Table 2.4.1 Chemical composition of substrate (g/kg milled silage DM)

	(g/kg DM (sd))	
Dry matter digestibility	658.0	(2.83)
Digestible organic matter	654.0	(13.95)
Crude protein	152.7	(2.87)
Ash	72.3	(0.47)
Neutral detergent fibre	576.3	(0.47)
Acid detergent fibre	358.0	(1.70)

Inoculum preparation

As detailed in Section 2.1.

In vitro technique

Modified Tilley and Terry (Section 1.4.2.1)

In vitro method

The experiment was completed in two blocks with all treatments incubated in each block. Experimental methodology for each experimental block was as detailed in Section 2.1 with the following modifications: cultures were sampled in triplicate 11 times over 96 h. The residues of all fermentation tubes were recovered by filtering through a 100 μm, with repeated washing or by washing *in sacco* bags in cold water until run off water was clear. Recovered residues were then dried at 40 ^{0}C over 48 h and weighed.

Statistical analysis

Data were analysed using the General Linear Model Procedure (Proc GLM) of Statistical Analysis Institute (1985). A model appropriate to a split-plot design was used with treatment and block in the main plot and time in the sub-plot.

Results and discussion

When the total substrate was incubated in free suspension (T1) or *in sacco* (T2) or sub-divided into two *in sacco* units within the one culture tube (T3), the digestion profile did not differ over time (**Figure** 2.4.1). The apparent DM disappearance profile of the incubated substrate was not affected by

containment within a nylon bag (SSC) when compared with a concurrent *in vitro* incubation of the substrate in free suspension (SSD, **Figure** 2.4.2). The apparent DM disappearance profile of the incubated substrate was not affected by containment within duplicate nylon bags (**Figure** 2.4.3).

Though the *in vitro* digestion profiles did not differ between any combination, concerns for the use of the *in sacco* procedure *in vivo* should be noted. Substrate digestion may be overestimated due to small particle wash out from the nylon bag post incubation (Huntington and Givens, 1995, Jouany *et al.*, 1998). Microbial population present with the nylon bag can be influenced by pore size (Carro *et al.*, 1995).

Conclusion

It is concluded that the *in vitro* apparent DM disappearance of the substrate was not impaired when incubated in one or two *in sacco* units per culture tube.

Implications

Since the *in sacco* containment of substrate did not affect the *in vitro* digestion profile this method could be used to distinguish between the digestion profiles of individual NDF substrates in an interactive *in vitro* environment.

Figure 2.4.1 Effect of incubation treatment (T1, T2, T3) on dry matter disappearance

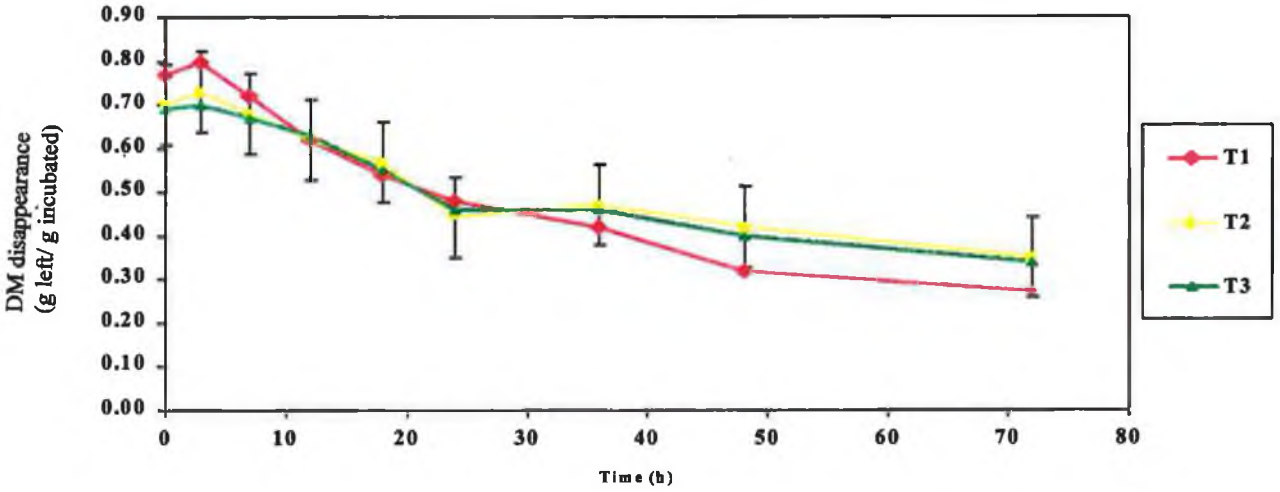


Figure 2.4.2 Effect of incubation treatment (in sacco, free) on dry matter disappearance

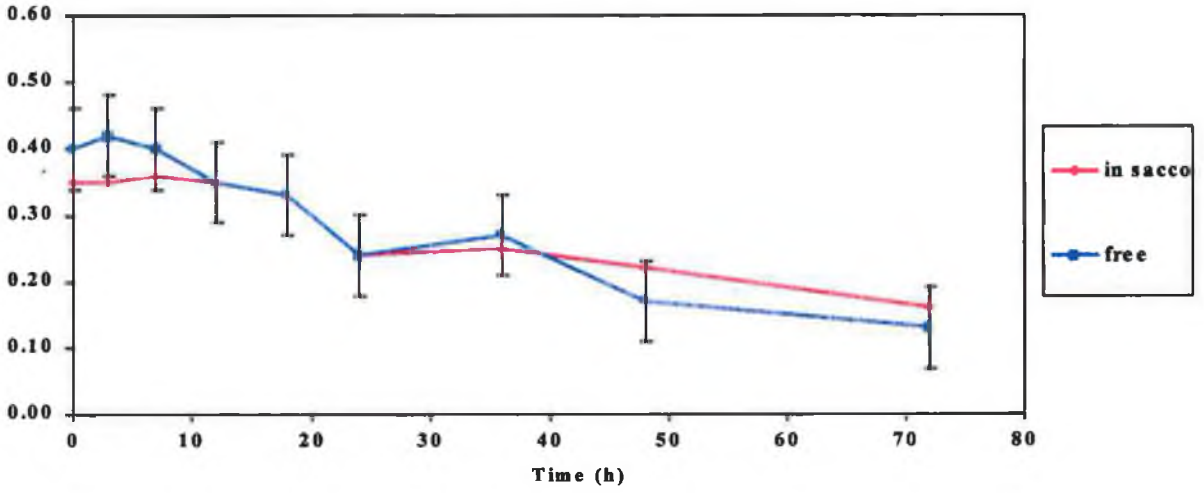
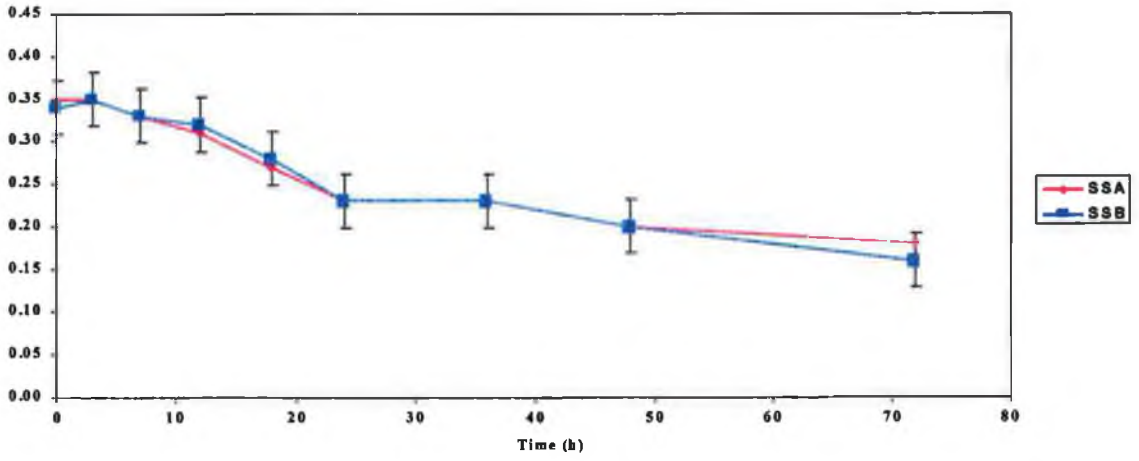


Figure 2.4.3 Effect of incubation treatment (SSA and SSB) on dry matter disappearance



CHAPTER 3

THE EFFECT OF ENSILING ON THE *IN VITRO* DIGESTION OF THE CELL WALL FRACTION FROM LATE SEASON PERENNIAL RYEGRASS

Introduction

The nutritive value of a forage is dependent on the voluntary DM intake and its subsequent nutrient utilisation in the host (Chesson *et al.,* 1990). The biochemical alterations of a forage due to ensiling are dependent on the preservation technique used (McDonald *et al.*, 1991) and minimum alterations in the chemical composition post-ensiling have been positively related to animal production (O'Kiely and Moloney, 1994, Cushnahan *et al.*, 1995a, Keady *et al.*, 1995). *In vivo* studies have also shown that when DM and digestible energy intakes on silage diets are comparable with those of the fresh herbage, production losses can still occur due to ensiling (Keady *et al.*, 1995, Keady and Murphy, 1998).

The fermentation of energy components during ensiling immediately reduces the energy potential of the soluble fraction for rumen microorganisms and this can potentially decrease microbial protein production (Chamberlain, 1987). Proteolytic activity during ensiling will breakdown soluble and structural proteins to peptides, amino acids and ammonia. The importance of ammonia alone or in association with amino acids and peptide sources for optimising cellulolytic digestion has been questioned (Satter and Slyter, 1974, Maeng and Baldwin, 1975, Argle and Baldwin, 1989, Merry *et al.,* 1990, Crutz Soho *et al.,* 1994, Griswold *et al.,* 1995). However, the three main cellulolytic bacteria are generally non-proteolytic in nature, while non-structural and readily degradable structural carbohydrate fermenting microbes have a requirement for peptide and amino acid nitrogen (Baldwin and Allison, 1983). Deficiencies in appropriate nitrogen sources can impair ruminal fermentation profiles.

It is possible that the biochemical alterations in the soluble fraction may influence rumen fibre digestion, which is important as DM intake is influenced by the fibre content of the diet (Steen *et al.*, 1998) and rate of fibre digestion (Gill *et al.*, 1969, Mertens and Ely, 1979). Microbial enzymatic activities are sensitive to environmental conditions many of which are mediated through the liquid phase i.e. pH (Russell *et al.,* 1979, Grant and Mertens, 1992, Grant and Weidner, 1992), soluble nitrogen and energy sources (Baldwin and Allison, 1983, Jung and Varel, 1988, Hoover and Stokes, 1991, Dore *et al.,* 1991), soluble organic acid concentration (Gorosito *et al.*, 1985, Jaakola and Huhtanen, 1992) and osmolarity (Peters *et al.,* 1989,

Carter and Grovum, 1990). The water-soluble fraction of a pre- and post-ensiled perennial ryegrass forage may differentially influence the rumen environment due to the different concentration and nature of soluble organic acids and protein fractions.

In vitro techniques allow the digestion of structural carbohydrates to be described when incubated in the presence or absence of the water-soluble fraction (Section 2.2). Using these techniques it is possible to determine if ensiling negatively affects the intrinsic rate of structural carbohydrate digestion in the rumen and to separate this effect into biochemical alterations of the structural and soluble components.

The experimental objectives were addressed in three experimental studies which were jointly discussed.

3.1 Objective

To determine the effect of ensiling on the digestion of the fresh and unfractionated perennial ryegrass cell wall fraction, by examining the *in vitro* digestion kinetics of the NDF component of the forages.

Materials and methods

Forage preparation

On the 18 August animals were removed from three perennial ryegrass swards and the excess herbage removed to a stubble height of 4 cm. All swards were cut on the 5 November and the fresh herbage (**G**) was precision chopped, pooled and ensiled for 8 weeks, with restrictive (**R** (5 ml 85 % formic acid/kg fresh grass)) or extensive (**E** (15 g sucrose/kg fresh grass)) preservation conditions imposed. For each treatment 6 mini silos were prepared (O'Kiely and Wilson, 1991).

Inoculum preparation

Rumen inoculum was prepared 1-week prior to the start of the *in vitro* study. On three consecutive days, a total of 9 l of rumen fluid and sufficient solid digesta was sampled pre-feed from three fistulated steers fed grass silage *ad-libitum*. Rumen fluid and digesta were prepared as described in Section 2.1. Once pooled and mixed the inoculum was placed into 500 ml containers under a CO_2 atmosphere and stored at – 20 ^{0}C. On any day of inoculation equal amounts of rumen fluid from any sample day were thawed at 39 ^{0}C, pooled under CO_2 and gently mixed.

In vitro technique

The Modified Tilley and Terry (Section 1.4.2.1)

In vitro method

On the day of harvest or silo opening, fresh or ensiled herbages were sampled for chemical analysis before pooling. After pooling of herbage or silo contents, a representative sample of the mixed forage was chopped to 1 cm using a paper guillotine. The DM of the herbage was estimated using a Sharp R-5A53 microwave. One gram of DM equivalent was weighed into each fermentation tube within 2 h of sampling. During this time all forages were maintained at 4 ^{0}C. Eighty millilitres of buffer and 4 ml reducing solution (**Table** 2.1.2) were then added to each tube under anaerobic conditions. Substrates were incubated under nitrogen-excess (N_e) and nitrogen-limited (N_l) conditions. For nitrogen-limited treatments, the NH_4HCO_3 was replaced with a molar equivalent of $NaHCO_3$ and casein was omitted. A control substrate (**Table** 3.1) was included in each *in vitro* run (G in run 1 and silages in run 2) as a nitrogen-excess treatment to monitor the consistency of inoculum activity.

Table 3.1 Chemical composition of dried milled control silages (g/kg DM (sd.))

	3.1		3.2	
Dry matter digestibility	776.0	(12.02)	658.0	(2.83)
Organic matter digestibility	714.0	(14.25)	654.0	(13.95)
Crude protein	187.3	(0.94)	152.7	(2.87)
Ash	83.3	(4.50)	72.3	(0.47)
Neutral detergent fibre	450.5	(1.50)	576.3	(0.47)
Acid detergent fibre	259.0	(2.0)	358.0	(1.70)

Fermentation tubes were inoculated under anaerobic conditions using a previously calibrated hand-held dispenser and incubated at 39 ^{0}C with agitation of the tubes maintained at 80 revs./min. Cultures were sampled in triplicate 11 times over 96 h. Each culture was sampled for VFA concentration. Blanks included under N_e and N_l restrictions were also sampled under anaerobic conditions at these time points to correct for background VFA production. The residues in all sampled cultures were recovered by filtering and washing contents, using a vacuum pump (Speed AC2, BOC) and filter (100 μm). Recovered residues were then dried at 40 ^{0}C for 48 h in an oven with forced air circulation and weighed. The NDF remaining at each time point was determined as described by Moloney and O'Kiely (1994).

Curve fitting

Curves were fitted to the data as described in Section 2.2

Apparent extent of digestion (AED)

The AED is an estimate of the extent of digestion in the rumen (Singh *et al.*, 1992) where

$AED = P \cdot (e^{-kp.L} \cdot (kd/(kp + kd))$ where

P = potentially digestible fraction (extent), e = a constant, L = lag of digestion, kd = rate of digestion and kp = rate of passage (assumed to be 0.03 /h, Mertens and Ely, 1979).

Chemical analysis

Herbages were characterised with respect to DM (40 ^{0}C for 48 h in an oven with forced air circulation) and lignin (quantified commercially in Analytical Chemistry laboratory, IGER). Dry matter digestibility, NDF, ADF, CP, DOMD and crude ash were analysed as described in Section 2. The water soluble fraction of grasses and silages were characterised with respect to water soluble carbohydrate (WSC, Birch and Mwangelvia, 1974), ammonia (NH_3, Sigma diagnostic method for plasma ammonia, Proc No. 171-UV), lactic acid (Boehringer UV-method for determination of lactic acid in foodstuffs and other materials, Cat No. 139084), VFA/ethanol (Ranfft, 1973) and total soluble nitrogen (Instrument Leco FP-428).

Statistical analysis

Data pertaining to the chemical composition of the herbages were analysed using the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985). Data obtained from the Gompertz equation were analysed using the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985) using a model appropriate to a split-plot design. Forage was in the main-plot and nitrogen supplementation in the sub-plot. Data relevant to the production of VFA were analysed with a model appropriate for a split-split-plot with forage in the main plot, nitrogen supplementation in the first sub-plot and time in the lowest sub-plot. Within significant interactions means were compared using the LSD test (Steel and Torrie, 1960).

Results

Chemical composition of forages

There was no effect of ensiling on *in vitro* DMD or DOMD while ensiling decreased the NDF content of the restrictively preserved forage ($p<0.05$) and increased the ADF content of the extensively preserved forage ($p< 0.05$, **Table** 3.2). Ensiling did not affect the lignin concentration.

The mean CP fraction was 263 g/kg DM. Crude protein ($p<0.05$) and soluble ammonia nitrogen concentrations ($p<0.001$) increased with ensiling with no significant effect on soluble nitrogen. The ADIN was not affected by preservation. The WSC fraction of grass was 57 g/kg DM and was reduced by ensiling ($p<0.001$), with the restrictively preserved forage having a greater residual fraction than extensive preservation ($p<0.05$). When compared with extensive preservation the restricted fermentation had lower levels of lactate ($p<0.05$) and TVFA concentrations ($p<0.001$). Acetic acid was the predominant VFA formed in both preservation systems, accounting for 98-99 % of TVFA. The ethanol concentration was not affected by preservation method.

In vitro control

There was no significant effect of inoculum preservation, on the apparent DM digestion kinetics between runs, of the control substrate (**Table** 3.3).

Neutral detergent fibre digestion and volatile fatty acid production from the fresh unfractionated forage

There was no significant interaction between forage type and nitrogen supplementation on any parameter of *in vitro* digestion (**Table** 3.4). The rate of NDF digestion was not affected by forage type or nitrogen supplementation. The lag of fermentation was increased by ensiling ($p<0.001$). There was no effect of nitrogen supplementation on the lag of forage NDF digestion. Ensiling decreased the extent of digestion ($p<0.01$) with the effect most severe for the restrictively preserved forage. Nitrogen supplementation did not affect the extent of digestion. Independently, ensiling ($p<0.001$) and nitrogen supplementation ($p<0.01$) decreased the AED.

Table 3.2 Chemical composition of fresh and ensiled perennial ryegrass

Component		Forage [a]				
		Grass	Restricted	Extensive	sig.	s.e.d.
Dry matter (DM)	(g/kg)	128.0	134.7	132.7	ns	4.46
Composition of dry matter (g/kg DM)						
Crude protein		257.0	267.3	264.7	*	2.06
Neutral detergent fibre		402.0	388.3	398.7	*	3.45
Acid detergent fibre		220.7	228.0	240.3	*	4.27
Acid detergent insoluble nitrogen		7.7	9.3	9.0	ns	2.19
Lignin		23.0	24.0	27.0	ns	0.047
Ash		158.7	160.0	165.3	ns	2.96
Water soluble carbohydrates		56.5	33.1	21.3	***	2.53
Digestibility (g/kg DM)						
Dry matter		716.0	703.7	705.7	ns	8.05
Organic matter		652.7	650.7	635.0	ns	7.75
Nitrogen fractions						
Total N (TN)	(g/kg DM)	41.1	42.8	42.3	*	0.34
Soluble N	(g/kg TN)	354.2	483.5	511.2	ns	49.30
NH_3-N	(g/kg TN)	1.73	40.3	47.9	***	1.57
Fermentation acids (g/kg DM)						
Total volatile fatty acids (TVFA)		ND	19.9	70.6	***	1.13
Acetate		ND	19.5	69.5	***	1.08
Propionate		ND	UD	1.1		
Butyrate		ND	0.1	0.3	**	0.01
Lactate		3.1	123.2	207.6	***	3.93
Ethanol		ND	33.5	30.4	ns	1.95

[a] Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

ND = not determined

UD = undetectable

Table 3.3 Kinetic parameters for the apparent dry matter (DM) digestion of control silage

Substrate for *in vitro* run	Grass	Silage	sig.	s.e.d.
Lag (h)	12.9	17.4	ns	3.26
Rate (/h)	0.15	0.09	ns	0.055
Extent (g/g DM)	0.76	0.78	ns	0.028

Table 3.4 The effect of forage type and nitrogen supplementation on the neutral detergent fibre digestion of fresh forages *in vitro*

Forage (F) [a]	Nitrogen (N)[b]	Rate (/h)		Lag (h)		Extent (g/g NDF)		AED (g/g NDF)	
Grass	Ne	0.08		5.6		0.93		0.61	
	N_l	0.10		7.3		0.91		0.61	
Restricted	Ne	0.11		18.7		0.83		0.32	
	N_l	0.09		17.7		0.82		0.41	
Extensive	Ne	0.05		18.2		0.90		0.39	
	N_l	0.07		15.6		0.87		0.44	
		sig.	**s.e.d.**	**sig.**	**s.e.d.**	**sig.**	**s.e.d.**	**sig.**	**s.e.d.**
	F	ns	0.028	***	2.68	**	0.021	***	0.024
	N	ns	0.023	ns	2.19	ns	0.017	**	0.024
	FxN	ns	0.040	ns	3.79	ns	0.030	ns	0.038

[a] Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[b] N_l refers to the nitrogen-limited treatment where all nitrogen sources in the buffer were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)

There was a significant three-way interaction for TVFA concentration, NGR, acetate, propionate and total branched fatty acid (**Tiso**) concentrations ($p<0.001$, **Table** 3.5). Total VFA was lower for grass at t=1 ($p<0.05$) and increased over time ($p<0.001$). Between 12-18 h, TVFA production was higher for all nitrogen-supplemented treatments. At 96 h, nitrogen supplementation had increased TVFA production for grass and the restrictively preserved forage but not for the extensive preservation ($p<0.05$). Total VFA concentration was greatest for the ensiled forages.

At t=1 the acetate concentration of grass was lower than restricted and extensively preserved forage but there was no effect of nitrogen supplementation. Nitrogen supplementation increased ($p<0.05$) the acetate concentration after 7, 7 and 18 h for the restricted, extensive and grass forage respectively. At 96 h nitrogen supplementation increased the acetate production for grass and restricted silage but not for the extensive silage. At t=1 the propionate concentration was lower for grass, which was also affected by nitrogen supplementation ($p<0.05$). Nitrogen supplementation differentially influenced fresh and ensiled herbages increasing the propionate concentration for grass at 18 h and decreasing the propionate content of the restricted and extensive forages after 72 and 12 h, respectively.

There was a significant F x N interaction for the butyrate concentration ($p<0.001$) as ensiled forages had a higher butyrate concentration than unsupplemented systems. There was a significant F x T ($p<0.001$) and N x T ($p<0.001$) interaction attributed to a decrease in butyrate concentration for grass at 96 h, and for nitrogen-supplemented systems at 36 and 96 h. At t=1 the Tiso acid concentration was not affected by supplementation or forage type but over time nitrogen supplementation increased the concentration of iso-acids and the effect was significant at t=18 h to the end of fermentation ($p<0.05$).

At t=1 the NGR was significantly affected by nitrogen supplementation and forage type as the NGR of nitrogen-supplemented grass was higher than unsupplemented ($p<0.05$) with the reverse true for the extensive silage ($p<0.05$). The NGR was greatest for the extensively preserved forage ($p<0.05$). Over time nitrogen supplementation increased the NGR of the preserved forages also but did not influence the NGR of grass after 12 h.

Table 3.5 The effect of forage type and nitrogen supplementation on volatile fatty acid production (mmol/l) during the digestion of fresh forages *in vitro*

Mmol /l	Forage [a] (F)	Nitrogen [b] (N)	Time (T)										Significance						
			1	3	7	12	18	24	36	48	72	96		*C2*	C3	*C4*	Tiso	*TVFA*	NGR
Total VFA	Grass	Ne	4.7	7.8	16.7	15.4	36.4	47.9	48.8	60.5	61.9	62.5	**F**	***	***	***	***	***	***
		N_l	8.2	12.1	18.1	18.6	23.8	30.5	41.1	45.0	50.0	50.8	**s.e.d.**	0.58	0.20	0.17	0.12	1.07	0.14
	Restricted	Ne	14.9	20.5	26.5	36.2	51.1	62.8	70.7	76.0	89.5	86.8							
		N_l	15.9	22.7	22.8	25.1	30.7	29.1	39.3	46.3	70.4	72.4	**N**	***	***	***	***	***	***
	Extensive	Ne	20.1	18.6	28.1	22.7	37.5	49.1	60.6	54.4	70.2	70.2	**s.e.d.**	0.23	0.09	0.08	0.07	0.39	0.10
		N_l	17.6	20.0	23.6	29.9	31.4	26.9	46.7	58.8	56.8	72.9							
													T	***	***	***	***	***	ns
Ethanoic (C2)	Grass	Ne	3.2	5.2	7.9	9.5	20.9	26.3	27.9	33.9	36.2	41.9	**s.e.d.**	1.05	0.41	0.24	0.25	1.87	0.22
		N_l	4.5	7.4	11.7	12.2	15.8	19.3	26.8	28.1	30.4	33.2							
	Restricted	Ne	9.2	13.4	18.2	21.3	27.7	35.0	39.6	45.2	52.0	51.6	**FxN**	***	***	***	***	***	***
		N_l	9.4	15.0	13.6	14.7	18.2	17.2	24.4	28.8	45.6	46.8	**s.e.d.**	0.64	0.23	0.20	0.15	1.18	0.18
	Extensive	Ne	15.6	14.6	19.5	17.0	23.4	28.2	32.9	29.4	40.8	40.9							
		N_l	14.6	16.0	16.7	20.2	20.8	18.4	29.4	34.5	35.5	47.7	**FxT**	***	***	***	***	***	***
													s.e.d.	1.82	0.70	0.43	0.43	3.25	0.38
Propanoic (C3)	Grass	Ne	0.8	1.1	1.8	2.4	5.8	8.3	8.3	11.4	12.6	10.8							
		N_l	2.5	2.9	3.8	3.5	3.8	5.5	8.0	9.5	10.9	10.4	**NxT**	***	ns	***	***	***	***
	Restricted	Ne	3.2	4.7	5.4	6.5	6.1	7.0	7.7	12.0	12.9	11.7	**s.e.d.**	1.43	0.55	0.33	0.34	2.54	0.31
		N_l	3.7	5.4	5.5	5.7	6.6	6.0	8.3	10.3	15.8	15.9							
	Extensive	Ne	2.7	3.0	4.8	2.5	3.7	4.6	6.8	7.7	11.1	16.7	**FxNxT**	***	***	ns	***	**	***
		N_l	1.9	3.7	3.5	5.0	6.0	4.7	10.7	12.9	13.2	15.6	**s.e.d.**	2.52	0.97	0.59	0.60	4.50	0.54
Butyric (C4)	Grass	Ne	0.5	0.9	1.7	2.0	5.3	6.4	5.2	6.4	6.2	2.4							
		N_l	0.5	1.0	0.7	2.2	3.2	4.3	4.8	5.4	5.7	4.5							
	Restricted	Ne	0.7	1.2	1.7	3.4	5.1	6.5	5.7	4.4	6.1	5.8							
		N_l	0.7	1.2	1.9	2.0	2.2	2.2	2.6	3.0	3.5	4.2							
	Extensive	Ne	0.7	0.8	2.1	1.3	3.2	4.9	3.6	3.4	3.9	3.7							
		N_l	0.4	1.0	1.3	1.5	1.9	1.4	2.6	3.0	2.9	3.9							
Total iso (Tiso)[d]	Grass	Ne	0.0	0.1	0.3	0.4	0.2	2.4	3.0	3.4	2.5	2.9							
		N_l	0.1	0.2	0.3	0.2	0.4	0.5	0.6	0.7	0.7	0.7							
	Restricted	Ne	0.8	0.5	0.2	1.2	4.2	5.1	6.6	5.8	7.5	7.0							
		N_l	0.8	0.3	0.5	0.8	1.2	1.2	1.3	1.3	1.7	1.6							
	Extensive	Ne	0.4	0.0	0.2	0.5	2.2	3.9	6.9	5.7	6.1	3.5							
		N_l	0.1	0.4	0.7	1.1	0.9	0.6	1.3	3.2	1.9	2.0							
NGR [c]	Grass	Ne	5.4	6.3	6.3	6.1	5.5	4.8	4.8	4.1	3.8	4.4							
		N_l	2.3	3.2	4.0	4.9	5.9	5.1	4.6	4.1	3.8	4.1							
	Restricted	Ne	3.3	3.4	4.4	4.4	6.2	6.9	6.7	4.8	5.0	5.4							
		N_l	3.0	3.3	3.2	3.3	3.4	3.6	3.6	3.4	3.3	3.5							
	Extensive	Ne	6.4	5.5	5.0	7.7	8.1	8.3	6.0	4.8	4.4	2.3							
		N_l	8.3	5.0	5.5	4.7	4.1	4.6	3.2	3.1	3.1	3.6							

[a]Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.
[b]N_l refers to the nitrogen-limited treatment where all nitrogen sources were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)
[c]Non glucogenic ratio (NGR) = [(Acetate + 2xButyrate)/Propionate)].
[d]Tiso refers to the sum of branched short chain fatty acids = (iso-butyric + iso-valeric acids)

3.2.1 Objective

To determine the effect of ensiling on the apparent digestion of the fractionated perennial ryegrass cell wall, by examining the *in vitro* digestion kinetics of the aqueously extracted component of the forages.

Materials and methods

Forage preparation

Fresh and ensiled forages from Section 3.1 were dried at 40 ^{0}C, chopped to 1cm and the aqueous insoluble fraction prepared (Section 2.2, **F70**).

In vitro technique

The Modified Tilley and Terry (Section 1.4.2.1)

Inoculum preparation

Inoculum was prepared on the morning of the *in vitro* run as described in Section 2.1.

In vitro procedure

One gram of F70 was weighed into fermentation tubes the day prior to inoculation and 80 ml buffer and 4 ml reducing solution (**Table** 2.1.2) were added under anaerobic conditions. Substrates were incubated under nitrogen-excess ($\mathbf{N_e}$) and nitrogen-limited ($\mathbf{N_l}$) conditions 18 h pre-inoculation. Inoculation and incubation conditions were as described in Section 3.1. Treatments were sampled in triplicate 11 times over 96 h. The residues of all cultures were recovered by filtering and dried at 40 ^{0}C over 48 h and weighed.

Curve fitting

Curves were fitted to the data as described in Section 2.2

Statistical analysis

Data pertaining to the chemical composition of the forages were analysed using the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985). Data obtained from the Gompertz equation were analysed with a model appropriate to a split-plot design. Forage was in the main-plot, and nitrogen supplementation in the sub-plot.

Results

There was a significant F x N interaction ($p<0.05$) for the rate of F70 digestion (**Table** 3.6). The rate was higher for the restrictively preserved forage when supplemented with nitrogen but lower for the extensive preservation ($p<0.05$). There was a significant F x N interaction ($p<0.05$) for the lag of F70 digestion as the lag for grass was higher and the lag of extensively preserved forage lower when supplemented with nitrogen ($p<0.05$). There was no effect of forage type or nitrogen supplementation on the extent of digestion. Restrictive preservation increased the AED of F70 digestion ($p<0.001$) when compared with grass and extensively preserved forage, and there was no effect of nitrogen supplementation.

Table 3.6 Effect of forage type and nitrogen supplementation on the apparent digestion of the fractionated cell wall fraction *in vitro*

Forage (F) [a]	Nitrogen (N) [b]	Rate (/h)		Lag (h)		Extent (g/g DM)		AED (g/g NDF)	
Grass	Ne	0.09		10.8		0.72		0.42	
	N_l	0.09		7.3		0.69		0.45	
Restrictive	Ne	0.11		8.6		0.77		0.50	
	N_l	0.08		9.1		0.76		0.47	
Extensive	Ne	0.07		7.4		0.73		0.45	
	N_l	0.10		12.1		0.68		0.40	
		sig.	**s.e.d.**	**sig.**	**s.e.d.**	**sig.**	**s.e.d.**	**sig.**	**s.e.d.**
F		ns	0.010	ns	1.17	ns	0.020	***	0.017
N		ns	0.007	ns	0.96	ns	0.017	ns	0.014
FxN		*	0.013	*	1.66	ns	0.028	ns	0.024

[a]Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.
[b]N_l refers to the nitrogen-limited treatment where all nitrogen sources were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)

3.2.2 Objective

To determine the effect of the water-soluble fraction pre- and post-ensiling on the apparent digestion of the aqueously extracted cell wall fraction of perennial ryegrass pre- and post-ensiling.

Materials and methods

Forage preparation

Fresh grass and silages from the experiment described in Section 4.1 were immediately frozen at – 20 ^{0}C for isolation of the water-soluble fraction (**W**). While frozen the herbage was chopped using a bowl chop (Type MKT 204 Special, Scarbrucken), then thawed at 4 ^{0}C. The WSC fraction was then isolated by compression. Extracted fractions were maintained at < 4 ^{0}C during isolation and subsequently pooled and frozen. The F70 fraction of fresh and ensiled forages from Section 3.1 were prepared as previously described in Section 2.2.

In vitro technique

The Modified Tilley and Terry (Section 1.4.2.1).

Substrate

Three *in vitro* incubations were carried out. In the first run, 1 g of grass F70 was incubated in the presence of the fresh weight equivalent of the grass water-soluble fraction ($\mathbf{W_G}$), the restrictively preserved water-soluble fraction ($\mathbf{W_R}$) or the extensively preserved water-soluble fraction ($\mathbf{W_E}$). In the second run, 1 g of restrictedly preserved F70 was incubated in the presence of the fresh weight equivalent of W_G or W_R. In the third run 1 g of extensively preserved F70 was incubated in the presence of the fresh weight equivalent of W_G or W_E.

Inoculum preparation

Inoculum was prepared on the morning of every *in vitro* run as described in Section 2.1. All inocula were collected within a 21-day period.

In vitro procedure

Fermentation tubes were prepared as described in Section 3.2.1. On the morning of inoculation, the relevant water-soluble fraction was thawed at 4 ^{0}C and added to the fermentation tubes, with the inoculum added in immediate succession before the cultures were incubated. A standard (dried milled silage, **Table** 3.1) was included into each run as a N_e treatment. Sampling of cultures was as described in Section 3.2.1.

Curve fitting

Curves were fitted to the data as described in Section 2.2.

Statistical analysis

Data pertaining to the chemical composition of the forages were analysed using the General linear model Procedure (Proc GLM) of Statistical Analysis Institute (1985). Data obtained from the Gompertz equation were analysed with a model appropriate to a split-plot design. In this model forage and water-soluble supplementation were in the main-plot, and nitrogen supplementation was in the sub-plot.

Chemical analysis

As described in Section 3.1.

Results

In vitro control

There was no significant effect of sample day on apparent DM digestion of the control (**Table** 3.7)

Table 3.7 Kinetic parameters for the apparent dry matter digestion of the control silage.

In vitro **run**	**1**	**2**	**3**	**sig.**	**s.e.d.**
Lag (h)	12.2	12.2	14.2	ns	1.64
Rate (/h)	0.10	0.10	0.10	ns	0.017
Extent (g/g DM)	0.45	0.41	0.47	ns	0.048

- **Restricted preservation**

The rate of digestion was not affected by any treatment (**Table** 3.8). There was a significant F x N interaction ($p<0.05$) for the lag of F70 digestion as the lag of grass was higher and that of the restricted preservation was lower when supplemented with nitrogen ($p<0.05$). Restrictive preservation increased the extent of F70 digestion ($p<0.001$), as did nitrogen ($p<0.05$) and W_G ($p<0.01$) supplementation. There was a significant three-way interaction ($p<0.05$) for AED such that there was a lower AED for grass when supplemented with W_R and nitrogen. Otherwise, ensiling increased the AED ($p<0.01$), nitrogen supplementation decreased the AED ($p<0.05$), and supplementation with W_G increased ($p<0.01$) the AED.

- **Extensive preservation**

There was a significant F x N interaction ($p<0.01$) for the rate of F70 digestion (**Table** 3.9) which reflected a decrease in the rate for the extensively preserved forage and an increase in the

rate of digestion for grass due to nitrogen supplementation. There was a significant three-way interaction for the lag of F70 digestion ($p<0.001$), which described a lower lag of F70 digestion for the extensively preserved forage when supplemented with W_G and with nitrogen. This effect was not evident for the F70 of grass. The lag of grass digestion was higher and the lag of extensively preserved silage was lower when supplemented with nitrogen ($p<0.05$). There was a significant F x W interaction ($p<0.01$) for the extent of F70 digestion. The extent of F70 digestion was lower when supplemented with W_E compared with W_G. There was a significant three-way interaction ($p<0.01$) for the AED such that there was a higher AED for the extensively preserved forage when supplemented with W_G alone or W_G and N. There was also a significant F x N interaction ($p<0.01$) such that the AED of grass and extensively preserved forage was lower and higher respectively when supplemented with nitrogen ($p<0.05$). A significant F x W interaction ($p<0.05$) may be attributed to a higher AED for grass when supplemented with W_G rather than W_E. A significant W x N interaction ($p<0.01$) described a higher AED when forages were supplemented with W_G and N rather than W_E and N.

Table 3.8 The effect of nitrogen and water-soluble fraction (W) supplementation on the digestion of the fractionated cell wall fraction of grass and restrictively preserved silage *in vitro*

Forage (F) [w]	W [x]	Nitrogen [y]	Rate (/h)	Lag (h)	Extent (g/g F70)	AED [z] (g/g F70)
Grass	W_G	Ne	0.12	10.6	0.65	0.41 [b]
	W_G	N_l	0.10	8.6	0.66	0.43 [ab]
	W_R	Ne	0.11	12.4	0.59	0.35 [c]
	W_R	N_l	0.10	9.2	0.64	0.41 [b]
Restrictive	W_G	Ne	0.09	9.4	0.71	0.44 [ab]
	W_G	N_l	0.11	9.8	0.74	0.47 [a]
	W_R	Ne	0.10	9.3	0.67	0.43 [ab]
	W_R	N_l	0.11	12.0	0.70	0.42 [ab]

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
F	ns	0.009	ns	0.79	***	0.010	**	0.008
W	ns	0.009	ns	0.79	**	0.010	**	0.005
N	ns	0.009	ns	0.79	*	0.010	*	0.007
FxW	ns	0.013	ns	1.11	ns	0.016	ns	0.009
FxN	ns	0.013	*	1.11	ns	0.016	ns	0.011
WxN	ns	0.013	ns	1.11	ns	0.016	ns	0.009
FxWxN	ns	0.018	ns	1.57	ns	0.021	*	0.014

[w] Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[x] The WSC fraction was extracted from the respective fresh herbages using a juice extractor and frozen. Supplementation described the re-addition of the WSC component to the fractionated cell wall on a fresh weight basis, immediately prior to inoculation. W_G refers to the grass WSC fraction and W_R refers to the silage WSC fraction

[y] N_l refers to the nitrogen-limited treatment where all buffer nitrogen sources were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)

[z] Means with similar subscripts are not significantly different ($p<0.05$).

Table 3.9 The effect of water-soluble fraction (W) supplementation on the digestion of the fractionated cell wall fraction of perennial ryegrass and extensively preserved silage *in vitro*

Forage (F) [w]	W [x]	Nitrogen [y]	Rate (/h)	Lag (h)	Extent (g/g F70)	AED [z] (g/g F70)
Grass	W_G	Ne	0.12	10.6 [b]	0.65	0.41 [bc]
	W_G	N_l	0.10	8.6 [c]	0.66	0.43 [b]
	W_E	Ne	0.11	11.2 [b]	0.63	0.39 [bc]
	W_E	N_l	0.13	9.8 [bc]	0.61	0.40 [bc]
Extensive	W_G	Ne	0.10	6.2 [d]	0.75	0.51 [a]
	W_G	N_l	0.12	14.8 [a]	0.76	0.43 [b]
	W_E	Ne	0.08	13.7 [a]	0.67	0.36 [c]
	W_E	N_l	0.13	14.6 [a]	0.64	0.37 [c]

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
F	ns	0.007	***	0.68	***	0.009	ns	0.007
W	ns	0.007	***	0.68	***	0.009	**	0.009
N	*	0.007	*	0.68	ns	0.009	ns	0.005
FxW	ns	0.010	ns	0.95	**	0.013	*	0.011
FxN	**	0.010	***	0.95	ns	0.013	**	0.009
WxN	ns	0.010	*	0.95	ns	0.013	**	0.010
FxWxN	ns	0.014	***	1.34	ns	0.017	**	0.013

[w] Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[x] The WSC fraction was extracted from the respective fresh herbages using a juice extractor and frozen. Supplementation described the re-addition of the WSC component to the fractionated cell wall on a fresh weight basis, immediately prior to inoculation. W_G refers to the grass WSC fraction and W_E refers to the silage WSC fraction

[y] N_l refers to the nitrogen-limited treatment where all buffer nitrogen sources were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)

[z] Means with similar subscripts are not significantly different ($p<0.05$).

General Discussion

Methodological considerations

Ensiling conditions were imposed with the aim of inhibiting or promoting the enzymatic breakdown of forage soluble and structural components during preservation. The immediate decrease in forage pH with formic acid addition to grass restricts enzymatic activities, giving a restricted preservation. Leibensperger and Pitt (1988), modelling the effects of sugar addition on ensiling proposed that there was little effect of sugar addition on pH and proteolysis when compared to the untreated herbage, as the time required for pH reduction was too long to prevent extensive proteolysis. Therefore the natural fall in forage pH for the extensive preservation was dependent on microbial enzymatic activities which convert soluble carbohydrates to organic acids (McDonald *et al.,* 1991). Lactate in the soluble pool was indicative of a lactobacillus dominated preservation, which is preferred as lactate can be used by ruminal microbes as a metabolic energy source (McDonald *et al.,* 1991).

The inoculum used in Section 3.1 differed in day of sampling and in method of preparation when compared with that of Section 3.2 and Section 3.3. Freezing of the inoculum can affect microbial enzymatic activity (Section 1.4.4.3 and Section 2.3). Duration of freeze storage can also affect cell viability (el-Kest *et al.,* 1991, el-Kest and Marth, 1992). In this study there was no effect of storage duration on the *in vitro* digestion kinetics of the control silage and it was concluded that storage conditions did not contribute to the extended lag for ensiled NDF preparations.

In section 3.1 and 3.2 fermentation profiles and subsequent curve fittings were described by the NDF and F70 residues, respectively. In section 2 it was concluded that expression of data sets as NDF or F70 disappearance would not affect the rate or lag of digestion but the former may under estimate the extent of digestion.

Forages were incubated *in vitro* in nitrogen-limited and nitrogen-excess conditions. In nitrogen-limited conditions the microbial population was dependent on the nitrogen supplied by the substrate (and rumen fluid) for their metabolic nitrogen requirements. Grant and Mertens (1991) showed the importance of nitrogen supplementation in the Goering and Van Soest buffer (1970) for the optimisation of *in vitro* cellulose digestion. Mertens (1993) states that to measure the true intrinsic digestion profile of structural carbohydrates, no parameter other than the biochemical and physical nature of the substrate should limit its digestion.

Therefore nitrogen was supplemented in excess in this study such that the nitrogen-excess treatment was defined by the Goering and Van Soest buffer (1970) which supplied 54 mg N/ g substrate incubated. Casein acid hydrolysate and urea are included at 39 mg and 15 mg /80 ml buffer respectively, such that the ratio of urea-N to AA-N in the buffer was 0.3.

Chemical composition

A low WSC concentration, reduced lignification of cell wall material and high protein content are characteristics of autumn grass, making it biochemically different from primary regrowth and early season grasses (Beever *et al.*, 1986, Lopez *et al.*, 1991, Givens *et al.*, 1993a, Sporndly and Murphy, 1996). Dry matter and organic matter digestibility values for the fresh herbage in this study are supported by previous findings for autumn grass (Beever *et al.*, 1986, Lopez *et al.*, 1991, French *et al.*, 2000) and silage (Lopez *et al.*, 1991, O'Kiely, 1993). The lignin concentration was low (2.5 % DM) which is similar to an early spring re-growth and typical of late season perennial ryegrass. The CP content for all herbages was high when compared with previous findings (Beever *et al.*, 1986, Lopez *et al.*, 1991) but supported by O'Kiely (1993).

The effect of ensiling on the CP content and the nitrogen fractional proportions of grass is well documented (van Vuuren *et al.*, 1990, Lopez *et al.*, 1991, Cushnahan and Gordon, 1995) with an increase in the nitrogen soluble fraction due to microbial and plant proteolytic activity, and an increase in ammonia content due to microbial deamination activity during the preservation process (McDonald *et al.*, 1991).

Ensiling decreased the NDF content of the restrictively preserved forage when compared with grass reflecting the acid hydrolysis of the NDF structure during preservation (Dewar *et al.*, 1963). The restrictive preservation also decreased the NDF content of the DM when compared with extensively preserved forage, but the latter retained a greater concentration of ADF suggesting that the hydrolysis of the NDF fraction was more severe for the extensive preservation. However the DMD and DOMD of the preserved forages did not vary reflecting the digestible nature of late season ADF (de Visser *et al.*, 1993).

The WSC fraction of the herbage was low (56.5 g/ kg DM) as the yearly mean was estimated at 200 g/ kg DM (McGrath, 1988) but again reflected the late harvest season as French *et al.* (2000) found WSC in autumn perennial ryegrass ranged from 42 to 109 g/ kg DM. These herbages are

characterised by low stem to leaf ratio and high nitrogen content which can decrease the WSC content of perennial ryegrass (van Vuuren *et al.*, 1990, McDonald *et al.,* 1991).

Restrictive preservation resulted in higher WSC retention, and lower lactate and TVFA concentrations when compared with extensive preservation, as previously shown by Cushnahan *et al.* (1995) and O'Kiely (1993). The lactate content of the extensively preserved forage was high (207 g/ kg DM) and indicative of a well preserved extensively fermented forage (McDonald *et al.,* 1991). The ethanol concentration was not significantly different between preservations and high levels have previously been reported (Henderson *et al.*, 1972, O'Kiely, 1993).

As both forages were well preserved (ammonia-N was <5 % of the total-N) the imposed restrictive and extensive preservation methods had influenced the biochemical composition of the forages without adversely influencing forage preservation. These forages were therefore considered suitable models with which to examine the effect of ensiling on the *in vitro* digestion of perennial ryegrass.

Short chain fatty acid production during in vitro digestion of fresh forages

Nitrogen supplementation increased the TVFA of all forages. Griswold *et al.* (1996) compared protein, peptides, amino acids and urea as nitrogen sources in continuous culture and found that the TVFA increased with peptide and AA supplementation when compared with urea, indicating greater OMD.

Romney *et al.* (1998) examined the effect of nitrogen supplementation on the *in vitro* cumulative gas profiles of feeds varying in CP content. Nitrogen supplementation increased gas production with the effect reduced as CP of the basal diet increased (37-201 g/ kg DM). It is unclear if this additional gas production was due to fermentation of the protein or improved digestibility of the basal diets as no reference was made to extent of organic matter fermentation.

The lack of effect of nitrogen supplementation in this study on lag and extent of NDF digestion would suggest that there was a positive effect of supplementation on TVFA production independent of NDF digestion. The increase in TVFA under conditions of excess ammonia and AA nitrogen are therefore attributed to proteolysis of the supplemented nitrogen due to restricted carbohydrate availability. This may also explain the findings of Romney *et al.* (1998).

The effect of nitrogen supplementation during the early hours of fermentation when TVFA concentrations were low, may reflect more the analytical rather than the biological system. The main volume of TVFA production in this study was associated with NDF digestion.

The increase in TVFA production from nitrogen supplementation was supported mainly by iso-acids, butyric acid and acetic acid. Griswold *et al.* (1996) found that peptide supplementation increased the molar proportion of acetate when compared with protein, protein and urea increased propionate when compared with peptides, while the butyrate ratio was unaffected. There was little effect of supplementation on the propionate concentration in this study.

The basal diet will dominate the VFA profile and that used in the latter had a 50:50 ratio of corn starch: oat straw which would support a propionate fermentation (Chamberlain *et al.,* 1983, Newbold *et al.*, 1987, Jaakola and Huhtanen, 1992). The current study examined forage F70 digestion which on fermentation would support an acetate profile, while the increase in the iso-acid content reflects a contribution of the carbon skeletons of AA to microbial metabolism (Baldwin and Allison, 1983).

The NGR, which is a calculated ratio, was very variable in the first 24 h of *in vitro* incubation. This was a period characterised by low TVFA concentrations and influenced by the soluble fraction of the incubated substrates. Increases in the NGR at the start of fermentation can be attributed to numerical but not significant differences in the VFA concentrations.

After 12 h a consistent trend had developed. Nitrogen supplementation increased the NGR reflecting the increase in acetate and butyrate production. In unsupplemented systems there was a trend towards a higher NGR for grass between 12 and 24 h. The NGR of ensiled forages was similar and forages had a mean NGR of 3.6 in the latter stages of fermentation.

In vivo studies have reported TVFA production dominated by propionic fermentation when lactate is digested in the rumen (Chamberlain *et al.,* 1983, Newbold *et al.,* 1987, Cushanhan *et al.*, 1995). The lactate concentration was greatest for the extensively preserved forage, while Syrjala (1972) concluded that the ruminal digestion of soluble sugars supported a butyrate fermentation. The butyrate concentration of grass was greater than restricted and extensively preserved forage after 24 h.

In this study, the overall molar ratios for acetate, propionate and butyrate at 96 h were 67:21:12, 71:23:8 and 73:20:7 for grass, restricted and extensive preservations, respectively. Cushanhan *et al.* (1995) reported molar ratios for acetate, propionate and butyrate of 64:22:11 and 67:20:11 for extensive and restricted preservation, respectively. Beever *et al.* (1991) and Sporndly and Murphy (1996) reported that the molar proportions of VFA in the rumen of dairy cattle grazing autumn grass was 66:22:12.

Direct comparisons between *in vivo* and *in vitro* VFA concentrations and proportions must be made with caution as the molar proportions of VFA *in vivo* are influenced by pH and individual short chain fatty acid absorption rates (Dijkstra, 1994). However the trends obtained in this study were quite similar to previous *in vivo* work.

The effect of ensiling and nitrogen supplementation on in vitro digestion of unfractionated and fractionated cell wall fractions

Though the NDF and F70 data sets are not directly comparable, the adverse influence of ensiling on the *in vitro* kinetics of digestion was not evident for the F70 fractions. The differences may be attributed to the effect the soluble pool on structural carbohydrate digestion *in vitro,* which may be independent of or interactive with, nitrogen supplementation.

Nutrient asynchrony is proposed to adversely affect microbial protein synthesis *in vivo* (Herra-Saldana *et al.,* 1990, Sinclair *et al.,* 1993, Henning *et al.,* 1993, Sinclair *et al.,* 1995). Optimum nutrient requirements *in vitro* have been defined as 20 mg (Henning *et al.*, 1991) to 25 mg (Newbold and Rust, 1992) of readily available N/g readily fermentable carbohydrate, which were supported by Czerkawski (1986).

Based on the date presented in **Table** 3.2, the ratio of (TN-ADIN)/ g DM for all fresh forages did not differ at 33 mg /g OM. If it is assumed that the soluble nitrogen is removed from the F70 fractions, the ratio for grass, restricted and extensively preserved F70 fractions were 19, 12.8 and 11.7 mg N (TN-ADIN-soluble N) /g substrate respectively. The effect of ensiling on structural proteins is seen in the reduced ratio of the F70 fractions of the restricted and extensive forage, which was below the recommended optimum pre-nitrogen supplementation.

There was no effect of ensiling or nitrogen supplementation on the rate of NDF digestion. Lopez *et al.* (1991) found that ensiling of autumn grass increased the *in vivo* rate of NDF digestion, but

in vivo estimations are reflective of the true interactive nature of the rumen environment. The rate of fermentation is controlled by substrate type and biochemical structure (Chesson *et al.,* 1986, Huhtanean and Kahili, 1992), and when lignification of the cell wall is low (Van Soest *et al.*, 1978), the intrinsic rate of NDF digestion which is that measured *in vitro* would not be expected to change.

Supplementation of the F70 with nitrogen did not affect the rate of digestion of grass indicating complementary nitrogen and energy availability within the structural fraction. The rate of digestion for the restricted silage was increased while the rate of the extensively preserved forage was decreased with supplementation. Therefore the proteolytic effect of ensiling can alter the available structural protein pool sufficiently to reduce the rate of microbial digestion. The negative effect of nitrogen supplementation on the rate of digestion for the extensively preserved silage indicates a nitrogen dependent inhibitory effect on microbial digestion, which is discussed later.

Ensiling increased the lag of NDF digestion with no difference between method of preservation, which is supported by Lopez *et al.* (1991). Nitrogen availability was not limiting the lag of digestion. The hydrolytic effect of acid and/or enzymes on the forage hemicellulose concentration is suggested to be an influential factor on the lag of NDF digestion by reducing the rapidly digestible proportion of the cell wall fraction.

This negative effect of ensiling on the lag of digestion was not apparent for the F70 fractions. The importance of NDF hydrolysis for the lag of autumn forage digestion may be questioned due to the potential digestible nature of the late season perennial ryegrass ADF fraction.

When isolated from the soluble component nitrogen became the dominant influence on the lag of F70 digestion as nitrogen supplementation decreased the lag of the extensively preserved forage. The lag of digestion for grass and restricted silage were unaffected. As extensive preservation allows for a greater degree of microbial proteolysis of structural proteins, the beneficial effect of nitrogen supplementation on the lag of F70 digestion would suggest that fibre digestion was restricted by amino acid and/or urea nitrogen availability.

Lopez *et al.* (1991) reported a reduced extent of digestion for ensiled forages and this reduction was evident only for the NDF digestion of the restricted silage in this study. With low degrees of

lignification the intrinsic extent of digestion would not be expected to vary. When isolated from the soluble component there was no effect of ensiling on the extent of F70 digestion.

Possible effects of the water-soluble fraction on the digestion of unfractionated cell wall in vitro

Fibre digestion can be adversely affected *in vitro* due to a deficiency in iso-acids, a negative effect of readily available carbohydrates, reduced pH and/or inhibition due to end-product formation. Based on the VFA analysis for NDF digestion, the concentrations of iso-acids for all forages was not deficient (0.3 mM are necessary for fibre digestion, Gorosito *et al.*, 1985).

Based on the chemical composition of the fresh herbages, the sugar content of the initial herbage was low. The amount of readily fermented carbohydrate present in the W_G, W_R and W_E was 0.15, 0.08 and 0.05 g/ g NDF respectively. The availability of non-structural carbohydrates can negatively affect the kinetics of fibre digestion *in vitro* (Mertens and Loften, 1980, Grant and Mertens, 1992) and *in vivo* (Noziere *et al.*, 1996). *In vitro,* Grant and Mertens (1992) found a negative effect of raw corn starch on alfalfa hay NDF digestion at 33 % inclusion, while Mertens and Loften (1980) concluded that 40 % inclusion of readily fermented carbohydrate negatively affected NDF digestion with the effects linear with greater inclusion rates. *In vivo,* a negative effect of readily fermentable carbohydrate on the NDF digestion is expected at levels higher than 300 g readily fermentable carbohydrate /kg DM inclusion (Noziere *et al.*, 1996). Therefore the WSC levels were not thought to be inhibitory to digestion.

The *in vitro* pH was maintained at 6.8 using the Goering and Van Soest buffer.

Inhibition of cellulolytic digestion by TVFA concentrations ≤100 mM have been reported and it is possible that the molar proportions of VFA present may also be influential (Peters *et al.*, 1989). However as the TVFA concentrations in this study were less than 25 mM at 3 h and less than 100 mM at 96 h it was unlikely that they would have exerted a negative effect on digestion.

Based on calculations using data from **Table** 2.2.1 and **Table** 3.2 the total ammonia nitrogen concentration (forage and buffer) for nitrogen-limited and nitrogen-excess systems at incubation were 0.7, 17 and 20 and 178, 194 and 197 mg ammonia nitrogen/l for grass, restricted and extensively preserved forage respectively. Though the unsupplemented levels are lower than

those recommended by Satter and Slyter (50 mg/l, 1974), nitrogen supplementation did not improve the lag of NDF digestion suggesting that ammonia was not limiting.

The higher levels are within the reported range of required ammonia nitrogen cited by Ricke and Schaefer (17 to 276 mg/l, 1996). They concluded that *S. ruminatium* growth was inhibited at concentrations of 165 mg/l but that optimum concentrations for maximum specific growth and ruminal microbial protein production differ amongst microbial species.

From this it may be deduced that though the levels are within physiological ranges initial concentrations or increases over time may have selectively restricted some microbial species, particularly NSC fermenting species. Though ammonia concentration was not measured *in vitro*, an increase in concentration as the fermentation proceeded may be indirectly deduced from the rapid increase in VFA from the metabolism of AA. This increase may have been quite significant as both forage cell wall digestibility and CP content were high.

The possibility of a negative interactive effect of TVFA and ammonia concentration on cellulose digestion *in vitro* was not discussed in any available literature.

Effect of nitrogen and water-soluble carbohydrate supplementation on digestion of fractionated cell wall fractions in vitro

If *in vitro* fractionation studies are to have merit, two assumptions must be made i.e. that extraction does not interfere with the biochemical composition of the isolated fraction and that the enzymatic activity of the microbial population is not affected. With these assumptions Stefanon *et al.* (1996) concluded that the *in vitro* microbial digestion profiles of forage NDF were influenced by an associative effect between the soluble and structural fractions.

Ensiling can alter the carbohydrate profile and the availability of peptide and amino acid nitrogen. In this study the *in vitro* digestion of the grass F70 was examined in the presence of W_G and the respective ensiled W fractions to determine if ensiling created a soluble fraction which was unfavourable for cell wall digestion.

- **Restricted fermentation**

When grass and restrictively preserved forage were compared, the rate of digestion of the F70 fraction from either forage was not affected by W or N supplementation.

The lag of F70 digestion for grass was increased with nitrogen supplementation irrespective of the soluble component. This may reflect a high ammonia level *in vitro*. The lag of F70 digestion for the restrictively preserved forage, supplemented with W_R was reduced by supplementation with nitrogen to levels similar to supplementation with W_G with or without nitrogen.

The proteolytic destruction of peptide nitrogen during ensiling may have adversely affected the lag of F70 digestion for the restrictively preserved forage. This limitation in nitrogen required for cellulolytic digestion, could alternatively be supplied via the W_G or by supplementation. However no significant effect of supplementation on the rate of digestion would suggest that in the absence of nitrogen supplementation of W_R the extended lag may allow for cell lysis and thus indigenous supply of the required nitrogen source.

Cushnahan *et al.* (1995) found a 20 % decrease in the sugar content of the water soluble fraction, on a DM basis when herbage was frozen and thawed for use during a production study. If this finding was to be applied to this study any beneficial effect of W_G supplementation would be attributed to a nitrogen rather than a carbohydrate supplementary effect. As the ammonia concentration of the W_G was low (0.7 mg/l, Table 3.2) this may suggest that the beneficial effect was AA or peptide in nature.

The extent of F70 digestion was greater for the restrictive preservation than grass, suggesting that the ensiling process predisposes the forage cell wall to more extensive rumen digestion, probably via a weakening of the associative bonds between structural molecules. Supplementation of F70 fractions with W_G increased the extent of digestion, which may be associated with the high CP content of the fresh forage and the rapid degradation of soluble protein (Broderick *et al.*, 1991).

This is apparently contradicted by the finding that nitrogen supplementation decreased the extent of F70 digestion. However the preferential use of soluble peptides/AA, supported by the increase in TVFA production, may decrease the extent of carbohydrate digestion. An inhibitory effect of excess-nitrogen supplementation is also possible.

- **Extensive fermentation**

The effects of supplementation on the *in vitro* digestion of F70 from the extensively preserved forage were more variable. As with the restricted silage, the rate of F70 digestion of the

extensively preserved forage was not affected by W supplementation. The rate of digestion for the extensively preserved forage was decreased with nitrogen supplementation. The extensive fermentation, unlike the restricted, may therefore have encouraged the metabolism of supplemented AA in preference to the structural polysaccharides and/or that the *in vitro* ammonia levels increased sufficiently to restrict the rate of digestion.

The lag of F70 digestion for grass was increased by N supplementation when supplemented with W_G. This effect was not present when supplemented with W_E The lag of digestion for the extensively preserved forage was reduced by nitrogen supplementation, and W_G and nitrogen supplementation. This would suggest that biochemical alterations due to proteolytic activity during the extensive preservation adversely affected the kinetics of digestion.

Whether microbial fibre digestion requires NAN nitrogen, and if this should be AA or peptide in nature has been a matter of some debate. Leedle and Hespell (1983) examined the effect of nitrogen source (urea, AA and protein) on the microbial fermentation of carbohydrate sources (glucose, cellobiose, starch, xylan and pectin) *in vitro* and concluded that 75 % urea nitrogen and 25 % AA-peptide nitrogen were optimum for cellulolytic fermentations, which was supported by Maeng and Baldwin (1975). Crutz Soho *et al.* (1994) found that urea but not AA and peptides, stimulated the growth of cellulolytic microorganisms on a cellulose substrate *in vitro*. Kernick *et al.* (1991, as cited by Griswold *et al.,* 1995) found that the *in vitro* digestibility of maize straw and alkaline treated wheat straw were not affected by peptide replacement of urea. These studies would suggest that when the basal diet is composed of a slowly degradable structural carbohydrate, fibre digestion is not limited when ammonia-nitrogen is available. Benefits of peptide supplementation to urea based diets are seen when the diets are composed of approximately 50 % rapidly degraded carbohydrate (Maeng and Baldwin, 1975, Argyle and Baldwin, 1989, Griswold *et al.,* 1995, Merry *et al.,* 1990) suggesting the improved growth of amylolytic bacteria.

The importance of nitrogen source for the lag of fermentation was not obvious for the extent of digestion but supplementation with W_G did improve the extent. This may suggest that a preferential use of AA did not impair the extent of digestion and/or that the inherent nitrogen content of the structural fraction was adequate for carbohydrate digestion. However the extent of F70 digestion was also increased by supplementation by ensiling, again highlighting the predisposition of CW to digestion post-ensiling.

The effect of supplementation on the in vitro AED of all forage fractions

The AED is an estimate of the apparent extent of digestion in the rumen using the combined effect of all kinetic parameters and an assumed outflow passage rate of solid digesta from the rumen. In this study the optimum AED is considered to be that of the original fresh forage and/or the F70 of grass when supplemented with W_G.

Ensiling decreased the AED of grass NDF digestion by 20 %, which is attributed to the extended lag imposed on NDF digestion. Nitrogen supplementation of the ensiled forages also decreased the AED by 7 % but did not affect grass.

The AED of restricted F70 fraction increased by 5 % when compared with the AED of grass F70, while the extensive preservation did not differ from grass and there was no effect of nitrogen supplementation on any AED. The negative effect of ensiling on the *in vitro* AED of NDF but not F70 highlights the influential interaction between the soluble and the structural fractions during *in vitro* digestion.

A significant three-way interaction was observed for the *in vitro* AED of all F70 fractions when supplemented with the respective W and nitrogen fractions. Nitrogen supplementation decreased the AED of grass supplemented with W_R by 6 %. Supplementation of the restricted F70 fraction with the respective soluble fraction removed the 5 % improvement seen in AED with F70 fractions in isolation, but did not infer the significant restriction on AED seen with the NDF fraction.

For the extensive preservation the mean AED of F70 supplemented with W_E was 5 % lower than the F70 AED of grass supplemented with W_G. Nitrogen supplementation was not influential in these situations. Supplementation with the soluble component again had a negative effect on the AED when compared with the AED of isolated F70 fractions but the adverse effect was not as severe as seen with the NDF fractions.

The AED of the extensive preservation was improved by 6 % when supplemented with W_G and improved by 14 % when supplemented with W_G and nitrogen. A 10 % increase for the AED of the extensively preserved silage F70 fraction under nitrogen and W_G supplemented conditions

would suggest that the NDF fraction of the ensiled forage was more susceptible to digestion than that of the fresh. Nitrogen supplementation had no inhibitory effects on the AED of digestion.

Conclusions

The apparent extent of digestion is a composite estimate of all kinetic parameters describing a digestion profile and their potential influences *in vivo*. Using late season perennial ryegrass it was concluded *in vitro* that

- The AED of the cell wall fraction, prior to isolation from the whole forage, was negatively affected by ensiling and nitrogen supplementation
- The AED of the cell wall fraction after isolation from the whole forage was not negatively affected by ensiling or nitrogen supplementation
- Supplementation of the fractionated fractions post-ensiling with the water-soluble fraction extracted from the herbage pre-ensiling improved the AED of the extensively preserved fractions. A positive interaction between AED and nitrogen supplementation suggested that the dominant negative effect of ensiling was the proteolytic breakdown of forage proteins.
- Nitrogen supplementation may have resulted in inhibitory levels of ammonia nitrogen, indirectly affecting the *in vitro* fibre digestion profiles.

Implications

The forage soluble component can be an important source of peptide and/or amino acid nitrogen requirements for cellulolytic digestion *in vitro.* The availability of nitrogen can be influenced by the preservation method, reflected in the improvement in the AED of extensively fermented silage only. However due to the closed nature of the batch system inhibitory levels of ammonia (and/or VFA) may affect the final digestion profiles reflected in the reduction of the AED of grass when supplemented with W_R and nitrogen. Such issues are best addressed using semi-continuous cultures where the possible negative effect of end-product build-up in batch systems can be removed.

CHAPTER 4

THE EFFECT OF MATURITY AND ENSILING ON THE *IN VITRO* DIGESTION OF THE CELL WALL FRACTION FROM PERENNIAL RYEGRASS

Introduction

Voluntary intake is one of the main factors influencing the nutritive value of a forage in ruminant rations (Steen *et al.*, 1998). Forage intake can be limited by its physical characteristics (Poppi *et al.*, 1981, Van Soest, 1982, Ulyatt *et al.*, 1986, Church, 1988) and it is well established that voluntary intake and subsequent animal production may be impaired as the ingested forage matures (Gordon, 1980, Steen, 1992, Givens *et al.*, 1993a). This negative impact has been associated with physical and biochemical alterations in the structure and proportions of the plant components (Chesson and Forsberg, 1988, Jung and Allen, 1995, Gordon *et al.*, 1995). An increase in the cell wall and lignin concentration of the DM with a concomitant decrease in the soluble carbohydrate and protein components, has been correlated with a decrease in ruminal and total tract digestibility of OM and CP (Van Soest 1982, Bosch *et al.*, 1992a, Sanderson and Weiden, 1989a).

Ensiling can affect the chemical composition of the herbage by converting readily fermentable proteins and carbohydrates to soluble ammonia and a heterogeneous mixture of organic acids (VFA and lactate) and residual sugars (McDonald *et al.*, 1991, Petit and Tremblay, 1992, Cushnahan and Gordon, 1995). A reduction in animal production has been associated with the ensiling of perennial ryegrass (Steen, 1992, Keady and Murphy, 1993). Alterations in the soluble component due to ensiling may be influential on ruminal cellulolytic activity, which can be dictated by pH, rumen turnover rates, microbial populations, end-products of fermentation and substrate availability (Russell and Wallace, 1988, Dore *et al.*, 1991, Hoover and Stokes, 1991, Grant and Mertens, 1992a, Weimer, 1992) and nutrient supply to the host with particular emphasis on microbial protein (Siddons *et al.*, 1982, Chamberlain, 1987, Gill *et al.*, 1989, Chamberlain and Choung, 1995).

The effect of ensiling on the biochemical composition of the forage will be dependent on the ensiling method used, as seen in Chapter 3. This work concluded that the AED of the fractionated cell wall fraction of a late season perennial ryegrass was not adversely affected by ensiling. Improvements in the AED of the ensiled fractionated cell wall post-supplementation suggested that proteolytic activity during ensiling and endproducts of fermentation (organic acids) may be contributing factors to poorer fibre digestion post-ensiling.

As perennial ryegrass matures the WSC and CP concentrations decrease with a subsequent increase in lignified cell wall material (Sanderson and Weiden, 1989b, van Vuuren *et al.* 1991). These alterations can negatively affect rumen digestion (Bosch *et al.*, 1992a, 1994). Though previous work has examined the effect of maturity on ensiled perennial ryegrass digestion *in vivo* (Rinne *et al.*, 1997a, b, Tamminga *et al.*, 1991, Steen, 1992), there is limited information available pertaining to the interactive effects of maturity and ensiling on the ruminal kinetics of unfractionated or fractionated cell wall digestion *in vivo* or *in vitro*.

The experimental objectives were addressed in two experimental studies using nitrogen-excess and nitrogen-limited *in vitro* conditions, and are jointly discussed.

4.1 Objective

To examine the effect of maturity and ensiling on the digestion of the fresh and unfractionated perennial ryegrass cell wall, by examining the *in vitro* digestion kinetics of the NDF component of the forages.

Materials and Methods

Sward management

Three perennial ryegrass swards differing in location were closed on 17 March after previously being grazed for 3 weeks. After closure all herbage was removed to a stubble height of 4 cm and each sward subsequently divided into 4 plots with nitrogen applied to all at 100 kg/Ha. Experimental treatments (**M1**=7, **M2**=10, **M3**=12 and **M4**=16 weeks re-growth) were randomly assigned to plots within each sward.

Sample preparation

On the day of harvest the herbage yield was estimated by cutting 3 plots (1.28 m x 5 m) to a stubble height of 4 cm, using an Agri-mower. A sub-sample was taken to measure morphological composition (leaf, head, stem, dead, weed, clover) of the herbage. Perennial ryegrass (**G**) was mixed, precision chopped and ensiled for 8 weeks in mini-silos where restrictive (**R**, 5 ml 85 % formic acid/ kg fresh grass) or extensive (**E**, 20 g sucrose/kg fresh grass) ensiling conditions were imposed (n=6, O'Kiely and Wilson, 1991). On the day of harvest or silo opening individual swards or mini-silos respectively were sampled for laboratory analysis, after which swards or respective mini silos for each forage were pooled and mixed.

In vitro technique

Modified Tilley and Terry (Section 1.4.2.1) (Goering and Van Soest, 1970)

Inoculum preparation

On five consecutive days 9 litres of rumen fluid and sufficient solid digesta were sampled pre-feed from three fistulated steers fed grass silage *ad libitum*. Sample collection, inoculum preparation and inoculum storage were as described in Section 3.1. On each day of inoculation equal amounts of rumen fluid from each sample day were thawed at 39 ^{0}C, pooled under CO_2 and gently mixed. Fermentation tubes were inoculated under anaerobic conditions using a previously calibrated hand-held dispenser.

In vitro method

Fresh forages were maintained at 4 ^{0}C and a representative sample of the forage chopped to 1 cm using a paper guillotine. The DM concentration of the forage was estimated using a Sharp R-5A53 microwave and 1 g DM equivalent was weighed into each fermentation tubes within 2 h of sampling. Fermentation cultures were prepared as described in Section 3.1 and a standard dried milled silage (**Table** 4.1) was included in each run as a nitrogen excess treatment to check for consistency in inoculum activity. Cultures were sampled in triplicate, 11 times over 96 h. Residues were recovered by filtration and washing and subsequently dried at 40 ^{0}C for 48 h and weighed. The NDF residue remaining at each time point was determined as described by Moloney and O'Kiely (1994).

Table 4.1 Chemical composition of standard milled silage (g/kg dry matter (sd.)

Dry matter digestibility	776.0	(12.02)
Organic matter digestibility	714.0	(14.25)
Crude protein	187.3	(0.94)
Ash	833.0	(4.50)
Neutral detergent fibre	450.5	(1.50)
Acid detergent fibre	259.0	(2.00)

Chemical analysis

Herbage DM were characterised with respect to DMD, DOMD, NDF, ADF, ADIN, CP and Ash, and water-soluble fractions were characterised with respect to NH_3, LA, VFA and TSN as described in Chapter 2.

Curve fitting

As described in Section 2.2

Apparent extent of digestion (AED)

As described in Chapter 3

Statistical analysis

Data were analysed using the statistical package of Genstat 5 (Lawes Agricultural Trust, 1990). Data pertaining to the chemical composition of herbages were analysed using a model appropriate to a split-plot, with harvest date in the main plot and forage type in the sub-plot. Within significant interactions the sums of squares were further separated using orthogonal contrasts into comparisons of linear, quadratic and cubic effects of maturity with reference made to the most appropriate relationship for the data discussed. Data pertaining to the kinetics of *in vitro* digestion were analysed using a model appropriate to a split-split-plot design. A covariate based on the kinetic parameters of the control for any given run was included in the model. The model used had terms for covariate and harvest date in the main plot, and forage type and nitrogen supplementation in the second split- and sub-plot respectively. Within significant interactions, means were compared using the LSD test (Steel and Torrie, 1960).

Results

Chemical composition

As the forage matured the yield increased (**Table** 4.2). The botanical composition altered as the leaf material decreased by 75 % over the harvest period and the head and stem material increased by 32 and 40 % respectively (**Figure** 4.1). Advancing maturity was also evident from the chemical composition of the fresh herbage (**Table** 4.3).

Figure 4.1 Botanical composition of perennial ryegrass harvested at different stages of maturity

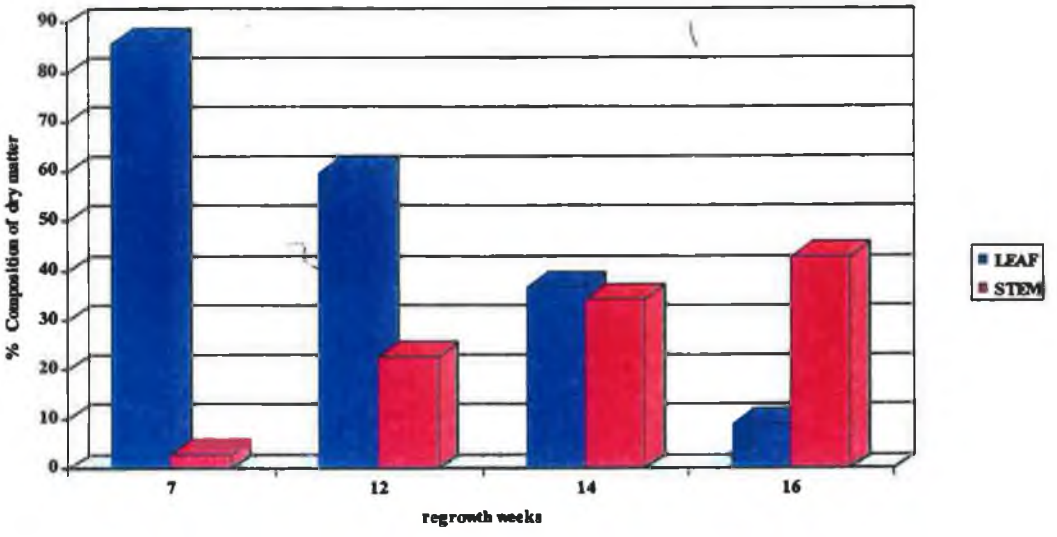


Table 4.2 Yield of herbage dry matter/hectare

Maturity[a]	Yield[b] kg DM/ Ha (sd)
1	4389 (335)
2	6618 (737)
3	9097 (912)
4	11493 (1270)

[a] Maturity refers to regrowth weeks where M1=7, M2=10, M3=12 and M4=16 weeks regrowth
[b]The conversion factor for kg/plot to kg/ha was 1562.

There was a linear increase in forage DM, NDF and ADF ($p<0.001$) from M1 to M4. The ash and WSC concentrations were variable over the harvest period, with the ash concentration greatest at M1 and the WSC concentration greatest at M2. As the cell wall fraction increased with maturity there was a linear decrease in the DMD ($p< 0.001$) and DOMD of the herbage ($p<0.001$). This reflects the linear increase in lignin concentration ($p<0.001$). Crude protein linearly decreased as the perennial ryegrass matured ($p<0.001$), but there was no effect of maturity on ADIN.

Forage preservation significantly altered the composition of the water-soluble fraction. The ammonia ($p<0.001$), TVFA, lactate ($p<0.001$) and ethanol ($p<0.001$) concentrations increased with ensiling when compared with fresh herbages and the WSC decreased ($p<0.01$). Restricted preservation retained more WSC than the extensive preservation, which had a higher concentration of lactate than the restricted preservation.

There was a significant MxF interaction for NDF ($p<0.001$) and ADF ($p<0.001$) concentration as the restricted preservation had a lower NDF and ADF content in M1 and M2, when compared with the extensively preserved forage but higher in later growths ($p<0.05$). Ensiling significantly increased the DMD of the herbage and there was a significant MxF interaction for DOMD, where the increase DOMD of perennial ryegrass in M2 was not reflected in the ensiled forages whose DOMD decreased linearly with maturity ($p<0.001$). There was a significant MxF interaction for lignin concentration ($p<0.01$) as there was no increase in lignin concentration for perennial ryegrass as the forage matured from M2 to M3. The lignin concentration increased at every stage of maturity for the ensiled forages ($p<0.05$).

Table 4.3 The effect of maturity (M) and ensiling (F) on the chemical composition of the fresh herbages (g/kg DM)

Harvest number (M) [a]	1			2			3			4			Significance			
Forage (F) [b]	G	R	E	G	R	E	G	R	E	G	R	E	M	F	[c] MxF	s.e.d
Dry matter (DM) (g/kg)	130.7	161.0	150.0	175.7	172.0	180.3	144.3	158.0	161.7	204.3	202.7	208.3	***	***	***	4.85
Digestibility (g/kg DM)																
Dry matter	792.0	808.3	810.7	759.7	785.7	787.0	692.3	710.0	701.0	565.7	570.7	594.7	***	*	ns	11.50
Organic matter	728.7	735.7	727.0	748.0	717.3	718.3	661.3	657.0	636.0	512.7	550.0	551.3	***	ns	**	13.10
Composition of DM (g/kg)																
Crude protein	182.3	198.3	179.3	163.3	168.7	162.0	111.3	130.0	122.0	101.7	108.7	114.7	***	***	***	2.46
Neutral detergent fibre	492.7	451.0	476.0	547.3	483.3	499.3	578.7	582.7	546.3	635.3	587.3	559.0	***	***	***	8.67
Acid detergent (AD) fibre	258.0	259.3	270.3	288.3	287.0	289.7	335.7	344.7	329.3	371.0	353.3	335.7	***	ns	***	5.36
AD insoluble nitrogen	2.7	4.0	4.3	3.7	3.3	4.0	2.6	3.3	3.7	4.7	4.7	4.0	ns	ns	ns	0.65
Lignin	0.18	0.20	0.19	0.25	0.22	0.26	0.27	0.33	0.31	0.46	0.47	0.46	***	ns	**	0.010
Ash	97.0	94.3	89.3	93.3	89.3	85.3	79.0	81.0	85.7	93.3	100.7	107.0	*	ns	ns	6.81
Water solubleCHO	51.2	31.3	11.3	61.1	48.2	15.2	53.5	33.7	17.3	58.8	19.7	10.1	*	***	*	5.53
Nitrogen fractions																
Total N (TN) (g/kg DM)	29.2	31.7	28.7	26.1	27.0	25.9	17.8	20.8	19.5	16.3	17.4	18.3	***	***	***	0.39
Soluble nitrogen (g/kg TN)	252.6	416.1	605.6	257.6	451.5	537.6	286.5	384.2	561.1	298.5	490.3	441.1	ns	***	*	44.7
NH_3 (g/kg TN)	3.8	20.3	51.0	4.5	39.7	57.3	11.8	31.6	57.7	3.9	54.7	57.4	***	***	***	1.72
Fermentation acids																
Total Volatile fatty acid	ND	9.8	28.8	ND	17.5	32.7	ND	8.8	39.2	ND	11.1	25.0	*	***	***	1.54
Acetate	ND	9.6	28.5	ND	16.9	32.2	ND	8.6	38.5	ND	11.1	25.0	**	***	***	1.36
Propionate	ND	0.17	0.33	ND	0.63	0.47	ND	0.17	0.68	ND	0.0	0.0	ns	**	ns	0.21
Butyrate	ND	UN	UN	ND	UN	UN	ND	UN	UN	ND	UN	UN				
Lactate	ND	67.1	119.2	ND	76.3	131.8	ND	54.2	124.1	ND	64.3	101.3	**	***	***	3.83
Ethanol	ND	48.9	49.5	ND	38.3	45.6	ND	50.4	64.2	ND	49.4	47.6	***	***	***	1.36

ND = not determined, UN = undetectable

[a] Maturity refers to regrowth weeks where M1=7, M2=10, M3=12 and M4=16 weeks regrowth

[b]Grass =G, Restricted preservation = R, Extensive preservation =E where grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[c]All significant F x M interactions were linear ($p<0.001$)

In vitro controls

The consistency of the *in vitro* activity of the preserved inoculum between runs was determined by describing the DM disappearance of a standard milled silage over 96 h. There was a significant effect of run for the lag variable of fermentation (**Table** 4.4). This data was subsequently used as a covariate in further analysis.

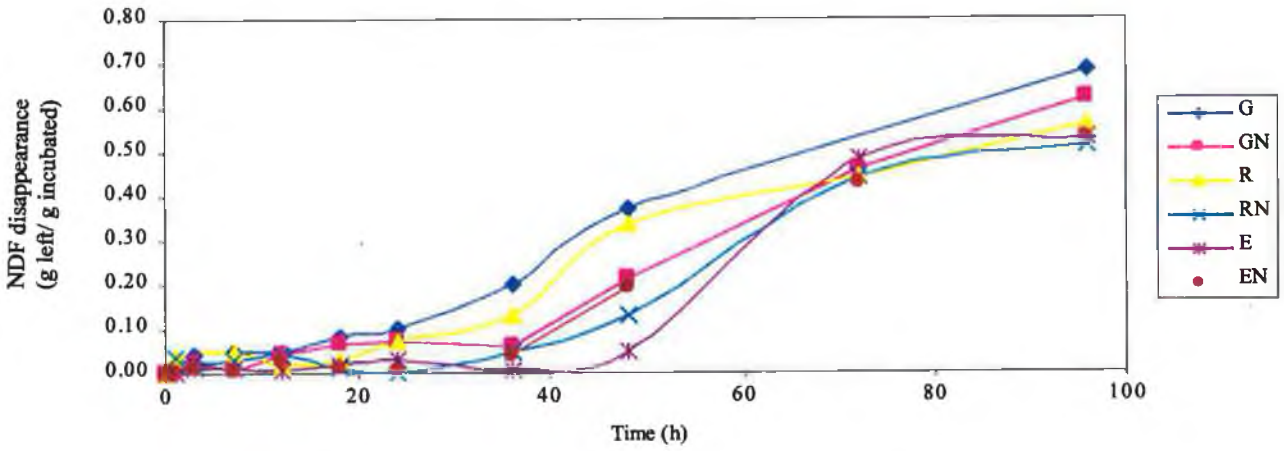
Table 4.4 Kinetic parameters for the apparent digestion of the standard silage over an experimental period of 8 *in vitro* runs

Incubation	1	2	3	4	5	6	7	8	sig	s.e.m
Lag (h)	8.5	10.6	15.4	8.3	19.5	16.7	16.1	18.7	**	2.72
Rate (/h)	0.11	0.13	0.10	0.11	0.10	0.11	0.11	0.12	ns	0.028
Extent (g/ g DM)	0.75	0.77	0.74	0.75	0.77	0.76	0.79	0.77	ns	0.020

Kinetic parameters for the digestion of the unfractionated cell wall fraction of fresh forages in vitro

The Gompertz model gave an unsatisfactory description of the data set for M4 due to an extended lag (appoximately 30 h) in NDF digestion (**Figure** 4.2). M4 was therefore omitted from any statistical analysis dealing with the effect of maturity and ensiling on the kinetic parameters of fermentation.

Figure 4.2 Neutral detergent fibre digestion of perennial ryegrass and silage harvested at a late stage of maturity (16-weeks regrowth) [G = grass, R= restrictedly preserved forage (5ml formic acid/kg fresh wgt.) and E= extensively preserved forage (20 g sucrose/kg fresh wgt.). With and without N (nitrogen) refers to *in vitro* supplementation of same]



There was a significant three-way interaction for the rate of NDF digestion ($p<0.05$, **Table** 4.5). The rate of digestion of perennial ryegrass decreased with maturity ($p<0.05$) and was not affected by nitrogen supplementation. Ensiling decreased the rate of digestion in immature forages (M1, $p<0.05$) but increased the rate in mature forages (M3, $p<0.05$). For ensiled forages, nitrogen supplementation increased the rate of the restricted silage in M1 and M3 and the rate of digestion for the extensively preserved forage in M3 ($p<0.05$).

There was a significant three-way interaction for the lag of NDF digestion ($p<0.01$). The lag of digestion of perennial ryegrass was increased with nitrogen supplementation in M1 ($p<0.05$) and the lag of the restricted and extensively preserved forage were increased with supplementation in M3 ($p<0.05$). The lag of NDF digestion was increased with ensiling ($p<0.001$). Maturity decreased the lag of digestion for perennial ryegrass ($p<0.05$). For ensiled forages, maturity increased the lag for the restricted and extensively preserved forage in nitrogen supplemented systems ($p<0.05$) only.

There was a significant three-way interaction for the extent of NDF digestion ($p<0.05$). The extent of NDF digestion decreased with maturity for all forages ($p<0.05$) though the extent of digestion of perennial ryegrass for M1 and M2 did not differ. Nitrogen supplementation did not affect the extent of digestion of perennial ryegrass but decreased the extent of the restricted preservation in M3 ($p<0.05$), and increased the extent of digestion for the extensively fermented silage in M1 and M2 ($p<0.05$). Ensiling decreased the extent, except for the restricted preservation in M1, where the extent was higher than perennial ryegrass and in M3 where it was similar to grass.

There was a significant three-way interaction for the AED of NDF digestion ($p<0.001$). The AED of all forages decreased with maturity, though perennial ryegrass had a higher AED in M2 ($p<0.05$). The AED decreased with ensiling. The AED of perennial ryegrass was decreased in M1 and M2 with nitrogen supplementation ($p<0.05$) with no effect in M3. Supplementation with nitrogen decreased the AED of restrictively preserved forage in M3 and increased the AED of the extensive preservation in M2.

Table 4.5 The effect of Maturity (M), Forage (F) and Nitrogen supplementation (N) on unfractionated cell wall digestion kinetics *in vitro*

M [a]	F[b]	N[c]	Rate (/h)	Lag (h)	Extent (g/g NDF)	AED [d] (g/ g NDF)
	Grass	Ne	0.11	9.9	0.83	0.51
		N_l	0.12	3.5	0.82	0.60
1	Restrictive	Ne	0.10	16.3	0.84	0.46
		N_l	0.07	11.8	0.88	0.49
	Extensive	Ne	0.06	14.8	0.86	0.41
		N_l	0.11	19.7	0.79	0.40
	Grass	Ne	0.08	0.0	0.80	0.55
		N_l	0.07	1.5	0.84	0.63
2	Restrictive	Ne	0.10	17.7	0.75	0.39
		N_l	0.11	21.1	0.76	0.37
	Extensive	Ne	0.07	11.1	0.79	0.45
		N_l	0.10	14.3	0.68	0.39
	Grass	Ne	0.06	1.3	0.72	0.49
		N_l	0.06	1.1	0.70	0.49
3	Restrictive	Ne	0.11	24.9	0.65	0.28
		N_l	0.08	14.8	0.73	0.38
	Extensive	Ne	0.13	24.0	0.62	0.28
		N_l	0.11	17.9	0.61	0.32

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
M	ns	0.008	*	0.76	***	0.008	***	0.006
F	ns	0.006	***	1.97	***	0.008	***	0.019
N	ns	0.005	**	0.75	ns	0.007	**	0.007
MxF	***	0.012	***	2.31	***	0.014	**	0.022
MxN	ns	0.010	**	1.19	**	0.011	**	0.010
FxN	*	0.009	ns	2.18	***	0.011	**	0.018
MxFxN	*	0.016	**	2.93	*	0.020	**	0.025

[a] Maturity refers to regrowth weeks of M1= 7, M2 =10, M3= 12 and M4= 16 weeks

[b]Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[c]N_l refers to the nitrogen-limited treatment where all nitrogen sources in the buffer were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)

[d]AED = Apparent extent of ruminal digestion assuming a flowrate of 0.03/h

4.2 Objective

To examine the effect of maturity and ensiling on the apparent digestion of the fractionated perennial ryegrass cell wall fraction, by examining the *in vitro* digestion kinetics of the aqueously extracted component of the forages.

Materials and methods

Sample preparation

Grass and silages from each harvest (**Table** 4.1) were dried at 40 ^{0}C and milled through a 2 mm screen (Christy Norris Laboratory Mill). The fractionated cell wall fraction of each forage was prepared using the procedure described in Section 2.2 (**F70**).

In vitro technique

The Gas pressure transducer (Section 1.xxx)

Inoculum preparation

Rumen fluid was sampled and prepared as described in Section 2.1. All inoculum collections were sampled within a 15-day period.

In vitro method

The F70 fraction of all forages from Harvest 1 to 4 were incubated *in vitro* (n=2) and the run was repeated within one week. One gram of substrate was weighed into each serum bottle and 85 ml of buffer and 4 ml reducing solution (**Table** 2.1.2) were added to each under anaerobic conditions. The serum bottles were sealed and incubated at 39 ^{0}C, 18 h prior to inoculation. Substrates were incubated under nitrogen-excess ($\mathbf{N_e}$) and nitrogen-limited ($\mathbf{N_l}$) conditions. Blanks for N_e and N_l treatments were included in triplicate to correct for the fermentation of residual feed in the inoculum. On the morning of inoculation 5 ml of inoculum was added to each bottle using a 10 ml syringe. Gas was released 10 min. after addition and time noted as t=0. Gas volumes were recorded and released, and pressure readings were recorded, such that the headspace pressure did not exceed 7 psi (Theodorou *et al.*, 1994). Serum bottles were inverted after every reading. At the end of an incubation period, all cultures were sampled for pH and VFA analysis and the residues recovered by filtration and washing. Residues were then dried at 40 ^{0}C for 48 h and weighed.

Curve fitting

As described in Section 2.2

Apparent extent of digestion (AED)

As described in Chapter 3

Statistical analysis

Data pertaining to the kinetics of *in vitro* digestion were analysed using a model appropriate to a split-split-plot design with harvest date and run in the main plot, and forage type and nitrogen supplementation in the second split- and sub-plot respectively. Within significant interactions, means were compared using the LSD test (Steel and Torrie, 1960).

Results

Kinetic parameters for the digestion of the fractionated cell wall fraction of forages in vitro

As the gas pressure transducer system was used in this section M4 generated an acceptable profile of substrate digestion for model fitting and was therefore included in the statistical analysis to examine the effect of forage maturity.

There was a significant M x F interaction for the rate of F70 digestion ($p<0.001$). The rate of F70 digestion for perennial ryegrass did not change with maturity but the rate of the restricted and extensive preservations decreased with maturity ($p<0.05$, **Table** 4.6). There was a significant M x N interaction for the rate of F70 digestion ($p<0.01$) as the rate increased with nitrogen supplementation for all harvests except M4 ($p<0.05$).

There was a significant three-way interaction for the lag of F70 digestion ($p<0.05$). Thus as nitrogen supplementation increased the lag of all forages except at M4. There was no effect of ensiling.

The extent is reported as ml gas/g F70 inoculated (**estimated**) and g digested/g F70 (**real**) incubated. The estimated extent was decreased by maturity ($p<0.01$), increased by ensiling ($p<0.01$) and decreased by nitrogen supplementation ($p<0.001$). The real extent was not effected by nitrogen supplementation but was decreased by maturity ($p<0.001$) and increased by ensiling ($p<0.05$).

There was a negative effect of maturity on the AED described by the estimated ($p<0.01$) and real ($p<0.001$) extent. Ensiling increased the estimated AED ($p<0.05$). There was a significant M x F interaction ($p<0.05$) for the real AED which described an increase in the AED for the extensive preservation in M1 and for restricted and extensive preservation in M2. Nitrogen supplementation had no effect on F70 digestion.

Volatile fatty acid concentration at 96 hour.

Total VFA concentration decreased with maturity ($p<0.01$). Nitrogen supplementation increased the TVFA concentration ($p<0.001$) and there was no effect of forage type (**Table** 4.7). Nitrogen supplementation increased the proportion of acetate ($p<0.001$), propionate ($p<0.001$), butyrate ($p<0.01$) and branched chain fatty acids ($p<0.001$). Maturity decreased the proportion of acetate ($p<0.01$), increased the proportion of propionate ($p<0.01$) and had no effect on butyrate or total branched chain VFA. The NGR was decreased by maturity ($p<0.01$) and increased by nitrogen supplementation ($p<0.001$).

General Discussion

Chemical composition

The botanical composition of perennial ryegrass is intended as an indication of the stages of maturity of perennial ryegrass. In the present study as the forage matured the proportion of leaf material decreased and the proportion of head and stem increased. Akin (1989) has shown that the lignin concentration is higher in stem than leaf, which is supported by the linear increase in lignin concentration. The linear decrease in forage digestibility may be attributed to the lignification of the structural cell wall material (Morrison, 1988).

The influence of advancing maturity on perennial ryegrass biochemical composition and forage digestibility are also supported by previous studies (Cherney *et al.*, 1993, Huhtanean and Jaakola, 1994, Rinne *et al.*, 1997a) which similarly reported a decrease in DMD and DOMD with an increase in NDF and ADF proportions. The lack of effect of maturity on the ADIN fraction despite a decrease in the

M	F	N	Rate (/h)	Lag (h)	Extent (ml gas/g F70)	Extent (g / g F70)	AED (ml gas/g F70)	AED (g/g F70)
	Grass	Ne	0.13	4.0	267.3	0.75	232.0	0.57
		N_l	0.07	0.0	289.1	0.75	212.3	0.55
1	Restrictive	Ne	0.16	5.1	279.2	0.76	213.0	0.58
		N_l	0.07	1.1	294.5	0.80	211.9	0.58
	Extensive	Ne	0.17	5.1	263.6	0.80	202.2	0.62
		N_l	0.08	0.7	289.4	0.80	216.6	0.60
	Grass	Ne	0.12	2.5	260.2	0.67	202.0	0.52
		N_l	0.07	0.0	282.1	0.65	203.5	0.47
2	Restrictive	Ne	0.14	3.5	272.0	0.70	211.3	0.55
		N_l	0.06	0.0	289.6	0.75	208.7	0.54
	Extensive	Ne	0.08	2.3	283.0	0.75	211.7	0.56
		N_l	0.10	0.2	277.6	0.71	203.2	0.53
	Grass	Ne	0.10	2.4	229.6	0.61	173.4	0.46
		N_l	0.08	0.5	248.6	0.61	185.1	0.45
3	Restrictive	Ne	0.11	2.9	258.9	0.62	190.6	0.47
		N_l	0.07	0.1	265.4	0.63	196.2	0.47
	Extensive	Ne	0.10	2.2	240.2	0.60	179.0	0.44
		N_l	0.06	0.0	253.0	0.60	181.4	0.43
	Grass	Ne	0.08	0.3	205.5	0.54	153.1	0.40
		N_l	0.06	0.0	221.2	0.53	158.0	0.38
4	Restrictive	Ne	0.08	1.3	217.4	0.55	159.9	0.40
		N_l	0.06	0.0	231.7	0.55	163.5	0.39
	Extensive	Ne	0.08	1.2	225.1	0.53	164.9	0.39
		N_l	0.06	1.3	231.6	0.54	159.2	0.38

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
M	*	0.007	ns	0.49	**	7.13	***	0.009	**	7.65	***	0.006
F	**	0.002	ns	0.25	**	2.44	*	0.008	*	2.36	*	0.007
N	***	0.005	***	0.20	***	3.09	ns	0.009	ns	1.34	ns	0.009
MxF	***	0.007	ns	0.64	ns	8.17	ns	0.016	ns	8.56	*	0.013
MxN	**	0.010	ns	0.56	ns	8.36	ns	0.016	ns	7.88	ns	0.014
FxN	ns	0.007	ns	0.35	ns	4.50	ns	0.014	ns	2.87	ns	0.013
MxFxN	ns	0.015	*	0.80	ns	11.13	ns	0.028	ns	9.17	ns	0.026

[a] Maturity refers to regrowth weeks of M1= 7, M2 =10 and M3= 12 weeks regrowth

[b]Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.

[c]N_l refers to the nitrogen-limited treatment where all nitrogen sources in the buffer were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)

[d]AED = Apparent extent of ruminal digestion assuming a flowrate of 0.03/h

Table 4.7 The effect of Maturity (M), Forage (F) and Nitrogen supplementation (N) on the volatile fatty acid proportions at 96 h post fractionated cell wall digestion *in vitro*

M [a]	F [b]	N [c]	Total concentration (mmol/l)	Acetate	Propionate	Butyrate	Tot-iso [d]	NGR [e]
	Grass	Ne	91.3	64.1	17.8	7.1	7.0	4.4
		N_l	72.6	69.0	22.1	6.6	0.8	3.7
1	Restrictive	Ne	76.8	64.4	17.5	7.1	7.0	4.5
		N_l	76.5	72.6	18.6	6.9	0.7	4.8
	Extensive	Ne	85.1	64.4	17.3	7.1	7.2	4.6
		N_l	61.1	69.6	22.4	6.0	0.7	3.7
	Grass	Ne	80.1	64.3	18.1	7.1	6.7	4.4
		N_l	58.2	68.6	22.2	6.8	0.9	3.7
2	Restrictive	Ne	83.4	63.9	17.8	7.4	7.0	4.5
		N_l	81.3	69.0	22.7	6.4	0.7	3.6
	Extensive	Ne	99.6	63.4	17.9	7.6	7.0	4.4
		N_l	79.4	70.1	21.7	6.5	0.6	3.8
	Grass	Ne	82.5	62.6	18.5	7.4	7.4	4.2
		N_l	57.7	67.3	23.8	6.7	0.9	3.4
3	Restrictive	Ne	82.3	62.5	18.6	6.9	7.5	4.1
		N_l	50.7	68.1	23.5	6.4	0.7	3.5
	Extensive	Ne	78.1	62.6	19.5	6.8	7.3	3.9
		N_l	59.0	68.6	23.6	5.9	0.7	3.5
	Grass	Ne	79.2	60.8	21.6	6.8	6.4	3.5
		N_l	50.6	69.4	21.1	7.1	0.8	4.1
4	Restrictive	Ne	77.0	63.4	16.9	7.7	7.9	4.7
		N_l	62.8	69.4	23.1	5.9	0.6	3.6
	Extensive	Ne	77.7	62.6	18.5	7.1	7.4	4.2
		N_l	53.1	65.5	28.4	3.0	0.5	2.6

	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.	sig.	s.e.d.
M	**	1.11	**	0.18	**	0.18	ns	0.29	ns	0.17	**	0.04
F	ns	4.15	ns	0.48	Ns	0.51	ns	0.34	ns	0.18	ns	0.15
N	***	2.28	***	0.33	***	0.54	**	0.27	***	0.15	***	0.13
MxF	ns	6.87	ns	0.80	Ns	0.85	ns	0.62	ns	0.34	ns	0.24
MxN	ns	3.40	ns	0.50	Ns	.089	ns	0.49	ns	0.27	ns	0.19
FxN	ns	5.00	ns	0.63	Ns	0.83	ns	0.48	ns	0.26	ns	0.22
MxFxN	ns	8.85	ns	1.13	Ns	1.57	ns	0.92	ns	0.50	ns	0.40

[a] Maturity refers to regrowth weeks of M1= 7, M2 =10, M3= 12 and M4= 16 weeks regrowth
[b]Grass was ensiled under restrictive (5 ml formic acid/ kg fresh weight) or extensive (20 g sucrose/kg fresh weight) ensiling conditions.
[c]N_l refers to the nitrogen-limited treatment where all nitrogen sources in the buffer were omitted, N_e refers to the nitrogen-excess treatment where nitrogen was supplemented according to Goering and Van Soest (1970)
[d] Total isoacids (**Tiso**) = iso-butyric + iso-valeric
[e] Non-glucogenic ratio (**NGR**) = [(Acetate + 2xButyrate)/Propionate)]

CP fraction may reflect the conversion of soluble nitrogen into structural NDF-based protein as the plant matures. This supports the negative effect of maturity on the readily available (R) protein:RCHO ratio discussed by van Vuuren *et al.* (1990). The WSC concentration of perennial ryegrass was low in this study when compared with the annual mean of McGrath (mean 20 %, 1988).

Ensiling conditions were imposed with the aim of inhibiting or promoting the enzymatic breakdown of forage soluble and structural components during preservation. The immediate decrease in forage pH with formic acid addition to fresh herbage restricted enzymatic activities. In contrast the natural fall in forage pH for extensive preservation is dependent on microbial enzymatic activities which convert soluble carbohydrates to organic acids (Leibensperger and Pitt, 1988, McDonald *et al.,* 1991). A rapid pH decline (within days) to pH 4.0 with a *Lactobacilli* microbial domination is necessary for a stable preservation and was evident from the high lactate concentrations of E.

All forages were well preserved with a low proportion of ammonia-N when expressed as a percentage of total-N (Byrant and Landcaster, 1970, Harrison, 1994). Ensiling increased the DMD and DOMD of the herbage which is supported by the work of O'Kiely and Moloney (1994). However these measurements did not reflect the negative effects of ensiling on the ruminal digestion of NDF.

Ensiling decreased the NDF content of herbages. The reduction in NDF content ranged from 14-62 g/kg DM ensiled and was not consistently affected by harvest date with the greatest losses at M2 and M4. Despite little alteration in the NDF and ADF content in M3 due to ensiling, the lignin concentration of the ensiled but not the fresh forages increased.

In previous *in vivo* studies, ensiling increased (Lopez *et al.*, 1991), decreased (Cushahan and Gordon, 1995) or had no effect (Cushnahan *et al.*, 1995) on the NDF content of herbages. The restricted preservation had a lower NDF content in M1 and M2 when compared with E, which may be attributed to the acid hydrolysis of the unlignified NDF component in the early harvests (Dewar *et al.,* 1961). This hydrolysis will release polysaccharide sugars into the soluble pool. The restrictive pH increased the

soluble sugars retained in the WSC fraction of restricted when compared with extensive preservation as supported by Rinne *et al.* (1997a, 1997b).

Proteolytic activity during the ensiling process increased the total nitrogen content of the soluble pool, which in well-preserved forages is reflected in a shift from soluble protein to amino acids, trace amounts of other organic nitrogen compounds (amines, nitrates, nitrites e.t.c.) and ammonia. The extent to which the soluble nitrogen pool will increase, and the WSC concentration will decrease, is dictated by preservation method as shown in this study. These effects have previously been reported (van Vuuren *et al.,* 1990, Cushnahan and Gordon, 1995).

The rapid absorption and/or dilution of the soluble ammonia nitrogen source in the rumen, bypassing incorporation into the microbial protein pool is seen to negatively effect the nutritive value of a preserved forage (Henning *et al.* 1993, Chamberlain and Choung, 1995, Van Vuuren *et al,* 1999). Urinary nitrogen losses were greater for extensively preserved perennial ryegrass when compared with perennial ryegrass or restricted preservation (Cushanhan *et al.*, 1995). Ensiling also influences the pattern of VFA production in the rumen (Cushnahan *et al.,* 1995, Keady *et al.*, 1995) and the pH immediately post-feeding (Cushnahan *et al.,* 1995).

Methodology considerations for the NDF digestion of fresh forages in vitro

In Section 2.3 inoculum preservation by freezing was recommended and used in this study to eliminate possible variation in inoculum activity during repeated sampling over a 3-month period. The negative effect of freezing on inoculum activity is discussed in Section 2.3. The lag of digestion increased as the duration of preservation increased (9.5 to 18 h over a 3-month period) and the kinetic parameters of the control silage were used as a covariate to correct for this.

Forages were incubated *in vitro* in nitrogen-limited conditions where the microbial population were dependent on the nitrogen supplied by the substrate for their metabolic requirements or in nitrogen-excess conditions as discussed in Chapter 3.

Methodological differences between the modified Tilley and Terry and gas pressure transducer technique

The modified Tilley and Terry technique was used in Section 4.1 as it was suitable for the incubation of wet forages *in vitro*, accommodating large substrate particle sizes and providing efficient agitation (Section 2.1). However as the modified Tilley and Terry technique relies on gravimetric measurements,

the extended lag in NDF digestion for M4 resulted in an insufficient number of data points obtained over 96 h to allow the data set to be described mathematically.

Gas measurements are sensitive to direct and indirect alterations in the fermentation environment as all direct and indirect gas produced within the system is incorporated into the mathematical description and expressed as the amount of gas /g total OM digested. The gas pressure transducer system is therefore suitable for monitoring forages of poor digestibility and was used in Section 4.2 to provide a sufficient number of data points for the curve fitting of M4. As the technique can also accommodate a large number of samples, the *in vitro* digestion of all treatments could be monitored in a single run eliminating the concern for inoculum variation.

Increases in gas production may be attributed to increased OM digestion. However it is important to consider the possibility that increases may also be attributable to the negative relationship between gas production and microbial protein production (Blummel *et al.*, 1997), to alterations in the VFA profile as slower microbial digestion patterns are often dominant in acetate production which will give higher yields of direct gas (Church, 1988) and/or to the negative relationship between ammonia production and indirect gas production (Cone and Van Gelder, 1999).

Microbial protein production was not measured. As the TVFA concentration and proportions of short chain fatty acids differed with maturity and nitrogen supplementation, treatment comparisons should be made with caution. Ensiling had no effect on the 96 h VFA concentration or proportions, which makes within harvest comparisons valid, noting of course the effect of nitrogen supplementation. The greater VFA concentration post-nitrogen supplementation may suggest a concomitant increase in ammonia concentration due to peptide/amino acid metabolism. This is supported by the increase in the proportion of total branched chain fatty acids.

Nitrogen supplementation was influential on most kinetic parameters for the gas pressure transducer technique. It is important therefore to understand the impact it may have on the interpretation of data derived from the gas pressure transducer system and subsequently on comparisons with the modified Tilley and Terry technique system.

As discussed in Chapter 3 the nitrogen-excess treatment was defined by supplemental nitrogen in the form of urea and AA, which can contribute to the ammonia pool immediately after addition or after metabolism by the microbial populations respectively. Nitrogen supplementation consistently increased the gas pressure transducer lag of *in vitro* F70 digestion.

Cone and Van Gelder (1999) state that protein metabolism *in vitro* can influence gas production directly and indirectly. An increasing ammonia pool reduces indirect gas production by binding H^+ ions, while an alteration in the stoichiometry of fermentation favoring branched chain fatty acids will affect the direct gas production. They elaborated on this finding to state that each 1 % of protein inclusion can decrease gas production by 2.48 ml/g fermented (1.77 and 0.71 associated with indirect and direct influences respectively).

Protein fermentation is a consideration when soluble concentrations are high and/or carbohydrate fermentation is limited (Cone and Van Gelder, 1999). Cone (1996) proposed that correction of gas data profiles may be necessary in these situations though no universal correction factor is available

Cone (1996) examined the effect of maturity and ensiling on NDF digestion using gas pressure transducer and the *in sacco* method, the latter being comparable to the gravimetric calculations of the modified Tilley and Terry technique. He observed a good relationship between the digestion rate determined by the *in sacco* technique and the second phase rate of the *in vitro* gas technique for perennial ryegrass and ensiled forages differing in maturity. The use of bi-phasic or multiphasic models to distinguish between the rapidly and slowly degradable phases in a gas production profile may clarify direct comparisons between gas pressure transducer technique and modified Tilley and Terry technique.

Multiphase models are complicated in nature and require very descriptive data sets normally obtained using automated sampling. In this study the data sets were too limited to be analysed by a multiphase model (Van Gelder 2000, personal communication). The Gompertz model will not adequately describe the different phases of digestion. Therefore caution should be used when interpreting the lag, extent and AED between nitrogen-limited and nitrogen-excess treatments, predicted using the gas production measurements. This went without comment in the work of Stefanon *et al.* (1996) who found that maturity decreased the lag for alfalfa forages but increased the lag for bromegrass forages when each had a CP range of 19-36 and 11-23 % DM respectively.

The AED of F70 digestion can also be predicted in the gas pressure transducer systems from the real extent, which is not indirectly influenced by the nitrogen soluble pool. However, the lag is an influential parameter on the determination of the AED (Singh *et al.*, 1992) and therefore the gas pressure transducer technique under conditions of nitrogen-excess may not adequately estimate the true AED of F70 fractions.

This issue was partially addressed by Blummel and Bullerdieck (1997) who suggested that the predictive ability of gas pressure transducer in relation to voluntary DM intake could be improved by using a correction factor, based on the ratio of gas produced to DM disappearance. However when the lag variable is the issue a correction factor based on a single time point measurement may not be sufficient.

- **Sample preparation differences between modified Tilley and Terry technique and gas pressure transducer technique**

The incubation procedures for wet and dried substrates differed. Dried materials were incubated 18 h prior to inoculation to simulate the water saturated nature of the fresh materials. Miller and Hobbs (1994) reported a significant decrease in the *in vitro* lag of meadow hay NDF fermentation when dried substrates were hydrated for up to 16 h prior to incubation, citing the conclusions of Fan *et al.* (1981) who stated that the activity of cellulolytic enzymes is dependent on an aqueous carrier. This was not supported by Corley *et al.* (1998) who found no effect of hydration for 7 days on the *in sacco* digestion of maize and soyabean meal.

In section 4.1 the fresh substrate was chopped to 1cm lengths and used to examine NDF digestion when incubated with the soluble fraction intact. For the gas pressure transducer system samples were milled for improved sample homogeneity. Milling reduces the particle size of the substrate, thus increasing the effective surface area for microbial degradation (Latham, 1978, Bauchop, 1981, Gerson *et al.,* 1988).

Uden (1992) using wheat straw of differing maturities found that the rate of *in vitro* NDF digestion was lower for particle sizes of 1-2 cm when compared with milled samples (4.5, 1 and 0.25 mm). There was a significant impact of maturity on the *in vitro* digestion in the study. For early cut forages the mean lag was less for 1-2 cm when compared with milled samples (2.1 vs. 3.1 h), but for late cut forages the lag was greater for 1-2 cm (33.5 h vs. 12.1 h). They concluded that particle size influenced the lag more than the rate or extent of *in vitro* NDF digestion. Lopez *et al.* (1995) found no effect of sample preparation (fresh (chopped), and freeze-dried (milled)) on silage DM disappearance *in sacco.*

The effect of maturity, ensiling and nitrogen supplementation on digestion of perennial ryegrass unfractionated and fractionated cell wall fractions in vitro

No available literature discussed the interactive effects of ensiling, maturity and nitrogen supplementation on *in vitro* or *in vivo* NDF digestion. Few authors have examined the *in vitro* fermentation of fractionated forage NDF. Stefanon *et al.* (1996) isolated the structural fraction by soaking forages in distilled water at 39 ^{0}C overnight and used gas pressure transducer to examine the effect of maturity on alfalfa (333-656 g NDF/kg isolated DM) and bromegrass (745-892 g/kg isolated

DM) NDF digestion *in vitro*. Doane *et al.* (1997a) discussed the main effects of maturity and ensiling on the *in vitro* digestion of fractionated NDF but did not report the kinetic data for the isolated fraction.

Therefore the results will be discussed in relation to the main effects of maturity and ensiling, with reference made to significant treatment interactions where necessary.

- **The effect of maturity and nitrogen supplementation on the *in vitro* digestion of forages**

The degree of lignification, the formation of lignin carbohydrate complexes and the cross-linking nature of the cell wall components are all controlling factors in cell wall degradation (Chesson *et al.,* 1986, Chesson, 1988). Disruption of ether linkages, which may be associated with lignin-carbohydrate cross-linking in mature cell walls, is essentially an aerobic process involving oxidative enzymes. Therefore lignification will negatively affect the extent of ruminal NDF digestion. Lignification may variably affect the rate of polysaccharide digestion by influencing the degree of substitution and the physical and/or chemical association of individual components within the structure (Moore *et al.,* 1994).

In the present study maturity decreased the rate of NDF digestion of the fresh herbage. This is supported by the *in vitro* work of Cherney *et al.* (1993) and Cone and Van Gelder (1999) and the *in vivo* work of Huhtanen and Jaakola (1994). Nitrogen supplementation had no effect on the rate of digestion of NDF from G.

Maturity decreased the rate of NDF digestion of ensiled forages *in vivo* (Bosch *et al.,* 1992b, Rinne *et al.*, 1997b). In this study ensiling decreased the rate of NDF digestion for immature but not mature forages, which may reflect biochemical differences in the structural fractions. Doane *et al.* (1997a) found no effect of maturity or ensiling on the rate of NDF digestion *in vitro*.

Stefanon *et al.* (1996) found that the trends observed in the rate of digestion of the unfractionated NDF were similar to that of fractionated NDF. When isolated from the water-soluble fraction, maturity did not decrease the rate of F70 digestion for G. This is unexpected if it is to be argued that lignification will affect the rate of F70 digestion. However the rate was also found to be dependent on nitrogen supplementation which increased the rate of digestion of all forages at all stages of maturity except M4. At M4, the lignification of the cell wall material dominated the rate of F70 digestion.

The lag of perennial ryegrass NDF digestion initially decreased with maturity then increased when the forage matured to an NDF concentration greater than 544 g/kg DM (M4). Cherney *et al.* (1993) found similar trends in the immature forages and suggested that the higher lag was due to a preferential

utilisation of abundant soluble and neutral detergent soluble carbohydrates in the earlier stages of growth.

The negative effect of maturity on the lag of NDF digestion reflects the reduction in the soluble and readily fermentable components and a deposition of lignin within the primary and secondary walls creating rumen indigestible moieties (Akin, 1993). Stefanon *et al.* (1996) and Doane *et al.* (1997a) found that the lag of NDF digestion increased with maturity but concluded that though statistically significant, it was numerically too small for any biological relevance. Huhtanen and Jaakola (1994) found no effect of maturity on the *in sacco* lag of perennial ryegrass NDF digestion.

The lag of immature forages was also higher when the F70 fractions were examined. Blummel and Bullerdieck (1997) suggest that a negative relationship exists between gas production and microbial synthesis. This may explain the increased lag of immature forages, not as a static period of fermentation but as a period of rapid microbial protein production.

Nitrogen supplementation differentially increased the lag in the modified Tilley and Terry and gas pressure transducer systems. Few rumen microbes can utilize amino acids alone for growth due to the low ATP generation (Gylwsky *et al.,* 1984, Russell and Wallace, 1988), but they may have preferentially used AA as a supportive energy source due to carbohydrate limitation, thus increasing the lag. As a nitrogen source, amino acids from casein are rapidly metabolized (< 1h, Broderick and Craig, 1989) increasing the *in vitro* ammonia concentration. This may have had inhibitory effects on microbial function (discussed in Chapter 3) or may reflect the indirect effect of ammonia on gas measurement as discussed by Cone and van Gelder (1999).

The extent of NDF and F70 (estimated and real) digestion decreased with maturity. Stefanon *et al.* (1996) found that the real extent of fractionated and unfractionated NDF digestion decreased with maturity while the estimated extent increased for fractionated but not unfractionated OM digestion. Bosch *et al.* (1992a, b), Huhtanean and Jaakola (1994), Cherney *et al.* (1993) and Doane *et al.* (1997a) all report a decrease in the extent of herbage digestion as the forage matures. This negative effect of maturity also applied to ensiled forages (Rinne *et al.*, 1997).

Nitrogen supplementation did not affect the extent of NDF digestion for G. For the real extent of F70 digestion, nitrogen supplementation was not influential. A decrease in the estimated extent may be related to the additional buffering capacity of the nitrogen pool and therefore not of biological significance.

- **The effect of ensiling and nitrogen supplementation on *in vitro* digestion**

In the present study ensiling decreased the rate of NDF digestion for immature forages but increased the rate of mature perennial ryegrass NDF digestion. This is supported by the *in vivo* work of Lopez *et al.* (1991) who found that ensiling increased the rate of late but not early season grass. Such results would suggest that the hydrolytic attack of the lignified cell wall during ensiling predisposed the lignified carbohydrate structure to cellulolytic digestion.

Other studies found that ensiling had an effect on the rate of forage digestion (Cushnahan *et al.*, 1995, Cushnahan and Gordon, 1995, Doane *et al.*, 1997a, b). Lopez *et al.* (1991) concluded that ensiling had little influence on DM degradability of forages but significantly altered the rate of protein solubilization and rumen degradation. They suggest that factors such as chemical and botanical composition of the fresh herbage may be more influential than ensiling on subsequent nutrient utilisation of the herbage. In Chapter 3 there was no effect of supplementation on the NDF rate of digestion.

The proteolytic effects of ensiling may have restricted microbial cellulolytic activity as nitrogen supplementation, which did not influence the rate of G, increased the rate of NDF digestion of restricted silage in M1 and M3 and the extensively preserved silage in M3.

In the absence of the water-soluble fraction, nitrogen supplementation increased the rate of F70 digestion for ensiled forages at all stages of maturity except M4. This suggests that the ensiled structural fractions were limited in nitrogen availability. Ensiling increased the rate of F70 digestion for immature forages. The predisposition of NDF in M3 to faster rates of digestion post-ensiling was not obvious in the absence of the water-soluble component.

The lag of NDF digestion increased with ensiling. Doane *et al.* (1997) found a significant increase in the lag of OM digestion with ensiling when compared to the freeze-dried (proxy fresh) sample. Cushnahan *et al.* (1995) found no effect of ensiling on the lag of ADF digestion and Lopez *et al.* (1991) found no effect on the lag of NDF digestion *in vivo.*

The hydrolysis of the NDF component during ensiling may enhance the lag caused by advancing maturity by reducing the readily available polysaccharide content of the cell wall and increasing the concentration of the lignin moieties. Rinne *et al.* (1996) however, found no effect of maturity on the *in sacco* lag of silage NDF digestion.

In the absence of the water-soluble fraction there was no effect of ensiling on the lag of fermentation, suggesting that the water-soluble fraction was hindering the initiation of ensiled cell wall digestion *in vitro* as discussed previously in Section 3. Nitrogen supplementation increased the lag of NDF digestion of ensiled forages in M3, and of all forages when the F70 fraction was incubated.

Ensiling generally decreased the extent of NDF digestion. Cone (1996) observed a trend for a reduction in extent of digestion with ensiling. Doane *et al.* (1997) found that ensiling decreased the estimated OM extent of digestion but did not influence the real extent of NDF digestion. *In vivo*, Lopez *et al.* (1991) and Cushnahan *et al.* (1995) found no effect of ensiling on the extent of NDF and ADF digestion respectively.

When the water-soluble fraction was removed ensiling increased both the estimated and real extents of F70 digestion. The inhibitory effect of the water-soluble fraction on the extent of NDF digestion is attributed to the extended lag.

Nitrogen supplementation improved the extent of NDF digestion of extensively preserved forage in the early harvests, while decreasing the extent of restricted silage in M3. Nitrogen supplementation did not influence the real extent of F70 digestion. The decreased estimated extent may be a due to high ammonia concentrations *in vitro,* as previously discussed.

- **The effect of maturity, ensiling and nitrogen supplementation on *in vitro* AED**

Maturity decreased the AED of NDF digestion for grass, restricted and extensively preserved forages by 6, 14 and 11 % over the first three harvests. Ensiling decreased the AED of perennial ryegrass by 9, 19 and 18 % in the first three harvests. This would suggest that ensiling had a greater effect on the AED of perennial ryegrass than maturity. When compared with the restricted fermentation, the extensive preservation had an adverse effect in M1 only. Cushnahan and Gordon (1995) found no effect of ensiling in a bunker or duration of ensiling on NDF AED while Keady and Murphy (1996) reported a decrease in the DM AED due to ensiling.

Nitrogen supplementation decreased the AED of NDF digestion for perennial ryegrass in M1 and M2 and the AED of the ensiled forages in M3 by approximately 10 %. This may be due to a negative effect of ammonia concentration on *in vitro* digestion as discussed in Chapter 3 and is supported by the fact that nitrogen supplementation had no effect on the AED of F70 fractions. Based on the ARC (1984) recommendations for optimal microbial activity (32 g-rumen degradable nitrogen per kg OMAD), Lopez

et al. (1991) concluded that early season grasses would be inadequate to supply this ratio (24 and 32 g N/kg OMAD for early and late respectively). This was not the case in this study.

For the isolated F70 fractions, maturity decreased the AED of grass, restricted and extensively preserved forages by 16, 18 and 23 % over the four harvests. Ensiling increased the AED of the extensively preserved forage in M1 by 4 % and both preserved forages in M2 by 5 %, with no effect in M3 and M4.

Conclusions

Using perennial ryegrass harvested at different stages of maturity it was concluded that

- The negative effect of ensiling on the AED of intact fresh, unfractionated perennial ryegrass cell wall digestion *in vitro* was greater than that of maturity.
- Nitrogen supplementation decreased the AED of *in vitro* cell wall digestion for all fresh, unfractionated forages
- When isolated from the soluble fraction maturity but not ensiling decreased the *in vitro* AED of perennial ryegrass digestion.
- Nitrogen supplementation had no effect on the *in vitro* AED of digestion for fractionated cell wall fractions.

Implications

When forage preservation conditions are good, maturity will have the greatest impact on the intrinsic ruminal digestion characteristics of the structural fraction. However it is important to recognise that ensiling may also influence forage palatability and the physiological control of intake (Steen, 1998) as decreases in DMI can be influenced by duration of ensiling (Cushanhan and Gordon, 1995) or preservation method (Fox *et al.*, 1971, Keady and Murphy, 1993).

Methodological practices such as nitrogen supplementation may interfere with the *in vitro* fermentation profile in both the modified Tilley and Terry and gas pressure transducer systems. Doane *et al.* (1997) concluded that ensiling decreased the rate of the neutral detergent solubles. This reflects the conversion of the fermentable sugars and proteins to lactic acid, VFA and non-protein nitrogen fractions respectively. In batch systems, where pH is controlled, such alterations in the soluble fraction may be sufficient to negatively affect fibre digestion, as they may enhance the rate of endproduct accumulation. These issues can be resolved in continuous fermentation systems where there is a continuous removal and replenishing of the fermentation liquids (Isaacons *et al.*, 1975, Meng *et al.*, 1989).

CHAPTER 5
EXPERIMENTAL METHODOLOGY
DEVELOPMENT OF A RUMEN SEMI-CONTINUOUS CULTURE

Introduction

The specific research objective and limitations of the available techniques will govern the methodological method used in studies on *in vivo* digestibility and nutrient supply to the ruminant. *In vivo* measurements can be subject to technical (Orskov *et al.*, 1986, Tamminga *et al.* 1989a, Tamminga *et al.*, 1989b, Illg and Stern, 1994) and animal variation (Mehrez and Orskov, 1977, Michalet-Doreau and Ouldbah, 1992). *In vivo* techniques can be expensive, time consuming and labour intensive with concerns that the welfare of fistulated experimental animals may be compromised by the need for invasive surgery. *In vitro* systems can be cheap and versatile and the continuous culture techniques have been developed as a means of studying rumen microbial metabolism in a system, which more closely models the *in vivo* environment. *In vivo* techniques are necessary to highlight animal-substrate interactions but only the controlled *in vitro* systems can be readily used to examine the influence of intrinsic properties of the substrate on the subsequent ruminal digestion profile (Mertens, 1993).

The three most cited rumen simulation models are the semi-continuous or Rusitec system of Czerkawski and Breckenridge (1977), the single flow semi-continuous system of Slyter *et al.* (1964) and the dual flow system of Hoover *et al.* (1976a). The design of these systems has remained relatively constant over time, though operational conditions such as flow rates, buffers, pH control and feeding regimes may have changed.

System choice will depend on the concerns and objectives of the experimental study. With a view to examining the influences of maturity and ensiling on the inherent ruminal digestion parameters of perennial ryegrass forages, the dual flow system with manual feeding to allow for diurnal variation was chosen. *In vivo,* maturity and ensiling will influence DM intake and particle retention time, microbial protein production and diurnal variations of soluble carbohydrate and nitrogen fractions in the rumen (Section 1), all of which have implications in the forage nutritive value. In attempting to quantify only the intrinsic characteristics of forage digestion, the control of the liquid dilution rate, solid dilution rate, feed input and pH is important. There were four progressive stages in the development of the rumen semi-continuous culture (**RSC**).

5.1 Objective

The objective was to establish an RSC based on the dual flow principle and to identify functional problems in the daily running of this system

Materials and methods

In vitro system

An *in vitro* system consisting of four fermentation vessels was prepared. Each fermentation vessel was made of glass (22 cm x 12 cm) with a working volume 1600 ml. The glass lid had three port-hole entries as shown in **Figure** 5.1 and was secured using a vaseline seal and a metal bracket which compressed the lid against the lip of the fermentation vessel. Each vessel was placed in an open water bath (**Figure** 5.2a) with the temperature controlled at 39 ^{o}C using a Grant 159 (SE15) heating element. Open orifices in the center of the waterbath accommodated the fermenter vessel overflow as described in **Figure** 5.2b. Anaerobic conditions were maintained by flushing the system continuously with nitrogen which was piped directly from a N_2 cylinder to the vessel with copper wire and controlled by a two-way valve. Portholes were sealed with butyl rubber stoppers. The central stopper had an additional gas seal on the outside surface (**Figure** 5.1) to prevent gas exchange through the hollow metal core, which facilitated an agitator shaft. An overhead agitation system was developed to simultaneously mix four fermentation vessels. The 4 rotary shafts were connected to an internal agitation arm in each vessel through the large central porthole. A solid paddle (3" x 1") was placed at the end of each shaft. Saliva was infused through the second porthole and the filtrate effluent removed through the third using a filter which was prepared as described by Hoover *et al.* (1976).

Operational conditions were based on the work of Hannah *et al.* (1986) and one fermentation vessel was prepared. Flow dynamics were controlled using a Whatmann peristaltic pump. Artificial saliva was prepared as detailed in **Table** 5.1, with urea supplement included at 0.5g/l. Rumen fluid was collected from 3 steers fed silage *ad-libitum* and was prepared as described in Section 2.1. The vessel was inoculated with rumen fluid 1 h after sampling and the agitation and peristaltic pump were switched on 1 h later. Agitation was continuous at 60 rev./min. and the liquid dilution (**LDR**) and solid dilution rate (**SDR**) were 0.1 and 0.5 /h, respectively. Thirty five grams of a milled silage (**Table** 5.2) were added to the fermenter at this stage and subsequently added at 12 h intervals.

Figure 5.1 Original fermentation vessel used in the development of the rumen semi-continuous culture

Figure 5.2a Original open waterbath used in the development of the rumen semi-continuous culture

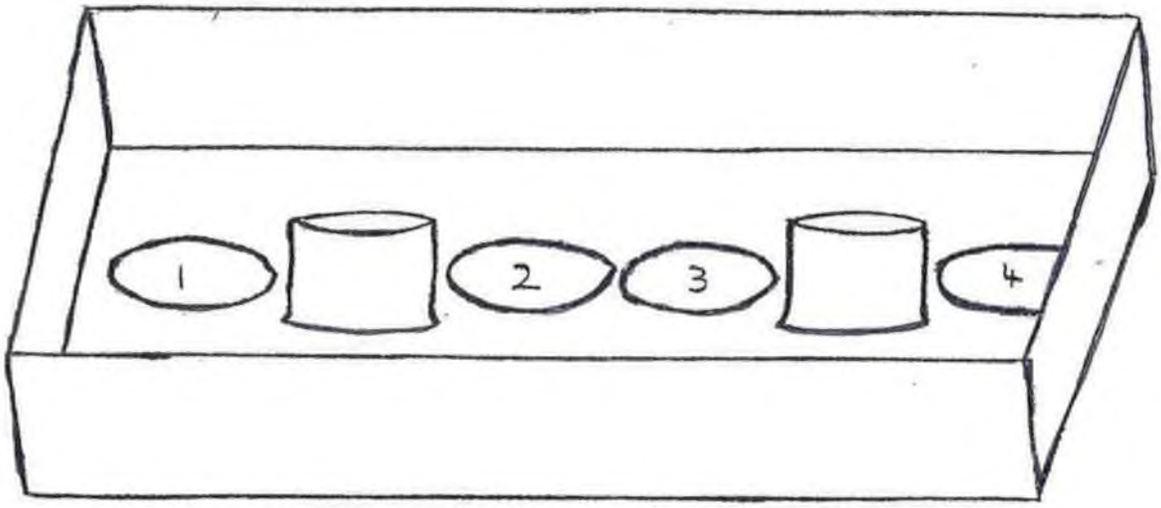


Figure 5.2b Original fermenter vessel overflow system in the development of a rumen semi-continuous culture

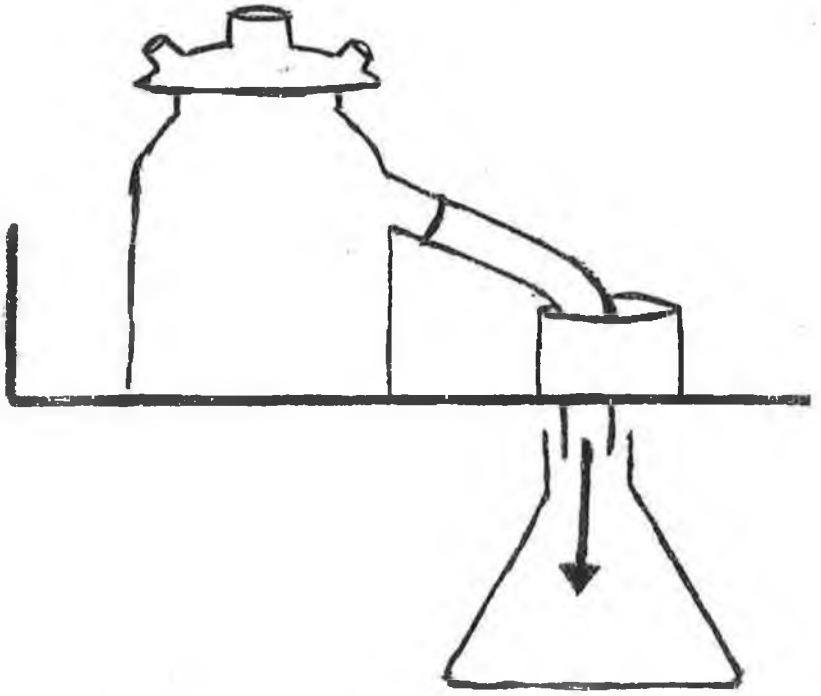


Table 5.1 Stern and Hoover mineral buffer (1976)

Distilled water (l)	**g/l distilled water**
Chemical	
Di-sodium hydrogen phosphate	1.76
Sodium hydrogen carbonate	5.0
Potassium chloride	0.6
Magnesium chloride	0.12
Potassium hydrogen carbonate	1.6
Urea [a]	0.4

[a] Urea is added at 0.5 g/l if the diet contains less than 15 % crude protein (DM basis).

Table 5.2 Chemical composition of dried milled silage (g/kg DM (sd.))

	g/kg DM	
Crude protein	187.3	(0.94)
Ash	83.3	(4.50)
Neutral detergent fibre	450.5	(1.50)
Acid detergent fibre	259.0	(2.0)
Digestibility		
Dry matter	776.0	(12.02)
Organic matter	714.0	(14.25)

Sampling

The pH of the system was measured by inserting an Orion (710A) pH probe into the vessel interior.

Calculating flow rates of fermenter digesta

Dilution rate (**D**) = percent of fermenter volume replaced /h

LDR = ((filtrate (ml /h) + overflow (ml /h))/fermenter volume (ml))*100

SDR = ((ml overflow /h)/ fermenter volume (ml))*100

Statistical Analysis

Data pertaining to pH measurements were not statistically analysed due to a lack of sufficient replication.

Results and discussion

The average pH of the buffer was 8.4. The pH of the vessel rose from 6.8 to pH 8.7 in <24 h (**Table** 5.3). Such conditions are outside the physiological range of the rumen and the optimum pH range for microbial activity (Church, 1988). As the system had no method of pH control it was decided to terminate the run at the end of Day 2.

Table 5.3 Periodic pH profile during *in vitro* digestion of a ground milled silage

Day	Time	pH
1	14.00	6.74
	15.00	7.23
	17.00	7.14
	22.00	7.88
2	08.00	8.26
	11.00	8.7
	13.00	8.6
	15.00	8.7
	17.00	8.3

Operational problems identified and later addressed were:

- *Insufficient mixing*

  Poor mixing within the reaction vessel allowed a dense mat to form at the surface of the inoculum, which subsequently interfered with the digesta flow at the overflow arm. Sparging nitrogen through the inoculum at feeding times assisted initial mixing, but the mat later reformed and when dried became partially solidified. The agitation paddle was redesigned to incorporate a foam breaker and a double paddle (**Figure** 5.3).

- *Insufficient control of N_2 flow*

  The simple 2-way valve tap gave insufficient control of nitrogen flow. This was modified so that N_2 flow was regulated at the cylinder and in the laboratory using an ISO 2000 approved system. The measured flowrate was 40 ml/min to each vessel as recommended by Stern and Hoover (unpublished).

- *Blocking of filters*

  This problem was attributed to poor mixing and small pore sizes (40 μm) of the nylon mesh. The filter was adapted to a single layer of nylon mesh of 100 μm pore size.

- *Fermentation vessel*

  The effective working area in the original fermentation vessel headspace was restricted due to the design of the vessel and lid and limited porthole entries. Both vessel (**Figure**

5.4a) and lid (**Figure** 5.4b) were altered. The modified vessel had a working volume of 1800 ml (13.7 cm x 12.5 cm)

- *Waterbath*

  The temperature of the waterbath and vessel contents was consistently 39-40 ^{0}C. However the design of the waterbath and central orifice to accommodate the overflow tubing restricted the flow of digesta to the collection container. Therefore the waterbath was re-designed (**Figure** 5.5).

Conclusion

The instability of the system was attributed to the poor performance of component elements used in its construction.

Figure 5.3 The re-designed agitation paddle which incorporated a foam breaker with double paddle to improve *in vitro* mixing.

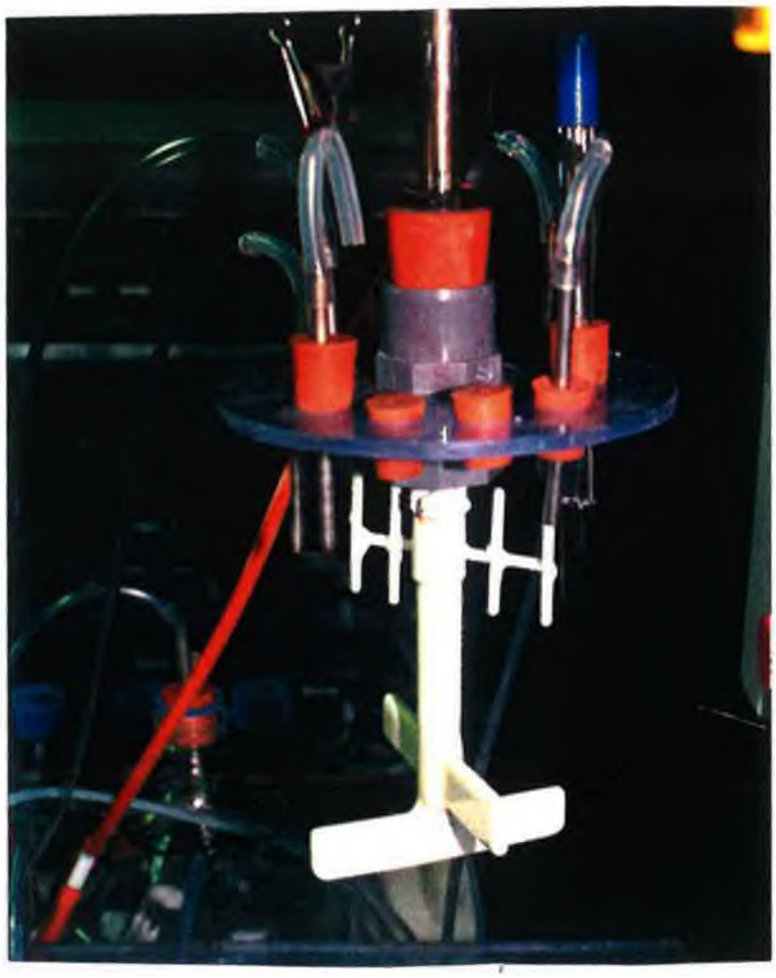


Figure 5.4a The altered fermentation vessel with increased internal effective working area

Figure 5.4b The altered fermentation vessel lid with additional portholes

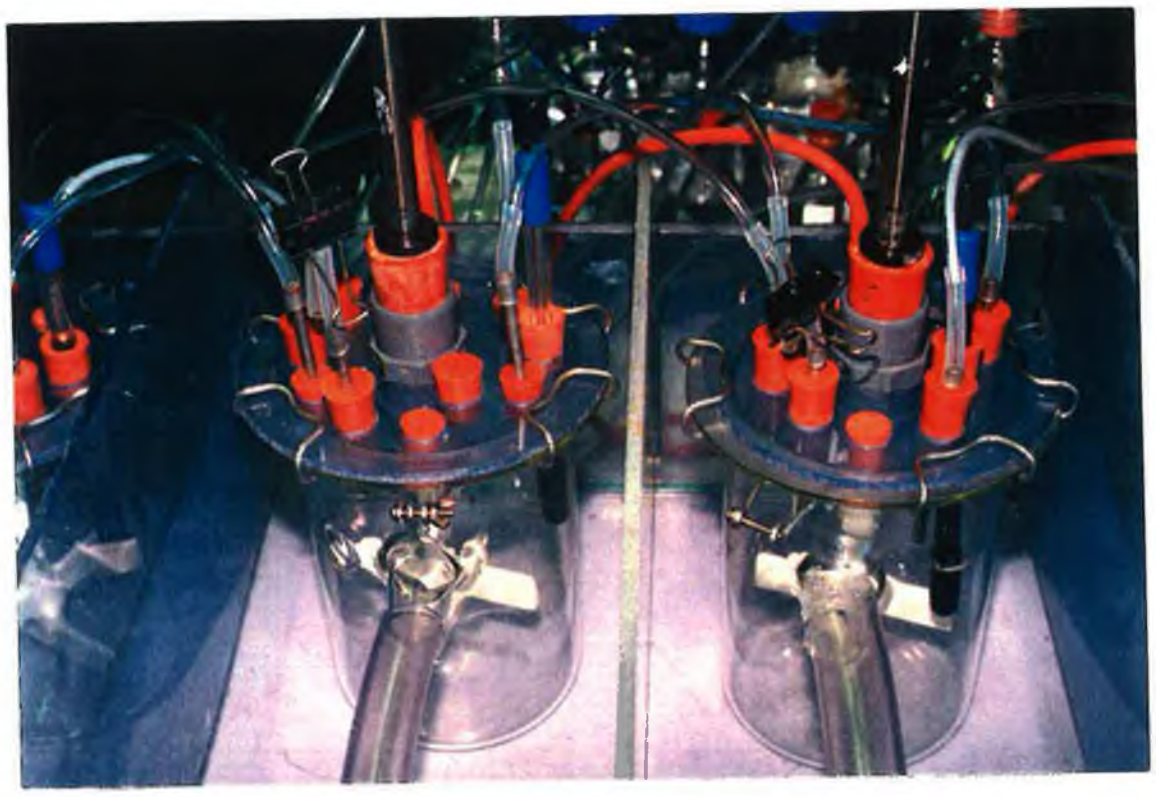
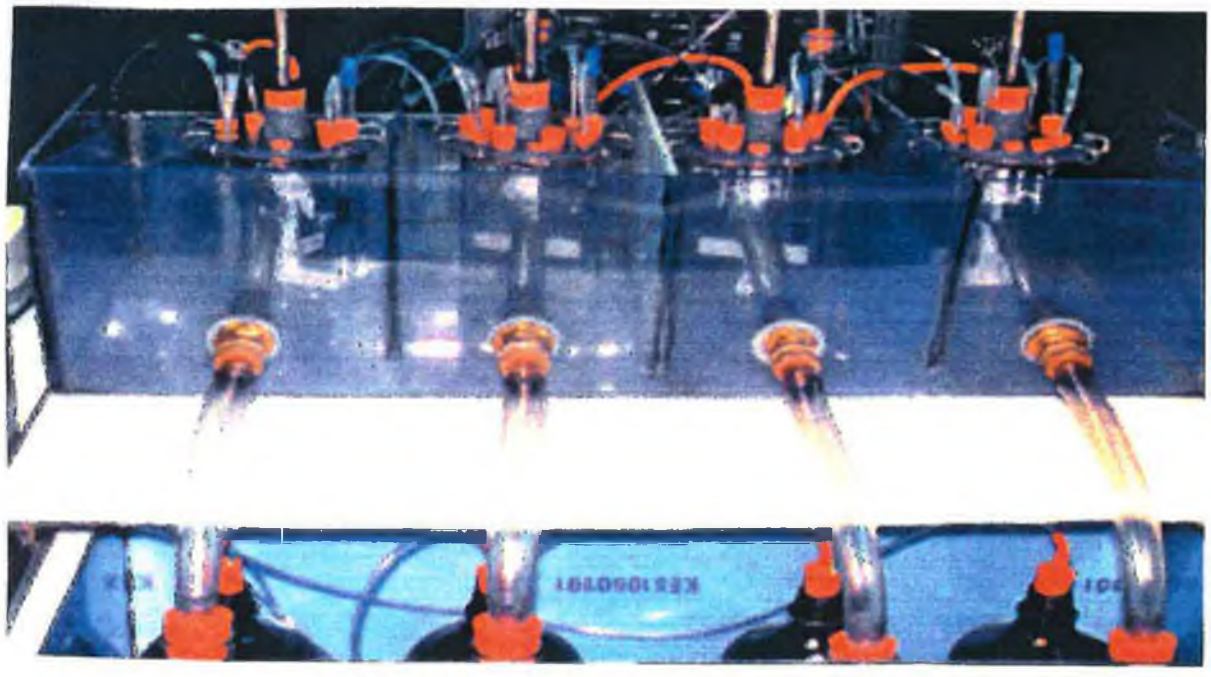


Figure 5.5 The redesigned waterbath to improve the flow movement of the overflow digesta



5.2 Objective

The objective was to examine the *in vitro* ruminal fermentation profile of a carbohydrate-based and fibre-based pelleted diet using the modified RSC system

Materials and Methods

In vitro system

Rumen fluid was collected from three fistulated steers fed silage *ad-libitum* and prepared as described in Section 2.1. Two fermentation vessels were prepared and inoculated as detailed in Section 5.1. The ingredients of the pelleted starch (**S**) and pelleted fiber (**F**) based diets, assigned to each vessel are shown in **Table** 5.4.

Table 5.4 Ingredient composition of starch (S) and fibre (F) rations

Item (%)	S	F
Barley	54.25	
Citrus pulp		20.70
Maize gluten	7.75	8.00
Dried grass		26.90
Soya hulls	2.32	
Soyabean		10.10
Sunflower	10.25	
Sugar beet pulp		30.30
Cotton extract	3.00	
Palm kernel	7.55	
Copra expeller	4.30	
Molasses	7.00	
Fat (tallow)	1.25	2.50
Lime flour	1.35	
Cattle minerals	0.30	1.50
Salt	0.65	

Operational conditions were based on Merry *et al.* (1984). The temperature of the water-bath was controlled at 39-40 ^{0}C. Each diet was assigned to a fermentation vessel and manually fed at 12 h intervals (9:00 and 21:00) when 22.5 g DM of the respective diets were added through the porthole furthest from the overflow exit. McDougalls buffer (**Table** 2.3.1) which was diluted 6:4

with distilled water and containing cysteine monohydrochloride (0.025 % w/v) was infused into each vessel to give an LDR of 0.06 /h and SDR 0.03 /h. Agitation was continuous at 60 revs/min. The collection vessels for displaced effluent (**DE**) and filtered effluent (**FE**) were stored in an ice-bath to minimize the fermentation of digesta. The pH was monitored 1 h after feeding and the pH adjusted to 6.5-6.8 with the addition of 3 M NaOH.

Sampling

At 8:00 daily, fermentation vessels were sampled for VFA anaylsis. Samples were acidified (10:1 with 5 M H_2SO_4) and frozen at - 20 ^oC. A sample was also removed for the estimation of protozoal numbers. Protozoa were counted without staining using a haemocytometer (Cockburn, personnal communication) for the first 5 days of fermentation. The agitation and peristaltic pumps were then switched off. Within an hour, the volume of inputs and outputs for the previous 24 h was recorded after which the agitation motor and peristaltic pump were switched on. Buffer was replenished daily and continuously mixed using magnetic stirrers. Triplicate samples of DE and FE were taken to estimate DM content (dried at 40 ^0C for 48 h). For 9 h post first feeding and prior to the second feed, each culture was sampled hourly for VFA and the pH recorded.

Chemical analysis

In vitro DE+FE samples were pooled for each vessel over sampling days for laboratory analysis. All samples were measured for DM, NDF, ADF, CP, crude and ash concentrations as described in Section 2. Concentrate feed samples were also characterised with respect to DMD (Section 2), digestible organic matter (DOMD, Alexander and McGowan, 1961), starch (Eoropean Communities Marketing of feedstuffs regulation, 1984- Statutory instrument no 200 of 1984) total sugar (Feeding stuffs (Sample Analysis) Regulations 1982 No. 1144) and oil B (Acid hydrolysis/ether extract, SI 200; 1984). Rumen fluid was characterised with respect to VFA (Ranfft, 1973).

Statistical analysis

No statistical analysis was done due to the lack of sufficient replication.

Results and discussion

The chemical composition of the pelleted starch (**S**) and pelleted fiber (**F**) based diets used are shown **Table** 5.5 .

Table 5.5 Mean (sd) chemical composition of the pelleted starch (**S**) and pelleted fiber (**F**) based diets

Component	S		F	
Dry matter (DM) (g/kg)	889	(4.9)	880.2	(9)
Dry matter digestibility (DMD, g/kg DM)	828	(12.3)	849.4	(5.2)
Composition of dry matter (g/kg DM)				
Crude protein	153	(4.2)	161.2	(2.9)
Ash	54	(5.3)	81	(1.5)
Starch	279.2	(18.7)	NA	
Sugar	55	(2.82)	110	(15.2)
Oil B	49	(1.5)	34.1	(3.4)
Neutral detergent fibre	250.2	(21.5)	307	(24.1)
Acid detergent fibre	115.8	(12.3)	162.4	(5.1)

NA = not assessed

The fermentation period lasted 8 days with SS assumed to be reached by day 5. Preliminary studies with this system, during development had shown a rapid decline in protozoal numbers using the higher dilution rates of Hannah *et al.* (1986). Merry *et al.* (1987) used lower LDR and SDR, which improved protozoal survival. In the present study there was a sharp decrease in protozoal numbers *in vitro* by day 5 (**Table** 5.6). A significant proportion are lost in the FE which may partially be attributed to increasing the pore size above that recommended by Hoover *et al.* (1976) but pore sizes less than 100 μm caused severe blocking in Section 5.1. The levels maintained at SS are less than the mean value of 1 x 10^5 of other reported studies using dual flow systems (Hannah *et al.,* 1986, Merry *et al.,* 1987, Mansfield *et al.,* 1995).

The agitation system successfully incorporated all pelleted feed into the inoculum maintaining a small mat of feed particles at the surface. This was partially attributed to the feed characteristics i.e. did not float due to density. The respective mean (sd.) requirements of 3 M NaOH addition at 11:00 for S and F diets were 11.12 (0.64) ml and 11.41 (0.45) ml daily and the mean pH of diet S and F from 8:00 to 22.00 h over 8 days is shown in **Figure** 5.6. There was an increase in pH with alkali addition directly post feeding, which subsequently decreased due to *in vitro* VFA production. This fluctuation would exceed the boundary limits of most controlled *in vitro* systems which maintain the pH in the range of 6.3 to 6.8 (Merry *et al.,* 1987, Mansfield *et al.,* 1996).

Table 5.6 The protozoa counts in the vessel , displaced (DE) and filtered effluent (FE) (x 10^5 ml) for the pelleted starch (S) and pelleted fiber (F) based diets

	S			F		
Day	**Vessel**	**DE**	**FE**	**Vessel**	**DE**	**FE**
1	10.6			10.6		
2	0.80	1.65	7.50	2.08	2.99	3.60
3	0.24	0.17	1.36	0.30	0.81	0.81
4	0.21	0.17	0.11	0.12	0.10	0.12
5	0.06	0.06	0.23	0.05	0.06	0.02

Figure 5.6 Mean pH profile during the digestion of starch and fibre diets in the rumen semi-continuous culture *in vitro*

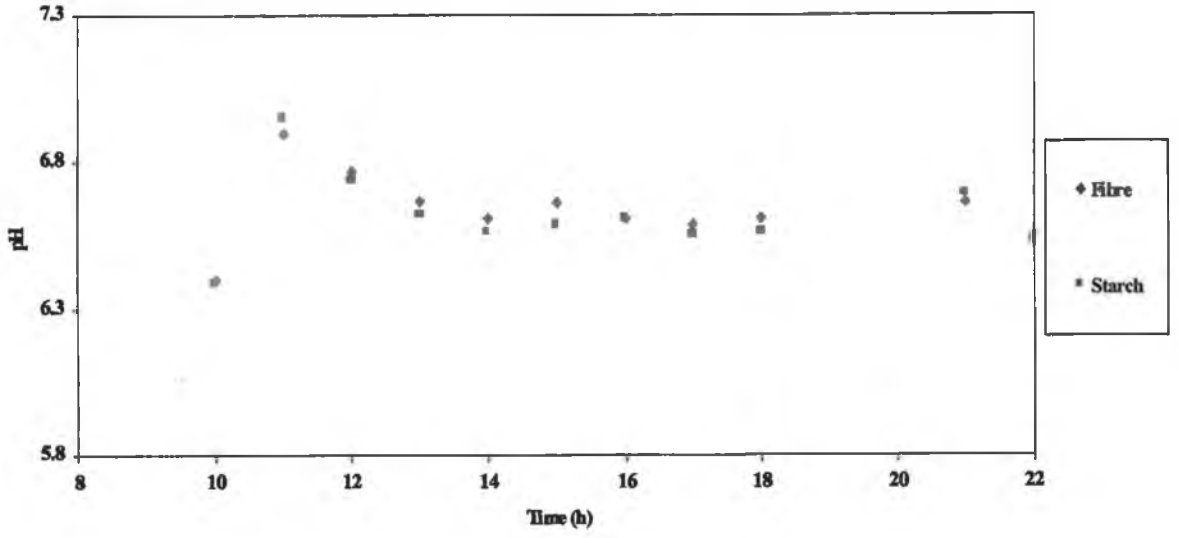


Figure 5.7 Daily non-glucogenic ratio for the digestion of the starch diet in the rumen semi-continuous culture *in vitro*

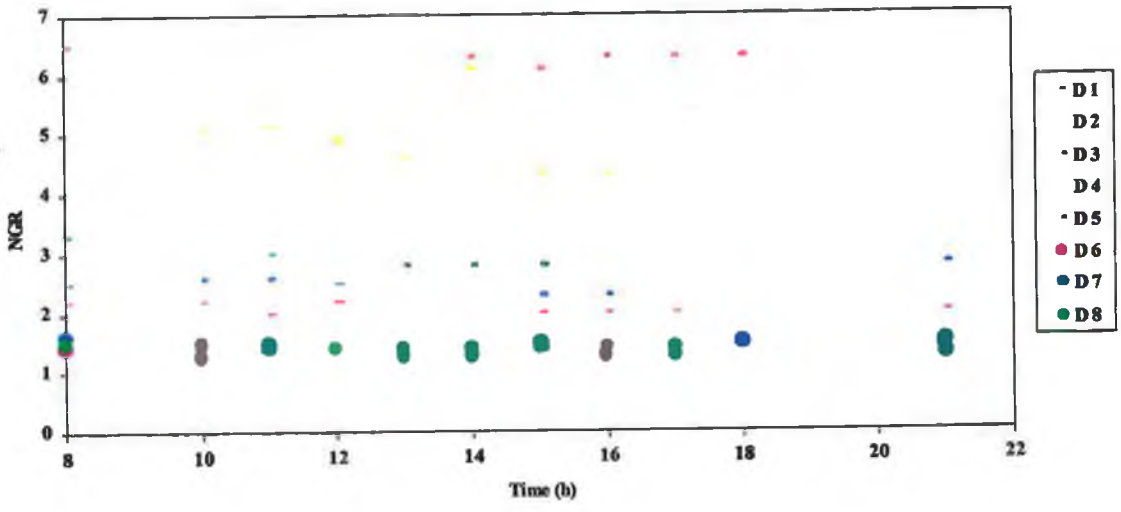
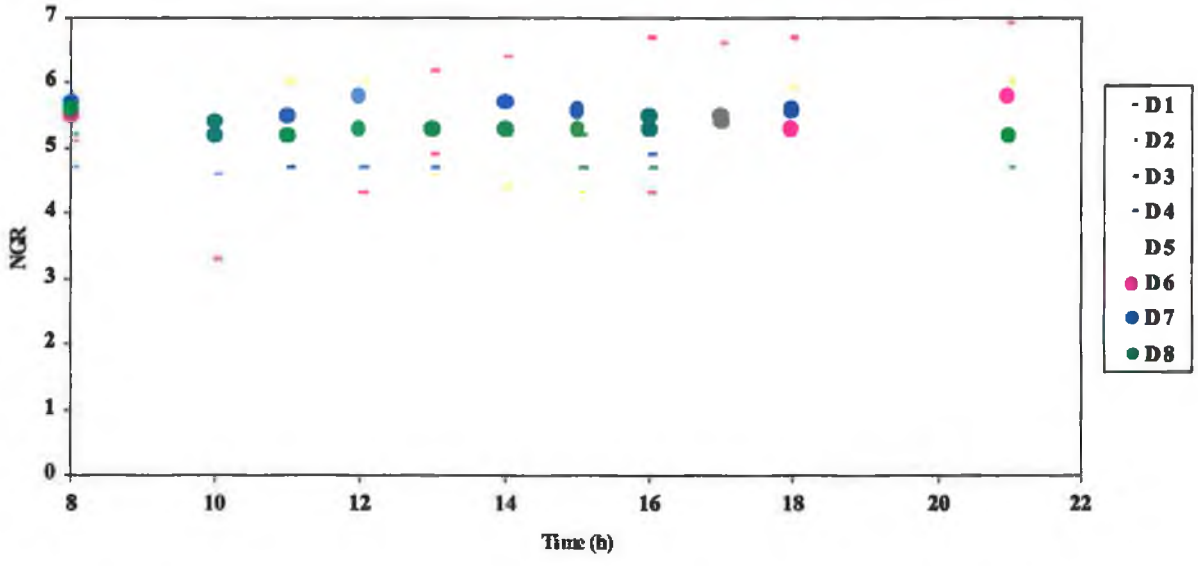


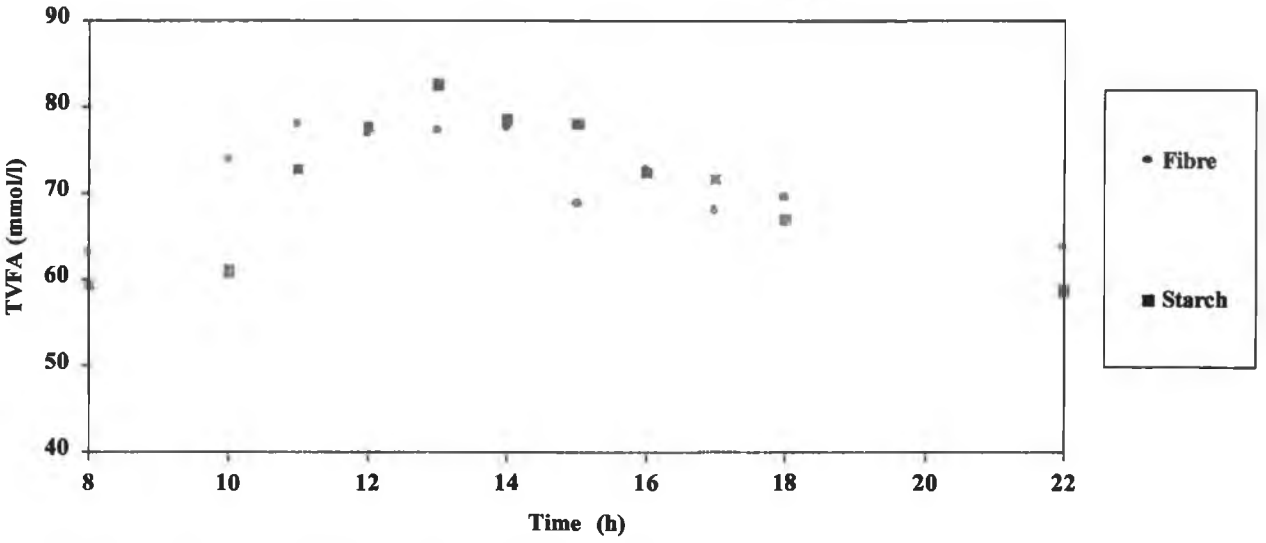
Figure 5.8 Daily non-glucogenic ratio for the digestion of the fibre diet in the rumen semi-continuous culture *in vitro*



Changes in the NGR for S and F diets over the experimental period are shown in **Figure** 5.7 and **Figure** 5.8 respectively, with much of the variation removed during the SS days. Variation in TVFA production was also minimal during SS. Similar TVFA levels were recorded for both diets with an increase in TVFA production immediately after feeding, returning to pre-feed levels before the second feed (**Figure** 5.9).

Figure 5.9 Mean total volatile fatty acid concentration for the digestion of the starch and fibre diet in the rumen semi-continuous culture *in vitro*

Figure 5.9 Total volatile fatty acid production for starch and fibre diets



The daily flow dynamics and apparent DM digestibility for both diets are shown in **Table** 5.7. The controlled dilution rates were very consistent through out the trial and considering the response of TVFA to feeding, the low apparent extents of DM digestion were attributed to poor sampling procedure rather than any serious functional problems with the system. The contribution of buffer to the DM of the effluents was estimated from the chemical composition, which may have been inadequate. Also the daily DM estimate of the DE may have been inaccurate due to displaced digesta lodging in the overflow arms.

Flow dynamics in the fermentation vessels could be improved by enlarging the over flow diameter to prevent blocking with solid digesta flow. The use of a forced air draft oven to dry a large volume of low DM samples required greater than 48 h. Samples were not treated prior to drying to prevent residual microbial fermentation during this time.

Table 5.7 Operational conditions (sd.) during, and apparent dry matter digestibility (sd.) for the *in vitro* digestion of the starch and fibre diet in the rumen semi-continuous culture

Diet	Starch	Fibre
LDR (%/h)	6.2 (0.12)	6.3 (0.31)
SDR (%/h)	2.9 (0.21)	3.0 (0.42)
Apparent dry matter digestibility [a]	390 (78)	367 (55)

[a] Calculation of Apparent DMD (%) = ((g dietary DM - (g effluent DM - g saliva DM))/g dietary DM)*100

Conclusion

In conclusion, daily fermentation in the RSC was stable. There was little variation in temperature and flow dynamics. The fermentation profile of each culture had attained steady state by day 6. *In vitro* fermentation patterns reflected the daily feeding pattern, with peak TVFA concentrations immediately post-feeding declining the pre-feed levels prior to the second feed. Protozoal numbers were quite low. All wet samples were to be freeze-dried in further studies. The requirement for pH control in the system was identified as the next phase of RSC development.

5.3 Objective

The objective was to install automatic pH control in the RSC system

Materials and methods

In vitro system

Operational conditions for 4 fermentation vessels were as described in Section 5.2. Two vessels were assigned to each diet, with the starch diet (**S**) fed to vessels (**V**) 1 and 2 and the fibre diet (**F**) fed to V3 and V4. Vessel 4 had a Syntex teflon pH probe submerged into the interior which was connected to a pH controller (Prosys, U.K.) preset to maintain a pH range of 6.3-6.8. Infusions of 5 M H_2SO_4 or 3 M NaOH were used to adjust the pH when necessary. The amount of acid/ alkali infused daily was recorded. The pH of all other vessels was monitored by submersing an external probe into the vessel contents and there was no addition of acid or alkali to these vessels.

Sampling

As described in Section 5.2. In addition two daily samples of un-infused buffer were taken for DM estimations during the SS days and protozoa numbers were not measured. The DM of all samples was measured by freeze-drying.

Statistical analysis

As pH control differed across treatments data were not analysed due to the lack of sufficient replication. pH results are presented as the mean of 8 days, while all other measurements are the mean of SS days only (3 days).

Results and discussion

Environmental pH can have a significant impact on the *in vitro* ruminal microbial fermentation of substrates. Without sufficient pH control the daily range may vary sufficiently within (diurnal variation due to feeding times) and between diets (variation due to metabolism of dietary components) to become a confounding factor within experiments. Most continuous fermentation systems have incorporated pH control between the range of 6.2 -6.8. Once these limits are exceeded in any vessel, acid or alkali is automatically added until the recorded pH is again within limits. In Section 5.2, 3 M NaOH was added manually 1 h after feeding. This maintained a relatively high pH initially but as the fermentation proceeded, the pH decreased. This system is laborious and fails to give adequate control, as shown in Section 5.2.

Preparatory work for Section 5.3 identified operational problems in preparing a system for pH control. These problems contributed to an unacceptable overloading of the system with acid and/or base. A lack of homogeneity in the vessel interior leads to pH gradients. To overcome this acid/ alkali additions were made at the center of the vessel where the mat was broken sufficiently by the agitation paddle to allow quicker incorporation into the vessel medium. Additions were also at the slowest possible speed (2.6 ml/min). Distortions in pH readings from the submerged Syntex pH ceramic probes were attributed to DM deposits around the protective rim of the submerged probes (**Figure** 5.10a). The protective rim was removed but the ceramic junction was rapidly contaminated with DM residue again leading to distortion in the pH readings (**Figure** 5.10b). Protective filters surrounding the probe head removed the level of DM in the immediate environment of the probe but also lead to very localized pH readings. This again resulted in an overloading with acid/base or failure to detect violation of the pH limits in the general vessel environment. These problems have not been highlighted in other validation studies. In each case the DM contamination made the probes redundant.

Teflon pH probes had previously been used to measure the pH of collected wastes and slurries in farmyard environments and were thought to be more resistant to contamination of the probe junction by DM particles. Due to financial considerations and the uncertainty of the teflon probe stability in the *in vitro* environment only one probe was prepared for this study. The teflon probe was washed and re-calibrated every morning during shut down to prevent any drift in readings and successfully maintained the pH of V4 over a 9 day period. The mean daily pH profile of all cultures is described in **Figure** 5.11. There was a greater decrease in pH post-feeding of the starch diet (V1 and V2) when compared with the fibre diet (V3) as there was no manual pH control imposed. The imposition of pH control apparently increased TVFA production (**Figure** 5.12) and the NGR albeit to a smaller extent (**Figure** 5.13) for the fibre diet.

When compared with Section 5.2 (**Figure** 5.9), the TVFA concentration for both diets had a higher pre-feed concentration and a higher peak TVFA concentration, which may be attributed to the higher LDR in the former experiment (5.6 vs. 6.3 /h, respectively). The diurnal pattern of TVFA production was very similar. The NGR ratios in Section 5.2. (**Figure** 5.7 and 5.8) had a daily mean of 1.5 and 5.8 for the starch-based and fibre-based diet respectively. The mean daily NGR in the current experiment was 1.0 and 3.2 for the starch-based and fibre-based diet respectively. Such difference may reflect the different operational conditions between the studies

i.e. pH profile, LDR and SDR, as other conditions i.e. feed input, feed composition, inocula source, temperature and anaerobic conditions were similar.

Figure 5.10 The pH probes used during the installation of pH control in the rumen semi-continuous culture were (a) ceramic probe with a protective lip, (b) ceramic probe without protective lip, (c) teflon probe without protective lip.

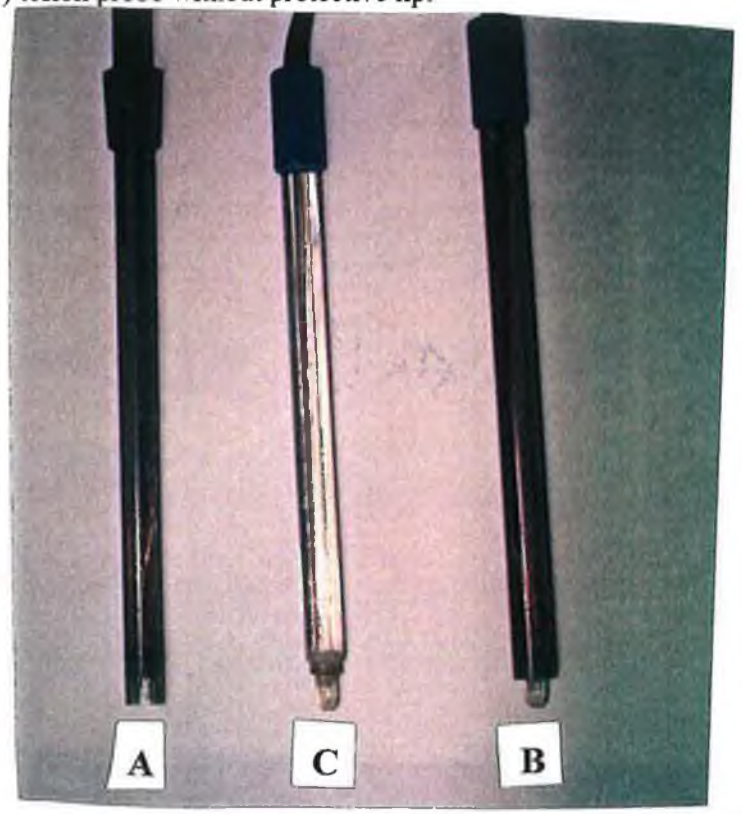


Figure 5.11 Mean pH profile of all cultures over a 9 day period, with pH control (using a telfon probe) in culture 4 only. A starch-based diet was fed to culture 1 and 2 and a fibre-based diet was fed to culture 3 and 4.

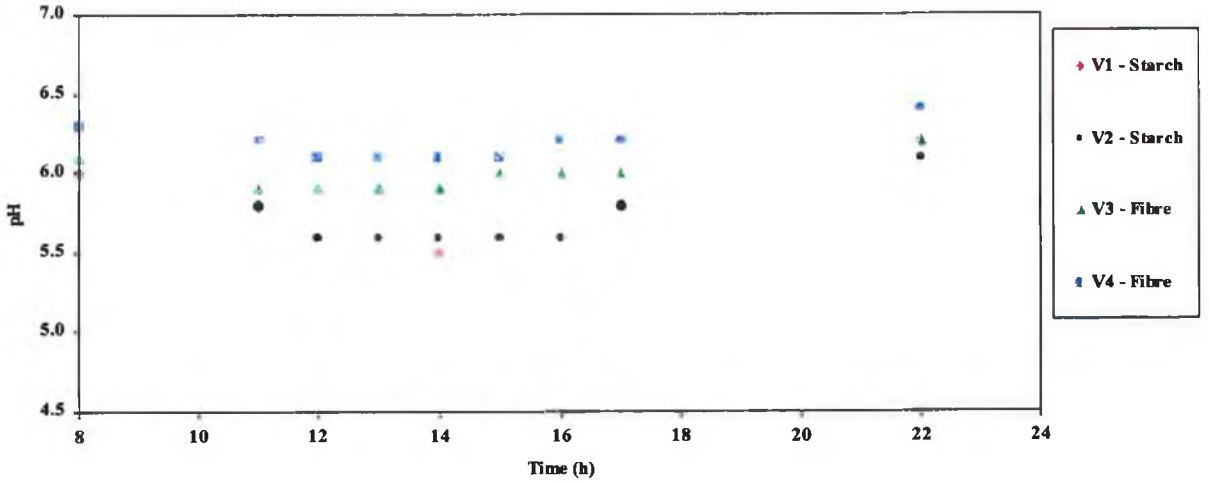


Figure 5.12 Mean total volatile fatty acid concentration of all cultures over a 3 day steady state period, with pH control (using a telfon probe) in culture 4 only. A starch-based diet was fed to culture 1 and 2 and a fibre-based diet was fed to culture 3 and 4.

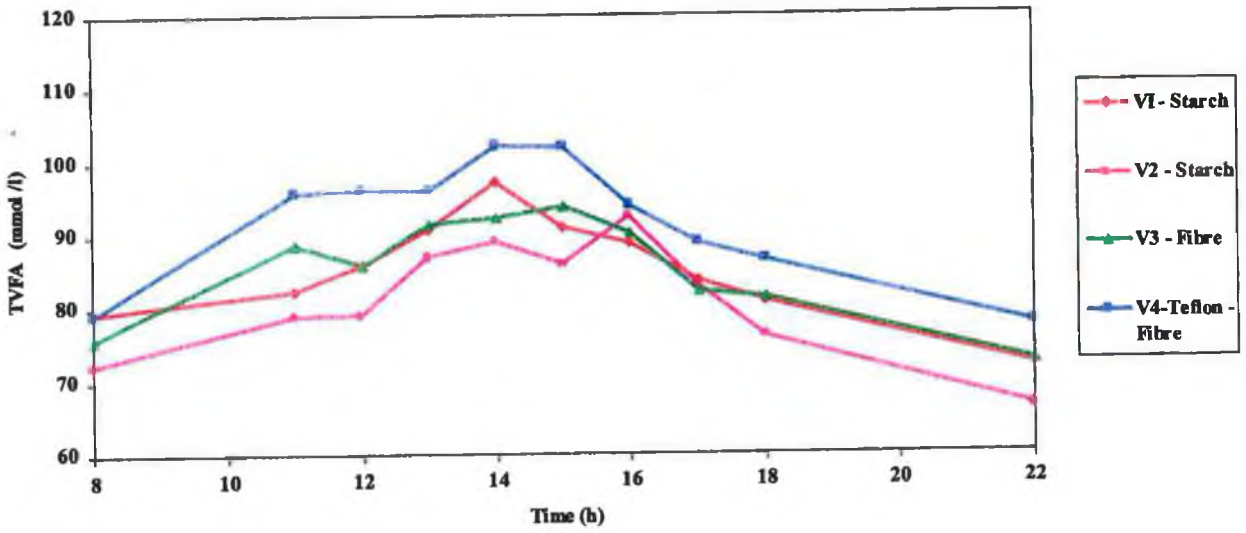
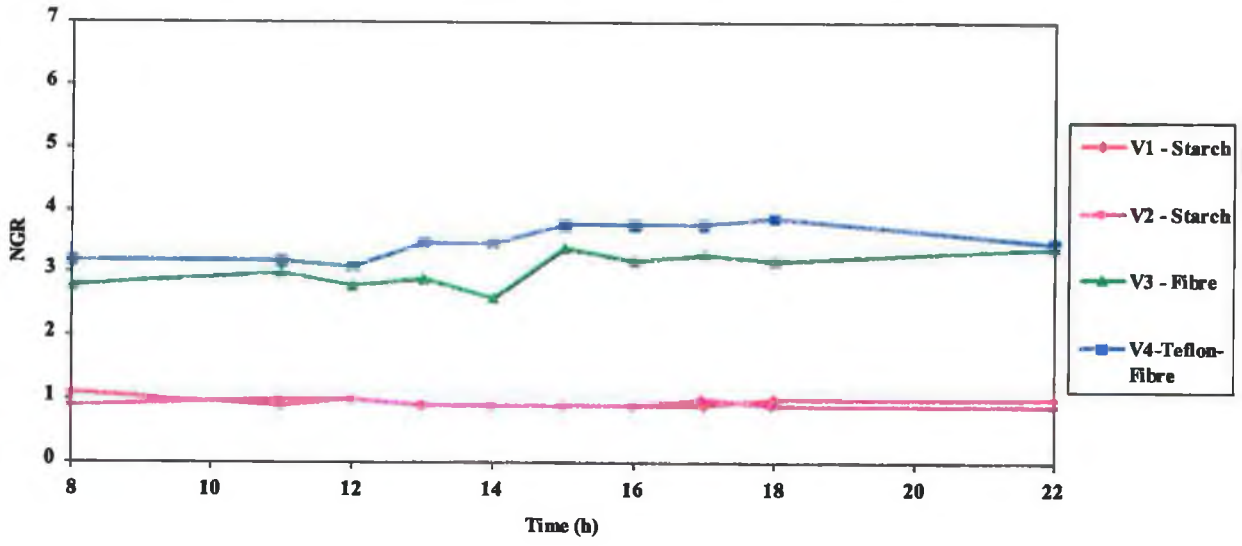


Figure 5.13 Mean non-glucogenic ratio of all cultures over a 3 day steady state period, with pH control (using a telfon probe) in culture 4 only. A starch-based diet was fed to culture 1 and 2 and a fibre-based diet was fed to culture 3 and 4.



The SDR of the system was lower than planned as the peristaltic tubing used was flawed which resulted in a rapid deterioration (within hours) in tubing integrity. Due to the restricted availability of replacement tubing the mean SDR established was 0.02 /h (**Table** 5.8). Peristaltic tubing needs to be checked frequently and replaced every second day to prevent problems with perishing and blocking due to small particles in the FE. These problems further reduced the mean estimate of SDR for V3 and V4. The alteration in SDR will confound most experimental comparisons as increasing the residence time can increase both DM digestibility and TVFA production (Hoover *et al.,* 1976a, Crawford *et al.,* 1980a, Crawford *et al.,* 1980b, Hoover *et al.,* 1982). The apparent DM digestibility estimate of each culture was improved when compared to Section 5.2, which may be attributed to the increased residence time due to lower SDR and to improvements in the sampling technique.

Table 5.8 The operational conditions (sd.) during and dry matter digestibility estimates (sd.) for the digestion of a starch-based and fibre-based diet *in vitro.*

Diet	**Starch**		**Fibre**	
Culture	**1**	**2**	**3**	**4**
LDR (%/h)	5.4	5.7	5.6	5.6
SDR (%/h)	2.4	2.0	1.5	1.2
Apparent dry matter digestibility (g/kg)	480	600	570	500

Conclusion

It is concluded that the teflon pH probe was not prone to pH drift and controlled the *in vitro* pH of the fibre based diet.

5.4 Objective

The objective was to examine the fermentation profiles of a starch and fibre diet as described by the *in vitro* RSC system and to compare the results with a concurrent *in vivo* digestibility study.

Materials and methods

In vivo

Six Friesian steers were surgically fitted with ruminal cannulas. Animals were fed twice daily at 8:00 and 22:00. Three steers were offered the starch pelleted based ration and three were offered the fibre-based pelleted ration described in **Table** 5.5. All animals had a DM intake of 8 kg concentrate and 2 kg hay (DM 876 g/kg, CP 86 g/kg DM, DMD 606 g/kg DM). The *in vivo* experimental period was 14 days in duration, with 10 days for adaptation to the diets and 3 days for sampling (Mansfield *et al.*, 1995).

On consecutive sampling days daily faeces output was recorded for each animal and a sample dried for DM estimation and chemical analysis. Rumen fluid was sampled pre-feeding and hourly for 7 h post feeding. Samples were withdrawn from four ruminal sites into a 250 ml collection vessel, using a rotary vacuum pump (Fullwood, Fullwood and Bland, England). After recording pH (Orion Digital Research Ion analyser 501 with a glass electrode) a 20 ml volume was then acidified using 1 ml 5 M H_2SO_4 and frozen at –20 ^0C for measurement of VFA, ammonia and lactic acid concentrations. Five daily samples of both concentrate diets were collected from day 5 to 10 of the *in vivo* experiment for use during the *in vitro* study (Mansfield *et al.*, 1995).

In vitro

Two RSC experimental periods were completed. Each period was 9 days in duration consisting of 6 days for adaptation to the diets and 3 SS days for sampling. Four fermenter flasks were prepared as described in Section 5.2. Ruminal fluid was sampled from each of the fistulated animals once they had adapted to their respective diets. For any dietary treatment the rumen inocula was pooled and the *in vivo* protozoal population was estimated (Section 5.3). After this inoculum from both dietary treatments was pooled and prepared in the laboratory as described in Section 2.1. The fermenter vessels were inoculated within 1 h of collection. A diluted mineral buffer solution (**Table** 2.3.1) was infused continuously into the fermenter flasks. There was no urea supplementation (Stern and Hoover, unpublished). Solid and liquid dilution rates were set at 3.0 and 6.0 %/h, respectively, by regulating buffer input and filtrate removal rates. Culture pH

was maintained between pH 6.2-6.8 in all vessels by the controlled infusion of 3 M HCl or 5 M NaOH, using Syntex teflon pH probes and on automatic pH controller. Fermenters were constantly purged with N_2 to maintain anaerobosis and temperature was maintained at 39 ^{o}C. Concentrate feeds sampled during the *in vivo* trial were pooled for each diet and the sample size of pellets reduced to 1cm or less. Two fermenter vessels were randomly assigned to each diet. Each culture received 22.5 g DM at 12 h intervals.

Sampling

Each morning the pH of each vessel was recorded using the internal probes and an external Orion probe, and a 20 ml sample of inoculum removed for protozoal estimations. The agitation and peristaltic pumps were switched off at :00. During shut down the volume of infused buffer, acid and base was recorded. The volumes of DE and FE were measured. A sample of buffer, DE and FE were frozen in duplicate for the estimation of DM content. Buffer was replenished daily and continuously mixed using magnetic stirrers. The agitation and peristaltic pumps were then switched on and feed added at 10:00. During SS days cultures were sampled for VFA, lactic acid and ammonia pre-feed and hourly for 7 hours post the morning feed. Samples (2 ml) were mixed with 200 µl 5 M H_2SO_4 and frozen at –20 ^{0}C. Displaced effluent and FE were combined on a volume ratio and 600 ml of the pooled sample was prepared for microbial protein measurement. The remaining volume was freeze-dried for subsequent laboratory analysis. Inoculum was centrifuged at 1000 g for 10 min using a Sorvall RC-3B centrifuge to remove feed residue and protozoa. The supernatant was then centrifuged at 20,000 g for 20 min., using a Sorvall RC-5B Refrigerated Superspeed centrifuge. The bacterial pellet was recovered and re-suspended in an equal volume of 0.9 % saline. Centrifugation and washing were repeated twice. The final pellet was washed in distilled water. On recovery the microbial pellet was freeze-dried and the DM measured.

Chemical analysis

Feaces samples and *in vitro* DE+FE samples were pooled for each animal and vessel, respectively , over sampling days for laboratory analysis. Concentrates were sampled during the *in vivo* trial, were pooled for laboratory analysis. All samples were measured for DM, NDF, ADF, CP, crude ash concentrations as described in Section 2. Concentrate samples were also characterised with respect to DMD, DOMD (Section 2), starch (European Communities Marketing of feedstuffs regulation, 1984- Statutory instrument no 200 of 1984), total sugar (Feeding stuffs (Sample Analysis) Regulations 1982 No. 1144) and oil B (Acid hydrolysis/ether extract, SI 200; 1984). Rumen fluid was characterised with respect to ammonia (NH, Sigma

diagnostic method for plasma ammonia, Proc No. 171-UV), lactic acid (LA, Boehringer UV-method for determination of lactic acid in foodstuffs and other materials, Cat No. 139084) and VFA (Ranfft, 1973). Microbial DM was characterised with respect to the nitrogen content (Association of analytical Chemists (AOAC) method 990-03)

Statistical analysis

Data were analysed using the statistical package of Genstat 5 (Lawes Agricultural Trust, 1990). The model used for non-periodic measurements was appropriate for a factorial analysis with terms for culture and diet, where culture refers to *in vivo* and *in vitro* systems. For periodic measurements the model used was appropriate for a three factor split-plot model with culture and diet in the main plot and time in the sub-plot. Within significant interactions, means were compared using the LSD test (Steel and Torrie, 1960).

Results

The chemical composition of the feed fractions are shown in **Table** 5.9.

The LDR and SDR, were close to the intended values and did not differ between diets (6.3 and 6.5, s.e.d 0.104 and 3.2 and 3.3, s.e.d 0.119, for starch and fibre diets respectively). There was no difference in the pH readings recorded using internal or external probes with a mean pH 6.4 ($p<0.064$). The protozoal population was significantly lower *in vitro* when compared with *in vivo* ($p<0.001$) with mean values of 0.42 and 10.5 x 10^5 cells/ml respectively (s.e.d. 0.110). There was a significant effect of time on the protozoal decline *in vitro* ($p<0.001$, **Figure** 5.14).

The effect of culture and diet on feed digestibility is shown **Table** 5.10. For *in vivo* data there were no feed refusals and digestibility results are for the complete diet (concentrate plus hay). The DMD of the fibre diet was greater in both cultures, when compared to the starch diet. Organic matter digestibility was higher in *vivo* than *in vitro* ($p<0.001$). Crude protein digestibility was lower for the fibre diet *in vivo* but was higher *in vitro* resulting in a significant culture x diet interaction. There was a significant culture x diet interaction for NDF ($p<0.05$) and ADF ($p<0.001$) digestibility which were higher for the fibre diet in both cultures, when compared with the starch diet. One animal showed a poor ability to digest NDF and ADF from the starch diet (303 and 170 g/kg DM respectively). When data from this animal were excluded, mean digestibilities *in vivo* were 434 and 302 g/kg DM for NDF and ADF respectively. There

was no significant effect of diet on microbial nitrogen produced/ day or on the efficiency of microbial protein production (g MN/kg OMD) *in vitro*.

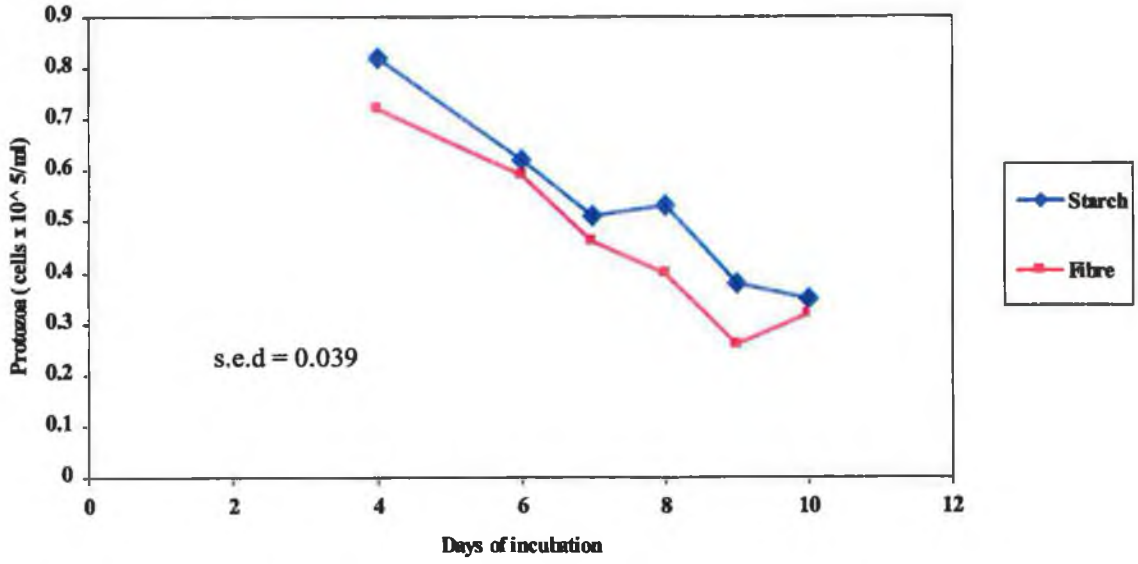
Table 5.9 Mean (sd.) chemical composition (g/kg) of starch (S) and fibre (F) diets

Component	S		F	
Dry matter (DM) (g/kg)	879	(7.8)	884.2	(10)
(g/kg DM)				
Dry matter digestibility	840.4	(16.7)	847.4	(5.8)
Organic matter digestibility	830.9	(23.2)	834.5	(6.02)
DOMD [a]	776.6	(16.9)	758.2	(4.57)
Composition of dry matter (g/kg DM)				
Crude protein	158	(5.4)	168.2	(3.5)
Ash	57	(5.4)	83	(1.2)
Starch	291.2	(15.9)	NA	
Sugar	51	(1.72)	113	(17.6)
Oil B	43.7	(1.8)	36.2	(3.8)
Neutral detergent fibre	253.2	(22.5)	301	(22.1)
Acid detergent fibre	121.8	(8.3)	159.4	(4.6)

[a] DOMD= digestible organic matter in the dry matter

NA = not assessed

Figure 5.14 The daily protozoal population decline *in vitro* during the digestion of a starch- and fibre-based diet



The effect of diet and culture on ruminal VFA production is summarised in **Table** 5.12. There was significantly greater TVFA produced during the fermentation of the starch diet ($p<0.001$). There was a significant culture x time interaction for TVFA ($p<0.001$), with the TVFA concentration higher *in vivo* between 2 to 5 h post feeding ($p<0.05$). The influence of diet on the NGR was not apparent *in vivo* but *in vitro* a greater proportion of non-glucogenic precursors (acetate and butyrate) from fermentation of the fibre diet raised the NGR. This was emphasised by a significant culture x diet interaction for acetate, propionate and butyrate ($p<0.001$, respectively), which were similar in description to that of the NGR.

The effect of culture and diet on the ruminal concentrations of lactic acid and ammonia is summarised in **Table** 5.12. Lactic acid concentration was significantly higher *in vivo* when compared with *in vitro* ($p<0.001$). An increase in concentration 1 to 4 h post feeding for the fibre diet only, to a maximum of 0.8g/l immediately after feeding compared with 0.2 g/l for the starch diet explained the significant diet x time interaction ($p<0.001$). The relative composition of lactic acid (ratio of D:L isomers, DLR) was higher for the starch diet *in vitro* ($p<0.05$) and higher for the fibre diet *in vivo* ($p<0.05$). There was a significant culture x time interaction due to a higher DLR *in vivo* immediately after feeding. This was subsequently lower than the *in vitro* DLR, 3 h post feeding. There was a significant diet x time interaction for ruminal NH_3 concentration due to the high NH_3 concentrations on the fibre diet up to 3-4 h post feeding after which there was no difference between diets. There was a significant culture x time interaction such that NH_3 concentration was significantly greater *in vivo* until 5 h post feeding when levels were similar to *in vitro* concentrations.

The *in vivo* pH profile (pH1, **Table** 5.12) showed a significant effect of time ($p<0.001$) with the pH decreasing after feeding to a minimum of pH 5.7, 4 h post feeding and rising again to pH 6.9 prior to the evening feed. The pH of the *in vitro* system was automatically controlled between pH 6.2-6.8, which caused a significant culture x diet x time interaction when compared with the *in vivo* profiles at comparable times (pH2, $p<0.001$, **Table** 5.12).

Table 5.10 Effect of culture (C) and diet (D) on the protozoal population and parameters of feed digestion and the effect of diet alone on *in vitro* microbial nitrogen production.

Culture	*In vivo*		*In vitro*		Significance[y]					
Diet	**S**	**F**	**S**	**F**	**C**		**D**		**[x]CxD**	
Protozoa (10^5 cells/ml)	10.63	10.3	0.47	0.37	***	0.110	ns	0.110	ns	0.155
Digestibility (g/kg)										
Dry matter (DM)	685	729	680	836	ns	25.8	**	25.5	ns	38.9
Organic matter (OM)	711	772	528	512	***	20.7	ns	20.5	ns	31.3
Crude protein	719[a]	658[b]	508[c]	669[b]	ns	46.7	ns	46.2	*	70.6
Neutral detergent fibre	391[d]	698[a]	495[c]	587[b]	ns	39.5	***	39.0	*	59.6
Acid detergent fibre	258[c]	650[b]	653[b]	723[a]	***	30.6	***	30.3	***	46.2
Microbial nitrogen (MN)										
g MN produced/ 45g DM			0.385	0.348			ns	0.036		
g MN produced/ kg OM digested			15.8	15.4			ns	1.34		

[x]Due to uneven replication (number of observations=4 *in vitro*, = 3 *in vivo*) the s.e.d quoted are for the minimum replicate number and thus the largest error. All other s.e.d are the min-max estimate.

[y] Means with similar subscripts are not significantly different ($p<0.05$)

Table 5.11 Effect of culture (C) and diet (D) on the volatile fatty acid (VFA) production from the ruminal microbial digestion of fibre- and starch-based diets

	Culture	Diet	Hours of sampling post feeding										Significance						
			0	1	2	3	4	5	6	7	8	12		NGR	TVFA	C2	C3	C4	Tiso
	in vivo	Starch	4.8	4.2	3.5	4.2	3.7	4.7	4.6	4.5	4.5	5.0							
Non glucogenic ratio		Fibre	5.6	4.3	4.2	4.5	4.5	4.2	4.5	4.9	5.3	4.4	**C**	***	***	***	***	***	***
(NGR)[a]	*in vitro*	Starch	1.9	1.9	1.9	1.9	1.8	1.8	1.8	1.8	1.8	1.9	**s.e.d.**	0.15	1.64	0.69	0.67	0.30	0.19
		Fibre	3.5	3.4	3.4	3.3	3.4	3.4	3.5	3.5	3.5	3.5							
													D	***	***	***	***	***	***
	in vivo	Starch	63.3	79.7	113.3	126.0	123.5	107.0	96.3	91.6	88.0	64.6	**s.e.d.**	0.15	1.62	0.69	0.66	0.30	0.19
Total VFA		Fibre	66.5	96.6	104.2	107.8	99.4	87.9	87.1	84.7	76.9	55.7							
(Mmol /l)	*in vitro*	Starch	68.2	73.6	79.6	81.1	83.1	82.8	84.0	76.7	77.7	69.3	**T**	ns	***	ns	ns	ns	ns
		Fibre	63.1	70.9	76.8	80.9	81.0	79.7	77.1	75.3	73.5	62.3	**s.e.d**	0.33	3.62	1.53	1.47	0.66	0.43
(mMol/100mol)																			
Ethanoic (C2)	*in vivo*	Starch	65.1	62.6	59.9	61.8	59.8	63.3	63.4	63.0	60.5	63.0	[c]**CxD**	***	ns	***	***	***	**
		Fibre	69.2	64.2	63.88	64.1	64.6	64.7	65.7	66.7	69.1	66.4	**s.e.d**	0.23	2.48	1.04	1.01	0.45	0.29
	in vitro	Starch	48.8	48.6	48.7	48.5	48.4	48.4	48.4	48.6	48.8	49.5							
		Fibre	59.9	59.4	58.5	58.3	58.8	59.4	59.6	59.8	59.9	60.4	[c]**CxT**	ns	***	ns	ns	ns	ns
													s.e.d	0.51	5.53	2.33	2.25	1.02	0.66
	in vivo	Starch	19.7	21.6	25.3	21.9	24.1	20.1	20.4	21.1	22.5	20.6							
		Fibre	16.5	20.9	21.3	20.3	20.0	21.1	19.9	19.0	17.3	19.9	**DxT**	ns	ns	ns	ns	ns	ns
Propanoic (C3)	*in vitro*	Starch	36.3	36.0	36.4	36.6	36.8	36.9	37.0	36.9	36..7	35.9	**s.e.d**	0.47	5.12	2.16	2.08	0.94	0.61
		Fibre	24.3	24.7	24.9	25.4	25.1	24.7	24.6	24.4	24.3	24.0							
													[c]**CxDxT**	ns	ns	ns	ns	ns	ns
	in vivo	Starch	10.3	11.7	11.1	12.9	12.1	12.9	12.5	12.0	12.2	11.7	**s.e.d.**	0.72	7.83	3.29	3.18	1.44	0.93
		Fibre	11.3	12.3	12.5	13.0	12.8	11.5	11.3	11.5	11.3	9.0							
Butyric (C4)	*in vitro*	Starch	8.6	8.8	8.7	8.8	8.5	8.4	8.5	8.4	8.4	8.2							
		Fibre	12.0	12.4	12.9	12.7	12.7	12.5	12.4	12.3	12.2	11.9							
	in vivo	Starch	3.4	2.5	1.8	1.7	2.1	2.1	2.0	2.1	2.8	3.2							
Total branched (Tiso)[b]		Fibre	2.4	1.6	1.4	1.5	1.3	1.5	1.6	1.6	1.6	3.2							
	in vitro	Starch	3.6	3.7	3.7	3.6	3.6	3.6	3.5	3.5	3.5	3.5							
		Fibre	2.1	1.9	2.0	2.0	2.0	2.0	2.0	2.0	2.1	2.1							

[a]NGR calculated as the {(acetate +2butyrate)/propionate]

[b]Total branched VFA calculated as the [iso-butyric + iso-valeric]

[c]Due to uneven replication (number of observations=4 *in vitro*, = 3 *in vivo*) the s.e.d quoted are for the minimum replicate number and thus the largest error. All other s.e.d are the min-max estimate.

Table 5.12 Effect of culture (C) and diet (D) on lactic acid (LA) concentration, ammonia (Amm) concentration and rumen pH during the ruminal microbial digestion of starch- and fibre-based diets.

	Culture	Diet	Hours of sampling post feeding										Significance					
			0	1	2	3	4	5	6	7	8	12		LA	DLR	Amm	pH1	pH2
														0.129	0.16	22.47		0.11
Lactic acid (g/l)	*in vivo*	Starch	0.12	0.30	0.19	0.20	0.13	0.16	0.13	0.12	0.11	0.11						
		Fibre	0.16	1.0	0.62	0.30	0.18	0.15	0.14	0.15	0.15	0.12	**C**	***	ns	***		ns
	in vitro	Starch	0.06	0.10	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.05	**s.e.d.**	0.027	0.03	4.70		0.05
		Fibre	0.07	0.56	0.36	0.17	0.10	0.09	0.08	0.08	0.08	0.07						
													D	***	ns	***	ns	ns
D + L ratio	*in vivo*	Starch	1.4	1.6	1.4	1.2	1.4	1.1	1.5	1.4	1.4	1.3	**s.e.d.**	0.027	0.03	4.65	0.14	0.05
		Fibre	1.4	2.0	1.4	1.3	1.4	1.4	1.5	1.4	1.4	1.4						
	in vitro	Starch	1.5	1.3	1.3	1.7	1.6	1.6	1.5	1.5	1.5	1.7	**T**	***	*	***	***	***
		Fibre	1.6	1.0	0.9	1.2	1.5	1.6	1.6	1.5	1.5	1.5	**s.e.d**	0.060	0.08	10.40	0.19	0.04
													[a]CxD	ns	***	ns		ns
Ammonia (mg/l)	*in vivo*	Starch	126.4	115.8	76.9	65.9	34.9	39.3	33.1	30.9	35.1	88.7	**s.e.d**	0.041	0.05	7.10		0.08
		Fibre	167.1	213.2	186.3	102.8	59.6	35.3	31.8	34.8	39.2	82.9						
	in vitro	Starch	33.7	45.8	35.0	25.6	20.7	23.5	25.2	26.0	30.0	41.0	**[a]CxT**	ns	***	***		***
		Fibre	48.1	66.5	65.7	56.9	42.4	29.2	26.1	25.6	30.3	50.7	**s.e.d**	0.091	0.12	15.89		0.08
pH1	*in vivo*	Starch	7.0	6.3	5.7	5.3	5.4	5.5	5.3	6.1	6.3	7.0	**DxT**	***	ns	*	ns	**
		Fibre	7.1	6.2	6.0	5.9	5.9	6.0	6.2	6.4	6.6	6.8	**s.e.d**	0.084	0.11	14.71	0.29	0.07
pH2	*in vivo*	Starch	7.0	6.3				5.5			6.3	7.0	**[a]CxDxT**	ns	ns	ns		***
		Fibre	7.1	6.2				6.0			6.6	6.8	**s.e.d**	0.129	0.16	22.47		0.11
	in vitro	Starch	6.5	6.3				6.3			6.4	6.6						
		Fibre	6.6	6.3				6.3			6.3	6.7						

[a]Due to uneven replication (number of observations=4 *in vitro*, = 3 *in vivo*) the s.e.d quoted are for the minimum replicate number and thus the largest error. All other s.e.d are the min-max estima

Discussion

The study of rumen digestion *in vivo* is complex due to the difficulty in accurately describing the influence of dependent and /or independent physiological processes on the measured parameter. *In vitro* methods are focused on experimental control and whether batch or continuous (Czerkawski, 1986, Stern *et al.,* 1997) the system should not be limited or altered by any experimental parameter other than that under examination.

The Rusitec system was designed as a closed system (Czerwaski, 1974). The feeding method of the system is such that each vessel contains a perforated polyethylene container which holds two nylon bags, one filled with rumen solid digesta and the other with the experimental substrate. This optimises the development of a uniform rumen microbial population by introducing solid-associated microbes, while the provision of a solid mat matrix enhances the survival of the protozoal population (Carro *et al.,* 1995). However, the LDR is directly related to the rate of saliva input, it lacks pH control and results can be influenced by method of *in vitro* feed containment (Carro *et al.,* 1995).

With a view to examining the influence of ensiling (and maturation) on the inherent ruminal digestion parameters of perennial ryegrass forages (Section 6.4) the dual flow system of Hoover *et al.* (1976a, 1976b) was chosen. In the dual flow system the LDR and SDR are independent and controlled by buffer input and a filtered withdrawal of vessel liquid, respectively. Manual feeding allowed for diurnal variations in the *in vitro* environment to be evaluated. The system allowed for solid feed input at variable rates without disruption of fermenter function. *In vivo,* maturity and ensiling will influence DM intake and particle retention time, microbial protein production and diurnal variations of soluble carbohydrate and nitrogen fractions in the rumen, all of which have implications for forage nutritive value. In attempting to quantify only the intrinsic characteristics of forage digestion, the control of LDR, SDR, feed input and pH was considered to be important.

The vessel contents are homogenous which allows for pH control but not the simulation of *in vivo* compartmentation (Czerkawski and Breckenridge, 1977). Due to the lack of sequestration protozoal numbers are always significantly lower during SS days than that measured in concurrent (Mansfield *et al.,* 1996) or reported (Hannah *et al.,* 1986) *in vivo* studies.

For validation, most systems have been compared with experimental data from published literature (Abe and Kumeno, 1973, Hoover *et al.* 1976a, Czerkawski and Brenkenridge, 1977, Estell *et al.*, 1982, Merry *et*

al., 1987). With concurrent *in vivo* validations the number of experimental parameters which were statistically compared varied between studies (Slyter and Putnam 1967, Hannah *et al.*, 1986, Mansfield *et al.*, 1994, Prevot *et al.*, 1994).

Environmental comparisons

In this study the *in vivo* and *in vitro* fermentation characteristics of two diets differing in carbohydrate composition were examined. *In vitro* environmental parameters such as LDR, SDR, temperature and pH were controlled and did not differ between diets. This is in contrast to the natural variation seen *in vivo*. The *in vivo* pH profile was significantly affected by time after feeding with a minimum pH reached 4 h post feeding. The continuous mixing within each culture in this study, like others (Hoover *et al.*, 1976, Hannah *et al.*, 1986, Merry *et al.*, 1987) creates an homogenous environment in the vessel interior. Work by Fuchigami *et al.* (1989) showed that intermittent stirring resulted in stratification of residues in the vessel interior with differential flow rates from 0.035 to 0.069 /h. Influential effects of stratification on ruminal flow dynamics is supported by the work of Czerkawski *et al.* (1991) using the Rusitec system and the *in vivo* work of Faichney (1986). Dual flow systems with continuous mixing therefore do not simulate the true rumen environment.

Microbial populations

The validity of any *in vitro* study will be dependent on the ability of the system to maintain a microbial population representative of the *in vivo* community. Differences in microbial ecology can affect total carbohydrate digestion, (Mendoza *et al.*, 1993), bacterial efficiency (Viera, 1986) and microbial composition and utilisation of nitrogen sources (Viera, 1986, Williams, 1986, Schadt *et al.*, 1999). Though the *in vivo* LDR and SDR were not measured in this study, previous work by Hannah *et al.* (1986) and Mansfield *et al.* (1995) suggest that the LDR and SDR of concentrate-fed bovines could be as high as 0.13 /h and 0.06 /h, respectively.

There is difficulty in maintaining protozoal numbers and populations in continuous systems due to lack of sequestration to facilitate their longer generation times relative to some bacteria, first noted by Weller and Pilgrim (1974). Optimising conditions to retain this population has been examined (Hoover *et al.*, 1976a, Merry *et al.*, 1983, Abe and Kuihara, 1984, Teather and Sauer, 1988, Fuchigami *et al.*, 1989, Broudiscou *et al.*, 1997). Levels of 10^4 to 10^5 have been achieved in most cases but holotrich species are nearly always lost (Slyter and Putnam, 1967, Abe and Kumeno 1973, Hannah *et al.*, 1986, Mansfield *et al.*, 1994).

Intermittent or slow agitation at 100 rev./min. appear to be the most advantageous treatments in dual flow continuous cultures for optimising protozoal retention.

The *in vitro* system in this study was operated at lower rates of dilution (Crawford *et al.,* 1980, Merry *et al.,* 1987) when compared with Hoover *et al.* (1976) and Mansfield *et al.* (1995) and low agitation speeds of 60 rev./min. to improve the retention of the protozoal population. The protozoal population declined significantly *in vitro* though the steady state values are similar to other *in vitro* studies (Abe and Kumeno, 1973, Hoover *et al.,* 1976, Merry *et al.,* 1987, Miettinen and Setala, 1989). A reduction in the protozoal population may support increased microbial efficiencies and viable bacterial counts *in vitro* (Mansfield *et al.,* 1994).

Bacterial populations were not examined in this study but Slyter and Putnam (1967) found no significant differences between *in vivo* and *in vitro* bacterial cultures with common changes between physiological groups and composition of these groups. Mansfield *et al.* (1995), examining the fermentation characteristics of 2 non-fibrous carbohydrates and 2 levels of degradable protein in a comparative study between *in vivo* and *in vitro* fermentations, found that though the total viable population of bacteria increased, the amylolytic and proteolytic populations were relative stable in number, while lower cellulolytic numbers *in vitro* were thought to reflect the negative effect of high dilution rates on slow generating cellulolytic bacteria. It may be assumed that in an *in vitro* environment with low dilution rates, the composition of the microbial population should not vary greatly from that *in vivo* though this remains to be confirmed.

Feed digestibility

In this study the *in vivo* digestibility values are estimates of total tract digestion while the RSC reflects ruminal digestibility only. Total tract digestion is the sum of microbial and acid hydrolysis of the ingested substrate in the rumen, small and large intestine. Galyean and Owens (1991) suggest that rumen, small and large intestine OMD digestibilities are approximately 56.2 to 64.4, 26.3 to 33.7 and 4.2 to 16.7 % of total organic matter digested. The small intestine is the main site of nutrient absorption (Church, 1988). Owens *et al.* (1984) suggest that microbial and feed nitrogen disappearance in the small intestine can be 68 and 73 %, respectively. A residual fermentation in the lower intestine will increase the microbial nitrogen content of voided faeces, which may affect *in vitro* and *in vivo* comparisons of CP degradability in the present study.

The DMD was significantly higher for the fibre diet in both cultures. The difference in feed digestibility *in vivo* was greater than predicted by the Tilley and Terry *in vitro* estimate (**Table** 5.5) but the mean *in vivo*

and *in vitro* Tilley and Terry total tract estimates of DOMD were similar (742 and 774 g/kg DM respectively, **Table** 5.10). The proportion of total tract digestibility attributed to the rumen for the starch and fibre based diets, according to Galyean and Owens (1991), would be 456 and 494 g/kg DM respectively, which are lower than *in vitro* findings.

Higher *in vitro* estimates of OMD have previously been reported (Hannah *et al.,* 1986). Mansfield *et al.* (1994) found that the *in vitro* OMD of diets with low nonstructural carbohydrate content (25 % NSC) were similar to *in vivo* measurements, but that this relationship did not hold for high (40 %) NSC diets where the *in vitro* DMD of NSC was > 90 %, with fibre digestion reduced. This was attributed to the gelatinization of the starch during pelleting and the increased susceptibility of the starch to rapid ruminal degradation, with a subsequent negative effect on fibre digestion. In this study the *in vitro* feed was not subjected to any additional processing. The greater OMD *in vitro* may reflect a greater residential time (33 h, SDR=0.03 /h) compared within *in vivo* estimates of 17 h as cited by Mansfield *et al.* (1994).

There was a significant culture x diet interaction for fibre digestion. Lower i*n vivo* NDFD and ADFD for the starch-based diet when compared with the fibre diet were exaggerated by very low estimates from one animal in particular. There was no effect of diet on ruminal pH *in vivo* eliminating an inhibitory effect of reduced pH on NDFD and ADFD. A constant DMI of 8 kg concentrate and 2 kg hay DM, with no refusals, for each diet would suggest that the *in vivo* LDR should not have differed greatly between animals. This animal showed no signs of poor health nor had any feed refusals during the complete trial. The lower *in vivo* estimates from this animal are therefore attributed to random animal variation. Animal variation is not an unusual phenomenon and may be addressed using a latin square designed study where the individual animal variation would be spread over diet type (Hannah *et al.,* 1986, Mansfield *et al.,* 1995).

Neutral detergent fibre digestibility was higher *in vivo* for the fibre diet and higher *in vitro* for the starch diet. *In vivo* estimates describe total tract digestion, therefore a lower *in vivo* NDF digestibility for the starch-based diet is surprising. This may be associated with the lower *in vivo* pH. Total VFA concentration was greater for the starch diet and a significant increase in TVFA concentration *in vivo* post feeding may suggest that the high DMI (relative to the *in vitro* system) may have caused extreme diurnal variations in readily available carbohydrate concentrations. High levels *in vitro* have been associated with the suppression of microbial colonisation of fibre, which is independent of pH (Pwionka and Firkins, 1993). The ADFD was higher for both diets *in vitro,* which suggests that the longer ruminal retention times were more effective at optimizing ADFD than lower tract fermentation in the *in vivo* situation. Crude protein

digestibility did not differ between diets *in vivo* but was higher for the fibre diet *in vitro*, which is supported by an increase in NH_3 concentration 3-4 h post feeding for fibre diets in both cultures. This is discussed later in relation to *in vitro* ammonia concentrations.

Soluble nutrients in the ruminal environment

Total VFA production was greater for the starch when compared with the fibre diet and was significantly higher *in vivo* than *in vitro* with a maximum peak *in vivo* 3 to 4 h post feeding. *In vitro* levels also reached a peak at 4 h post feeding, similar to results found in the preliminary developmental trials. The *in vitro* levels are similar to those reported by Merry *et al.* (1987). The TVFA concentration *in vivo* is partially regulated by the absorption of volatiles across the rumen wall (Chamberlain *et al.*, 1983, Gaebel *et al.*, 1987, Dijkstra, 1994) and in the absence of this physiological absorption it may be expected that the *in vitro* levels should exceed those *in vivo* (Hannah *et al.*, 1986, Mansfield *et al.*, 1995). However the higher *in vivo* values reflect the higher DMI intake relative to the *in vitro* system and the rapid microbial breakdown and metabolism of the ingested feeds, which is supported by the lactic acid data. All of the VFA proportions and the NGR had a significant culture x diet interaction, which may represent the influence of *in vivo* absorption that does not apply *in vitro*.

The digestible carbohydrate fraction of the fibrous diet (beet pulp, dried grass and citrus pulp) supported a greater increase in the lactic acid concentration in both cultures when compared with the starch diet. There was no effect of the elevated lactic acid concentration on the NGR *in vivo* but there was a significant increase in non-glucogenic precursors *in vitro*. Lactic acid is quickly metabolised in the rumen supporting a propionic type fermentation (Chamberlain *et al.*, 1983, Newbold *et al.*, 1987), and was metabolised on a molar basis, in the rumen of silage-fed steers to 0.21 acetate, 0.52 propionate and 0.27 butyrate (Jaakola and Huhtanen, 1992). Gill *et al.* (1986) concluded that lactate was metabolised in the rumen of sheep fed perennial ryegrass at hourly intervals to 0.6 acetate, 0.35 propionate, 0.05 butyrate. Lactic acid may also be absorbed directly from the rumen (Waldo and Schultz, 1956 cited by Gill *et al.*, 1986).

A high NGR may reflect the influence of the residual protozoal population as lactate fermentation in the rumen may be 15 times greater for protozoal populations than bacterial (0.133 - 1.12 g/g protozoal protein/h), with metabolism associated only with entodiniomorphid species (Newbold *et al.*, 1987). Protozoal populations could be responsible for 30 % of VFA production from lactate (Newbold *et al.*, 1986, Newbold *et al.*, 1987), producing mainly acetic and butyric acids (Chamberlain *et al.*, 1983). As there is no

selective utilisation of d- or l-lactate by rumen microorganisms (Chamberlain *et al.*, 1983) the significant culture x diet interaction may represent the influence of *in vivo* absorption.

The CP content was 16 and 17 g/kg DM for starch and fibre, respectively and therefore the urea supplement was not included in the infused buffer, based on the recommendation of Mansfield and Stern (unpublished), who suggest inclusion if CP is lower than 15 %. There was a significant culture x time and diet x time interaction in this study for ammonia concentration but the culture means were low at 80 and 37 mg/l for *in vivo* and *in vitro* respectively.

Previously reported NH-nitrogen concentrations *in vitro* were higher than reported here (206 mg/l, Merry *et al.,* 1987, 141 mg/l, Mansfield *et al.,* 1995). Mansfield *et al.* (1994) reported *in vivo* concentrations of 156 mg/l and *in vitro* concentrations of 141 mg/l, with urea supplementation *in vitro*. When the recommended urea supplement is omitted Schadt *et al.* (1999) studying the *in vitro* digestion of alfalfa hay, reported ammonia concentrations as low as 12.2 mg/l, with dietary CP of 15.7 g/ kg DM. Satter and Slyter (1974) suggest that 50 mg NH-nitrogen/l is the minimum level for optimum cellulolytic activity, which would suggest that fibre digestion *in vitro* may have been limited. However a restriction on digestion *in vitro* is unlikely due to the high NDFD and ADFD ruminal estimates obtained. Many studies have shown that for diets composed of a digestible NDF fraction, peptide supplementation rather than urea supplementation optimises *in vitro* ruminal digestion (Maeng and Baldwin, 1975, Argle and Baldwin, 1989, Merry *et al.*, 1990, Griswold *et al.,* 1995) which may have been applicable in this study as the CP content is presumed to be readily available due to the high DMD (**Table** 5.10).

Ammonia concentration was influenced both by culture type as concentrations were greater *in vivo,* and by the effect of dietary source on the diurnal variation. Both cultures showed diurnal variation, as ammonia concentration increased 1 h post-feeding and subsequently declined with NH_3 reaching a minimum 4 h post feeding for the starch diet and 6 h post feeding for the fibre diet. However, higher *in vivo* concentrations and an increase in the *in vivo* pre-feed NH_3 concentration, that was not simulated *in vitro*, may be attributed to urea recycling and/or microbial protein recycling *in vivo*, in the absence of available dietary nitrogen. Urea recycling may be expected to make a large contribution to immediate pre-feed values as mastication and prevention of ruminal acidosis causes an increased influx of saliva, which contains soluble urea. A five to six fold decrease in *in vivo* ammonia concentrations 5 h post feeding to levels similar to *in vitro* would suggest the influence of absorption (greater at pH<6.5), and dilution from the rumen or microbial utilisation. The higher ammonia concentration on the fibre diet may reflect the CP intake.

Microbial protein production

The efficiency of microbial protein production was lower than values normally quoted for ruminal digestion (mean 32 g MN/kg OMD, ARC) but not outside the range of values reported in *in vitro* studies. Microbial protein yields are dependent on the system and the maintenance energy demands it places on the population. Meng *et al.* (1999) reported levels as low as 23.6 and 18.9 g MN/kg OMD for a basal diet of soya hulls and ground corn respectively, at a dilution rate of 0.05 /h. Schadt *et al.* (1999) found that microbial efficiency decreased from 29.9 to 20 g MN/kg OMD as the SRT increased from 10 to 30 h at a dilution rate of 12 %/h. As yields decrease with decreasing dilution rate this would suggest that yields at a dilution rate of 0.05 /h would be lower again. In batch systems examining nitrogen preferences, Maeng and Baldwin (1975) found MN production increased as amino acid nitrogen replaced urea, quoting levels of 13.2 to 15.8 g MN/kg OMD. Argle and Baldwin (1989) found that microbial nitrogen yields on purified substrates (glucose, cellobiose, pectin, starch) were 5.2 g N/kg OMD (urea nitrogen only) up to 20.4 g MN/kg OMD (amino acids and peptide nitrogen).

Microbial protein was estimated by measuring total nitrogen in the isolated microbial pellet, as previously reported (Hoover *et al.,* 1984). Alternative methods for microbial protein estimation are diaminopimelic (**DAPA**) and aminoethylphosphate acid (**AEP**, Czerwaski, 1974) for bacteria and protozoa respectively, purine content (Zinn and Owens, 1986), external markers such as N^{15} and P^{32} (Merry *et al.,* 1984, Calsamiglia *et al.,* 1996) and D-Alanine (Garrett *et al.*, 1987).

The accuracy of any method depends on obtaining a representative relationship between the marker and total microbial nitrogen. The ideal microbial marker should 1) not be present in feed, 2) be biological stable, 3) have a relatively simple assay, 4) occur in similar percentages for all microbes, 5) be a constant percentage of the microbial cell at all growth stages. Aminoethylphosphate acid has been found in bacterial cells (Whitelaw *et al.,* 1984) and DAPA may vary with substrate (Schadt *et al.,* 1999). Garrett *et al.* (1987) compared D-Alanine and DAPA as bacterial markers and found that the coefficient of variation for the marker:N ratio was less with D-alanine but concluded that the cellular ratio was not consistent within *in vitro* incubations and between *in vitro* and *in vivo* microbial samples from similar dietary sources. Purine concentration can vary with sample preparation (Ha and Kennelly, 1984), sampling time after feeding (Cecava *et al.,* 1990) and microbial species (Firkins *et al.,* 1987). Digestion of feed purines has been found to vary *in vivo* (Djouvinov *et al.,* 1998) but not *in vitro* (Calsamiglia *et al.,* 1996). The purine assay is complex, labour intensive and has been adapted on many occasions (Ushida *et al.,* 1985, Obispa and

Dehority, 1992, Calsamiglia *et al.*, 1996). As all methods are dependent on an initial estimation of the total nitrogen content of a sampled population it was decided to use a measurement of Kjeldahl nitrogen as the estimate of microbial protein production.

Without a marker, microbial protein may be overestimated due to feed contamination (Van Soest, 1994) as ruminal feed particles can exist in the size range of bacteria (Pichard, 1977). The mean nitrogen content of all isolated microbial DM fractions was 7 % DM, which is supported by the study of Merry *et al.* (1987). A lack of variability in the ratio between studies, and within treatments would suggest little if any feed nitrogen contamination. Low yields of microbial nitrogen were attributed therefore to low DM yields. The isolated pellet was washed three times to remove residual nitrogen contamination. It is unlikely that repeated washing steps would result in excessive losses of DM as this procedure has been used by other authors without comment (Schadt *et al.*, 1999, Meng *et al.*, 1999). It is concluded therefore that these low yields are representative of the true microbial protein yield in the system.

Microbial protein synthesis calculated for the *in vitro* system and protozoal numbers did not differ between diets. This would indicate that differences in protein degradability between the two diets had no effect on microbial recycling or efficiency in microbial production.

Conclusion

It is concluded from 5.4 that

- the RSC controlled all environmental (LDR, SDR, pH) conditions without significant variation and was not subject to the unplanned influences, such as animal variation as seen *in vivo*
- the operational conditions of the RSC maintained protozoal numbers at levels which are typical for *in vitro* dual flow systems
- the RSC can qualitatively simulate the ruminal diurnal trends in the *in vivo* soluble pool post feeding for TVFA, LA and ammonia. Quantitative differences are attributed to the effect of absorption, dry matter intake and flow dynamics *in vivo*.

Implications

Due to the obvious design and operational conditions the *in vitro* system was not expected to simulate directly *in vivo* fermentation, rather it is a system designed to describe a process of digestion that is influenced only by the inherent nature of the substrate or the specific operational conditions of the system.

This is in agreement with the conclusions of Tamminga and Williams (1998) such that 'the role of *in vitro* methods in the prediction of nutrient supply probably lies more in helping to elucidate the mechanisms underlying digestive processes than in giving straight forward predictions of nutrient supply'.

The application of this system to the study of fresh silages is unlikely due to the difficulties in fresh forage input and the potential difficulties in solid digesta flow dynamics. Fresh forages can be used in the Rusitec system. However to examine the effect of forage maturity and ensiling on *in vitro* digestion kinetics the control of pH, LDR and SDR are important which necessitate a dual flow system.

CHAPTER 6

THE IMPACT OF ENSILING *PER SE* ON THE *IN VITRO* FERMENTATION OF PERENNIAL RYEGRASS WATER SOLUBLE CARBOHYDRATE AND CELL WALL FRACTION

Introduction

In Chapter 3 and Chapter 4 it was concluded that ensiling did not affect the ruminal AED of the isolated structural carbohydrate fraction. It was also concluded that supplementation with the soluble fraction and nitrogen pre- and post- ensiling influenced the AED of the structural carbohydrate fraction. The latter work suggested that the while beneficial effects of supplementation may reflect peptide restriction in the substrate, the adverse effects on cell wall digestion may have been artifacts of the batch *in vitro* systems.

Microbial fermentation of carbohydrate and protein fractions during ensiling creates a pool of short chain fatty acids and proteolytic endproducts (McDonald *et al.,* 1991). These alterations may decrease the nutritive potential of the soluble pool (Chamberlain, 1987). The effect of ensiling on MP and VFA production from the soluble pool was examined in Section 6.2.

To study the effect of ensiling on the nutrient potential of a perennial ryegrass soluble fraction, a solution representative of the WSC fraction pre- and post-ensiling was prepared from the work of O'Kiely (1993). In preliminary studies this substrate had a pH <1.0 due to high VFA concentrations. Microbial activity can be influenced by pH and VFA concentration making it difficult therefore, to examine and characterise the *in vitro* microbial fermentation of the isolated WSC fraction post-ensiling (Johnson *et al.,* 1958, Peters *et al.,* 1989, Grant and Mertens, 1992c, Grant and Weinder, 1992, Getachew *et al.,* 1998). In preliminary studies the use of buffers with a high buffering capacity (Piwonka and Firkins, 1996) was not sufficient to stabilise the pH. Decreasing the substrate to buffer ratio decreased the initial VFA concentration of the system, but not sufficiently to stabilise the *in vitro* pH. Therefore in order to examine the nutrient potential of the soluble fraction post-ensiling it was necessary to develop a method of neutralisation of the substrate prior to inoculation (Section 6.1).

Biochemical alterations can influence the digestion of the structural fractions *in vitro.* Fibre digestion can be adversely influenced by VFA concentration (Johnson *et al.,* 1958, Peters *et al.,* 1989) and the associated decrease in environmental pH (Mould *et al.,* 1984, Russell, 1987, Grant and Mertens, 1992c). As described in Chapter 3 and Chapter 4 these factors may potentially confound batch studies, thus distorting the true effect of ensiling on the *in vitro* digestibility of the cell wall fraction. The objective of

section 6.3 was to assess the importance of the soluble fraction for perennial ryegrass digestion. The RSC *in vitro* system was used to alleviate endproduct inhibition. To assess the importance of ensiling on the soluble fraction and subsequent ruminal digestion of NDF, the cell wall fraction was defined as F20 and not the F70 aqueous extract (See section 2.3). The *in vitro* systems would therefore more closely simulate the total nutrient intake of ingested perennial ryegrass forage and subsequently describe the ruminal nutritive potential of the experimental treatments. To assess the importance of proteolytic alterations during ensiling on subsequent ruminal digestion and MP production, the system was operated under ammonia-excess conditions, with peptide nitrogen availability defined by the experimental treatments solely.

6.1 Objective

To develop a system of substrate neutralisation, which would stabilise the *in vitro* pH of a simulated silage water-soluble carbohydrate fraction pre-inoculation and to determine if substrate neutralisation altered the subsequent *in vitro* fermentation pattern of the residual water-soluble carbohydrate fraction.

Materials and methods

Substrate preparation

The ratio of carbohydrates in the water-soluble carbohydrate fraction of perennial ryegrass was assumed to be 2.81:1.51:2.25:14.3 for fructose, glucose, sucrose and fructan (degree of polymerisation =25), respectively (McGrath, 1988) (**GS**). The chemical composition of the simulated substrate for the water-soluble fraction of silage is described in **Table** 6.1. Substrates were prepared in a 400 ml volume of Buffer 1 (**Table** 6.2) and were stored at 4 ^{0}C.

Table 6.1 The chemical composition of the water-soluble carbohydrate (WSC) fraction of ensiled perennial ryegrass[a]

	WSC	Lactic Acid	Acetic Acid	Propionic Acid	Butyric Acid	Ethanol
Residual g/75 g WSC ensiled	11.4	29.5	37.1	8.6	26.6	11.4
Equivalent to 0.5 g sugar	0.08	0.2	0.25	0.06	0.18	0.08

[a] Based on the work of O'Kiely (1993) and prepared in 10 ml of Buffer 1.

Table 6.2 Chemical composition of *in vitro* buffers[a].

Components (g/l)	Buffer 1	Buffer 2
$NaHCO_3$	11.50	9.80
Na_2HPO_4	2.28	1.43
KH_2PO_4	2.48	1.55
$MgSO_47H_2O$	0.12	0.15
Micromineral	1.00	
Casein	5.00	
NH_4HCO_3	1.80	

[a]All buffers were gassed for 3 h using CO_2

Substrate neutralisation

One hundred millilitres of the simulated substrate (**Table** 6.1) was titrated with 1M NaOH and the pH recorded 5 min. after alkali addition. This was repeated until pH 5.0 was reached (**NaES, Table** 6.3). The NaES was then added to a fixed volume of Buffer 1 based on a 1:8 ratio respectively (Goering and Van Soest, 1970). The pH drift of the solution was recorded until it became stable (**pHB**). To examine the effect of NaOH inclusion on the *in vitro* fermentation, the simulated silage water soluble fraction was prepared and diluted to an equivalent volume as NaES with distilled water (**ES**).

Inoculum preparation

Inoculum preparation was as described in Section 2.1

In vitro technique

The gas pressure transducer (Theodorou *et al.*, 1994, Section 1.4.2.2).

In vitro procedure

Serum bottles were prepared 18 h before inoculation. Under anaerobic conditions, 85 ml Buffer B and 4 ml reducing solution (**Table** 2.1.2) were added to each and the bottles sealed. Serum bottles were incubated at 39 ^{0}C until inoculation. Blanks were included in triplicate to correct for the fermentation of residual feed in the inoculum. On the morning of inoculation, 5 ml of inoculum was added to each bottle. Immediately after this 12.5 ml of ES and NaES were added to the appropriate cultures. Gas was released 10 min after addition and the time noted as t=0. Gas volumes were recorded and released, and pressure readings were recorded, such that the headspace pressure did not exceed 7 psi (Theodorou *et al.*, 1994). Cultures were inverted after every reading. At 0, 7, 10, 23 and 26 h serum bottles from each

treatment were removed in duplicate. The pH of each culture was recorded (Orion pH probe) and then sampled for VFA analysis (Ranfft, 1973).

Statistical analysis

Data were analysed using the Statistical package Genstat 5 (Lawes Agricultural Trust, 1990). A model appropriate for a factorial split-plot design was used with simulated substrate in the main plot and time in the sub-plot.

Results and Discussion

Methodology

Microbial activity was inhibited when VFA concentrations were high (90-100 mmol/l) but not when the concentration was decreased to 62 mmol/l (Johnson *et al.,* 1958). Reducing the substrate to buffer ratio from 1 g/100 ml to 0.5 g/100 ml decreased the initial VFA concentration from 139 to 69 mmol/l thus reducing or removing the inhibitory effect to microbial activity. The initial concentration of individual acids can also influence the subsequent fermentation profile (Peters *et al.,* 1989).

Substrate neutralisation

Though the microbial populations responsible for the fermentation of soluble carbohydrates are more tolerant of low pH than cellulolytic bacteria, little metabolic or microbial growth is expected at pH<5.0 (Hungate, 1966, Russell and Domobrowski, 1979). *In vitro* gas production is also pH sensitive (Getachew *et al.,* 1999). In preliminary studies, buffers normally employed to maintain an environmental pH 6.8 in situations of active fermentation (Piwonka and Firkins, 1996) were not sufficient to stabilize the *in vitro* system. Sodium hydroxide is used as an external buffer in many continuous fermenter studies and was therefore incorporated into the buffering system to stabilise the *in vitro* pH pre-inoculation.

There were two potential buffering stages during the preparation of the simulated silage soluble fraction. The first was at the mixing of individual solutions in Buffer 1 and the second was at the pre-incubation stage where the simulated silage soluble fraction substrate is added to the *in vitro* buffer at a ratio of 1:8. When 1M NaOH was used in the titration of the simulated silage soluble fraction (**Table** 6.3.1) the low molarity of the alkali required large volumes to neutralise the fraction. Therefore 5 M NaOH was used in subsequent titrations (**Table** 6.3.2). The pHB using 30 ml 5 M NaOH was too high (**Table** 6.3.2). From experimental trials the optimum pH at the first phase of neutralisation was pH 6.0 or less, as the solution gained approximately 0.6 pH units on addition of 80 ml of Buffer 1. The initial reading of pHB did not account for the gradual rise in the recorded values after approximately 30 min. No importance was attached to this increase, as the production of VFA *in vitro* would reduce the pH profile over time. From experimental titrations substrate neutralisation pre-incubation was achieved from the addition of

25 ml 5 M NaOH to 100 ml of the simulated silage soluble fraction (**Table** 6.3.3). Therefore from every 500 ml final volume of NaES (1: 4 of NaOH: simulated silage soluble fraction) 12.5 ml was to be added to each culture.

Table 6.3 Neutralising 100 ml of a simulated silage soluble fraction with Sodium hydroxide (NaOH)

Section	6.3.1			Section	6.3.2			Section	6.3.3		
Part A		Part B		Part A		Part B		Part A		Part B	
1M NaOH (ml added)	pH [a]	Time (min)	pHB[b]	5M NaOH (ml added)	pH	Time (min)	pHB	5M NaOH (ml added)	pH	Time (min)	pHB
0	3.3	0	6.7	0		5	7.3	25	5.6	0	6.7
4	3.4	15	7.0	5	4.3	20	7.5			5	6.8
10	3.6	20	7.5	10	4.7	60	7.9			15	6.9
20	3.8	150	8.6	15	5.0	100	7.9			30	7.2
40	4.2			20	5.5	180	8.0			60	7.2
50	4.4			25	5.8						
60	4.5			30	7.0						
70	4.6										
80	4.8										
90	4.9										
100	5.0										

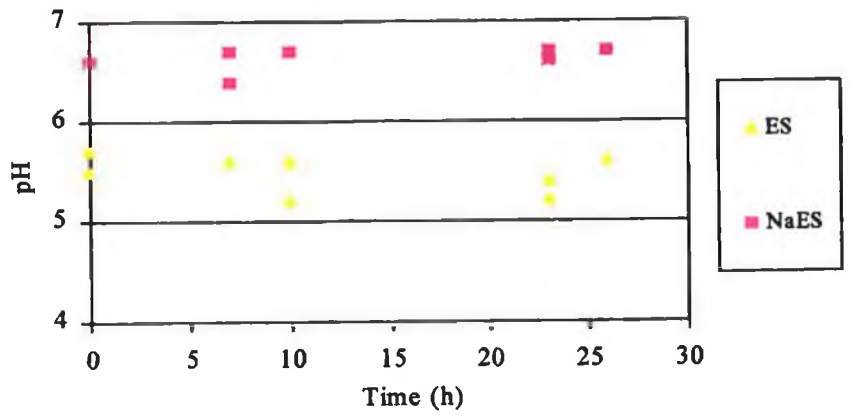
[a] One hundred millilitres of a simulated silage soluble fraction (Table 6.1) was titrated with 1M NaOH and the pH recorded 5 min after alkali addition. This was repeated until a pH5.0 (6.3.1) or pH6.0 (6.3.2 and 6.3.3) was reached.

[b] NaES (see materials and method, Section 6.1) was then added to a fixed volume of Buffer 1 based on a 1:8 ratio respectively (Goering and Van Soest, 1970). The pH drift of the solution was recorded until it became stable (**pHB**)

Effect of neutralisation on the in vitro fermentation of a simulated silage water soluble fraction

The pH profiles of all incubations are shown in **Figure** 6.1. The pH of ES was lower than NaES ($p<0.001$) reaching a minimum of pH 5.2 at 24 h and never rising above pH 6.0. Sodium hydroxide inclusion stabilised the *in vitro* pH of SS.

Figure 6.1 pH profile of simulated silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions



The gas profiles of ES and NaES, corrected for residual gas production using appropriate blanks, are shown in **Figures** 6.2. Sodium hydroxide inclusion depressed gas production, which would be expected due to the neutralisation of the acids pre-incubation. At 26 h the cumulative gas volume of ES was twice that of NaES.

Figure 6.2 Cumulative gas production from simulated silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions

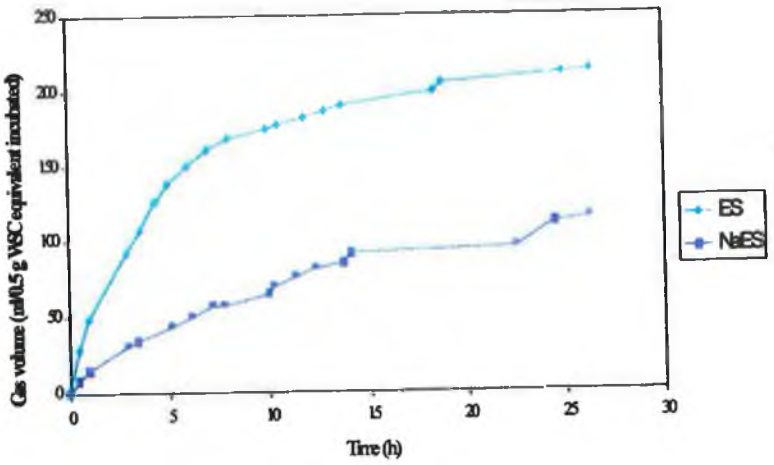


Table 6.4 Effect of sodium inclusion on the endproducts of simulated silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions

Variable analysed	Substrate (S) [a]	Time (T) (h)					Significance			
		0	7	10	23	28		S	T	S x T
TVFA (mmol/l) [b]	ES	99.6	114.2	114.7	112.8	118.1	**TVFA**	***	***	ns
	NaES	109.2	127.7	130.6	127.0	126.7	**s.e.d**	0.98	1.54	2.18
%TVFA	ES	100	115	115	113	119	**%TVFA**	ns	***	ns
	NaES	100	117	120	116	116	**s.e.d.**	0.96	1.51	2.14
NGR [c]	ES	7.8	7.9	7.9	8.2	7.8	**NGR**	***	***	***
	NaES	8.2	7.8	7.7	7.0	7.1	**s.e.d.**	0.05	0.18	0.12

[a] ES and NaES refer to the simulated silage water soluble fraction as described in Materials and Methods

[b]TVFA refers to total volatile fatty acid concentration (mmol/l)

[c] NGR refers to the non-glucogenic ratio [(acetate +2butyrate)/propionate]

Total VFA production increased during the first 7 h ($p<0.05$). The increase in TVFA was also expressed as a percentage of the t=0 concentration. This parameter was termed % TVFA and allowed for comparisons in TVFA production over time between treatments. The greater concentration of VFA for NaES may be attributed to t=0 differences as %TVFA was only affected by an increase over time ($p<0.001$). The NGR ratio was dominated by the high acetic acid content of the substrates and a significant S x T interaction ($p<0.001$) highlighted a tendency for propionate production for NaES in the latter stages of fermentation.

Conclusion

It was concluded that

- total VFA production from a simulated silage soluble fraction was not influenced by neutralisation, which stabilised the pH and reduced indirect gas production from the simulated silage soluble fraction.

6.2 Objective

To examine the effect of ensiling *per se* on the microbial utilisation of the water-soluble carbohydrate fraction.

Materials and methods

Substrate preparation

Substrates were prepared as detailed in **Table** 6.5.

Table 6.5 Composition of the substrates representative of simulated grass (GS), silage (ES) or neutralised silage (NaES) water-soluble carbohydrate fractions (g/ 400ml Buffer 1B).

	GS	**ES**	**NaES**
WSC (g)	20.00	3.04	3.04
Lactic acid (ml)		7.8	7.8
Acetic acid (ml)			9.88
Butyric acid (ml)			7.0
Propionic acid (ml)			2.28
Ethanol (ml)		3.04	3.04
5M NaOH (ml)			100.00
Distilled water (ml)	100.00	100.00	

Inoculum preparation

Inoculum was prepared as detailed in Section 2.1.

In vitro technique

Gas pressure transducer system (Theodorou *et al.,* 1994, Section 1.4.2.2)

In vitro method

The studied was carried out in two replicated blocks and all systems were examined under nitrogen-excess conditions (see Chapter 3). Serum bottles were prepared 18 h prior to inoculation as detailed in Section 6.1 and incubated at 39 ^{0}C overnight. Blanks were included to correct for residual gas and VFA production from the inoculum. On the morning of inoculation 400 ml of simulated water-soluble fractions of fresh (**GS**) and ensiled forages (**ES** and **NaES**) were prepared. The MP concentration of the inoculum was kept constant between blocks. To facilitate this a MP pellet was isolated from 500 ml of inoculum under anaerobic conditions at 39 ^{0}C. Inoculum was centrifuged at 1000 g for 10 min using a Sorvall centrifuge to remove feed residue and protozoa. The supernatant was then centrifuged at 20,000 g for 20 min, using a Sorvall RC-5B Refrigerated Superspeed centrifuge. The bacterial pellet was recovered and re-suspended in an equal volume of 0.9 % saline, preheated to 39 ^{0}C. Centrifugation and washing were repeated. On recovery, the microbial pellet was re-suspended in preheated Buffer 2 (**Table** 6.2) to give a protein concentration of 3 mg MP/dl. Inoculum (5 ml) and substrates (12.5 ml) were added in quick succession to appropriate bottles. All cultures were vented 10 min after substrate addition and

the time noted as t=0. The recording frequency of gas volume produced and venting was dictated by the pressure within the serum bottle, which was not allowed to rise above 7 psi (Theodorou *et al.*, 1994). Serum bottles were removed in duplicate, at intervals over 48 h. The pH of each culture was recorded and a sample removed for VFA analysis. A sample was also removed from each culture to measure MP concentration as described according to the procedure of Makkar *et al.* (1982).

Statistical analysis

Data were analysed using the statistical package Genstat 5 (Lawes Agricultural Trust, 1990). A model appropriate to a factorial split-plot design was used with substrate and block in the main plot and time in the sub-plot.

Results and discussion

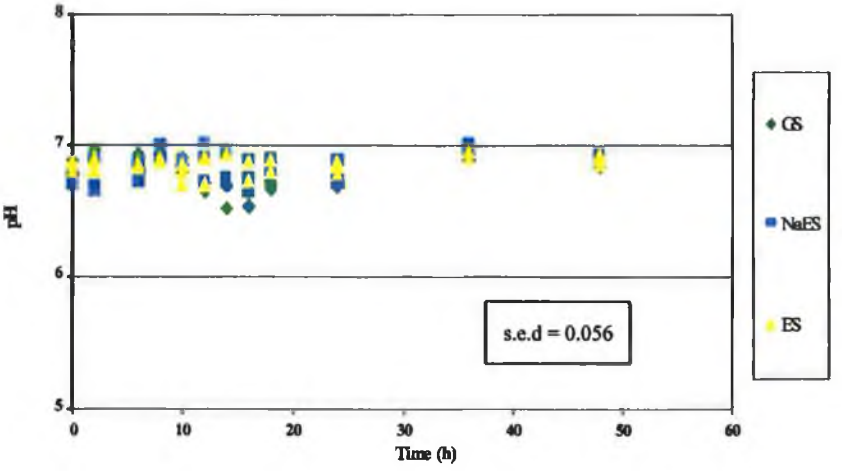
Methodology

The fermentable energy components of ES were incubated without the addition of the organic acids to examine the microbial fermentation of the residual energy components. As neutralisation of ES with NaOH was not found to affect VFA production in Section 6.1, the ES component was neutralized with NaOH to examine the effect of the organic acids formed during ensiling on subsequent VFA and MP production from the residual energy components.

In vitro fermentation

There was a significant substrate x time interaction for *in vitro* pH ($p<0.001$), which is described in **Figure** 6.4. Though there were significant fluctuations in values, these were thought not to be of biological importance as the pH range was controlled and narrow (pH 6.5-6.9) across treatments. This indicated the successful neutralisation of the organic acids of fermentation.

Figure 6.3 pH profile of simulated grass (GS), silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions during *in vitro* fermentation



The NGR was significantly affected by substrate ($p<0.01$) due to the high initial VFA concentration of the NaES treatment, but was not affected by time and there was no substrate x time interaction (**Table** 6.6). This allows for comparisons of GS and ES gas production profiles (**Figure** 6.5). There was a lag in gas production for all treatments of approximately 8 h. The GS had a significantly greater and more rapid fermentation when compared to ES after 10 h. Though residual substrate was not measured, it is assumed that the dilute soluble sugars are rapidly and completely fermented within 48 h. The final extent of gas production was proportional to initial WSC concentration at 151 and 23 ml gas for GS and ES, respectively. The initial gas production for NaES was thought to be indirect in nature due to the initial acid added and the extent was 46 ml/substrate incubated, supporting the findings of Section 6.1.

Figure 6.4 Cumulative gas production of simulated grass (GS), silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions during *in vitro* fermentation

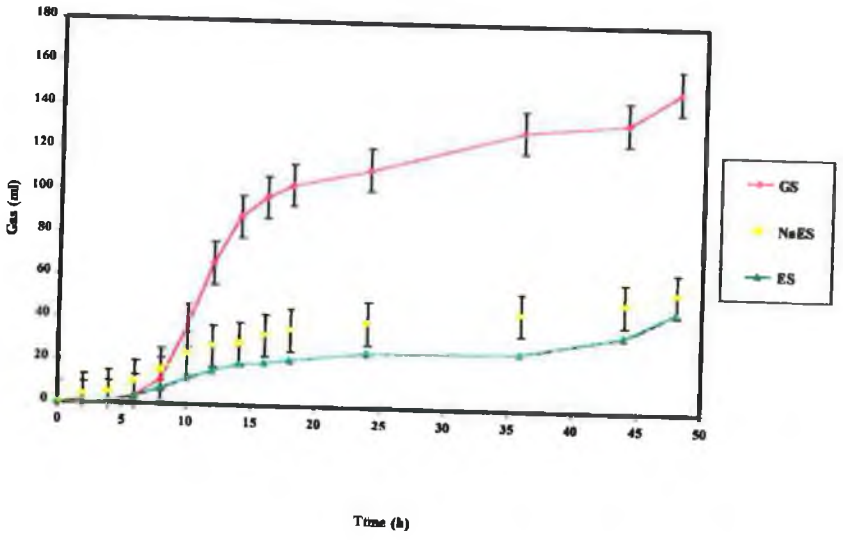


Table 6.6 Effect of substrate and time of sampling on volatile fatty acid concentration (VFA) from the fermentation of simulated grass (GS), silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions *in vitro*

Variable analysed (mmol/l)	Substrate (S) [a]	Time (T) (h)												Significance					
		0	2	4	6	8	10	12	14	16	24	44	48		TVFA	NGR	C2	C3	C4
Total VFA (TVFA)	GS	5.4	5.0	5.8	6.2	5.4	3.7	10.8	11.1	13.2	17.6	30.4	28.3						
	NaES	60.8	56.5	63.5	63.5	59.7	64.5	67.8	63.7	70.9	67.7	86.2	87.8	**S**	**	**	**	ns	***
	ES	6.5	8.3	6.0	6.9	9.1	11.0	14.5	13.4	19.1	23.6	49.4	30.6	**s.e.d**	3.50	0.25	1.44	0.44	0.47
Acetic acid (C2)	GS	1.9	1.6	2.1	2.3	1.5	0.8	5.5	5.2	6.1	6.4	8.8	7.1						
	NaES	35.1	33.1	36.6	36.9	35.0	37.4	39.2	37.0	41.0	39.0	47.3	48.8	**T**	***	ns	**	***	***
	ES	3.3	4.5	3.4	3.5	5.0	6.5	7.4	7.6	10.8	11.4	15.5	13.4	**s.e.d**	4.14	0.29	2.40	0.77	0.78
Propanoic acid (C3)	GS	2.5	2.7	3.0	2.7	2.8	1.9	3.1	3.7	4.3	7.0	14.1	13.8						
	NaES	8.7	8.1	9.5	9.2	9.1	9.6	10.4	10.3	11.2	10.8	13.6	14.2	**SxT**	ns	ns	ns	ns	ns
	ES	2.1	2.5	2.1	2.6	3.0	3.5	4.8	4.0	5.5	7.8	7.0	9.0	**s.e.d**	7.56	0.60	3.45	2.61	1.54
Butyric acid (C4)	GS	0.6	0.4	0.3	0.5	1.0	1.2	2.1	2.2	2.5	3.1	4.5	4.1						
	NaES	17.1	15.1	17.0	17.2	15.5	16.9	17.6	15.7	18.0	16.7	21.3	20.9						
	ES	0.6	1.0	0.4	0.6	0.8	0.8	1.4	1.4	2.2	2.6	7.4	3.4						
NGR [b]	GS	1.2	0.7	0.9	1.2	1.4	1.6	3.4	1.6	1.6	1.8	1.3	1.1						
	NaES	8.0	7.8	7.5	7.8	7.3	7.4	7.1	6.7	7.0	6.8	6.6	6.4						
	ES	2.2	2.6	2.1	1.9	2.3	2.4	2.4	2.6	2.8	2.4	2.2	2.3						

[a] GS, NaES and ES refer to the simulated water-soluble substrates as described in **Table** 6.5

[b] NGR refers to the non-glucogenic ratio = [(acetate +2xbutyrate)/propionate

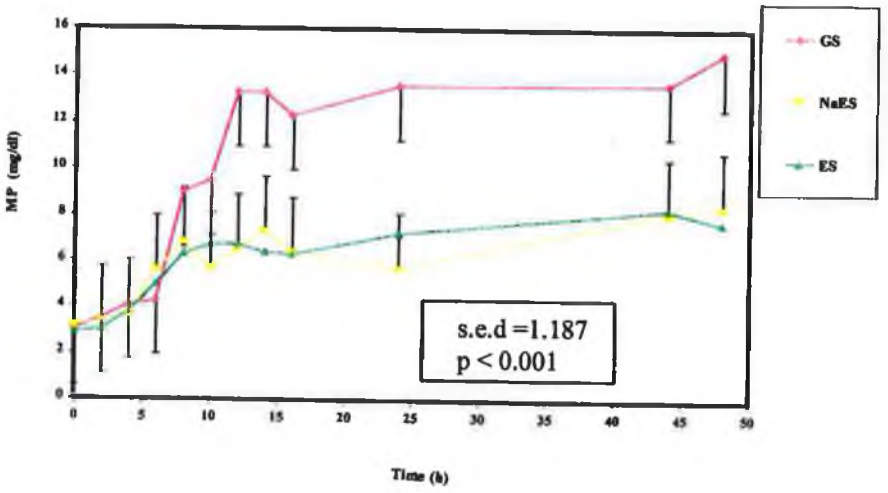
There was a significant effect of substrate ($p<0.01$) and time ($p<0.001$) on TVFA concentration. Total VFA production increased over time ($p<0.001$) and GS and ES did not differ in mean TVFA production. The TVFA concentration was higher for NaES ($p<0.05$) as expected. Over the 48 h incubation period ES and NaES produced 24.1 and 27.8 mmol/l of TVFA which would suggest that an initial VFA concentration of 60.8 mmol/l was not inhibitory to VFA production. This is supported by Johnson *et al.* (1958) who concluded that microbial activity was not influenced by an initial VFA concentration of 60 mmol/l but negatively affected when the initial concentration was increased to 90 mmol/l.

Acetate concentration increased as fermentation proceeded ($p<0.01$) and was higher for NaES ($p<0.01$). The acetate concentration of GS was lower than ES ($p<0.05$). An increase in acetate concentration for all substrates during the later hours of fermentation, was more notable for ES and NaES and may reflect the residual fermentation due to cell lysis which would be more advanced when compared with GS due to earlier substrate depletion. Propionate was not affected by substrate but increased over fermentation time ($p<0.001$). Butyrate increased over time ($p<0.001$) and was greater for NaES when compared with ES and GS as expected ($p<0.001$). The NGR was influenced by the initial VFA proportions of NaES as stated earlier. The NGR did not differ between GS and ES and the low value (mean 2.0) when compared with NaES reflected the formation of glucogenic precursors supported by hexose and lactate fermentation.

There was a significant two-way interaction for MP concentration ($p<0.001$, **Figure** 6.6). MP concentration was greater than 3 mg/dl for all treatments at 6 h ($p<0.05$). At 10 h, the MP of GS was greater than NaES and ES and did not increase again after 12 h until the end of fermentation at 48 h ($p<0.05$). When compared, NaES and ES did not differ in MP concentration, reflecting no inhibitory effects of initial VFA concentration or Na inclusion on MP synthesis. Within substrates, ES increased at 8 h and then both ES and NaES were stationary until a final increase at 44 h ($p<0.05$). The final increase in MP concentration was in the later hours of fermentation and may be attributed to cell lysis and nutrient recycling (Cone and van Gelder, 1999).

Nitrogen was supplemented in excess (164 mg N/g carbohydrate) of the recommendations of Czerkawski (1984). The protein-N:ammonia-N ratio was 2:1 which is in accordance with the recommendations of Russell *et al.* (1983). It may be assumed since nitrogen was supplied in excess, that carbohydrate availability limited MP production for GS after 12 h and for ES and NaES after 6-8 h. The efficiency of MP production 30 and 28.9 mg MP/ g of carbohydrate incubated for GS, and the mean of ES and NaES, respectively. There was no difference in TVFA production between GS and ES which suggests a negative relationship between MP and VFA production (Kristnamoorthy *et al.*, 1991b, Blummel *et al.*, 1997).

Figure 6.5 Microbial protein production from the *in vitro* fermentation of simulated grass (GS), silage (ES) and neutralized silage (NaES) water-soluble carbohydrate fractions



Maintenance energy requirements will affect bacterial Y_{ATP} and are thought to be generally higher for bacteria fermenting non-structural carbohydrates than those fermenting structural carbohydrates (0.3 and 0.1 mg CHO/mg protein/h, Russell *et al.*, 1992). Henning *et al.* (1991) and Newbold and Rust (1992) concluded that the maintenance energy demands of bacteria in batch systems between synchronous and asynchronous situations are not greatly different. If expressed in relation to the ATP production from the incubated substrates (Chamberlain, 1987) the efficiency of MP synthesis in this study for GS and ES was 1.3 and 3.1 mg MP/mmol ATP, respectively. Though the MP production was greater for GS, the production efficiency was numerically much lower. This would suggest a greater maintenance energy requirement to support the initial rapid increase in the microbial population when GS was metabolised. However, the apparently higher maintenance energy requirements may not apply *in vivo* where liquid associated microbial populations are rapidly washed from the rumen to the lower intestine.

Conclusion

It was concluded that

- Ensiling *per se* decreased the MP production of the water-soluble carbohydrate fraction by a factor of 2.
- The efficiency of MP production (mg MP/ mmol ATP was lower for the grass simulated water-soluble carbohydrate substrate when compared with the silage equivalent which was attributed to an increase demand in *in vitro* maintenance energy.
- Ensiling *per se* did not affect the final concentration or proportions of VFA produced from the fermentable energy components

- Buffering with 5 M NaOH stabilised the *in vitro* pH of a simulated silage water-soluble fraction
- Total VFA production from the fermentation of simulated silage water-soluble carbohydrate substrate was not affected by an initial concentration of VFA of 60 mmol/l.
- MP production from the fermentation of simulated silage water-soluble carbohydrate substrate was not affected by neutralisation or the initial concentration of VFA.

6.3 Objective

To examine the effect of the water-soluble fraction pre- and post-ensiling on the ruminal digestion of a perennial ryegrass structural carbohydrate fraction pre- and post-ensiling using the *in vitro* RSC system

Materials and methods

Substrate preparation

The fresh grass and extensively fermented silage of Harvest 3, as previously described in Chapter 4 were used in this study. The cell wall fraction of the grass (**G**) and extensively fermented silage (**E**) was extracted as described in Section 2.2 and subsequently dried at 45 ^{0}C for 48 h and milled to 2 mm. Based on the chemical analysis of the fresh herbages (**Table** 6.7) the respective simulated soluble (**W**) fractions ($\mathbf{W_G}$ and $\mathbf{W_E}$, **Table** 6.8) were prepared daily prior to feeding.

Table 6.7 Chemical composition of fresh and ensiled perennial ryegrass

	[a]Grass	Ensiled	Sig.	s.e.d
Dry matter (DM) (g/kg)	144.3	161.7	**	2.85
Composition of DM (g/kg)				
Dry matter digestibility	692.3	701.0	ns	11.29
Digestible organic matter	661.3	636.0	ns	9.87
Crude protein	111.3	122.0	ns	5.14
Neutral detergent fibre	578.7	546.3	*	9.50
Acid detergent fibre	335.7	329.3	ns	7.88
Acid detergent insoluble nitrogen	2.6	3.7	*	0.12
Ash	79.0	85.7	ns	2.33
Water-soluble carbohydrate	53.5	17.3	*	7.26
Nitrogen fractions				
Total N (TN) (g/kg DM)	17.8	19.5	*	0.38
Soluble nitrogen (g/kg TN)	286.5	561.1	ns	73.6
NH_3 (g/kg TN)	11.8	57.7	***	1.58
Fermentation acids				
Total Volatile fatty acid	ND	39.2		
Acetate	ND	38.5		
Propionate	ND	0.68		
Butyrate	ND	UN		
Lactate	ND	124.1		
Ethanol	ND	64.2		

ND = not determined; UN = undetectable

[a] Perennial ryegrass was ensiled after a 10-week regrowth period under extensive (20 g sucrose/kg fresh weight) ensiling conditions.

Table 6.8 Simulated water-soluble carbohydrate composition for Grass (W_G) and silage (W_E) (equivalent to 22.5 g forage DM (g/10ml distilled water))

Component	W_G	W_E
Hexose [a]	1.2	0.4
Lactic acid	-	2.8
Ethanol	-	1.5
Acetic	-	0.87
Butyric	-	
Propionic	-	0.02
Casein [b]	0.93	0.5

[a] Mixture was 9.9 g fructose, 80.1 g glucose and 10 g sucrose
[b]Soluble protein was estimated from the extracted soluble fraction and substituted on an equal nitrogen weight basis with casein. Casein had a 12.8% nitrogen content (Sigma). The ammonia content of the soluble fraction was omitted

In vitro system

The RSC and its operational conditions, sampling and laboratory analysis were as outlined in Section 5.4 with the following modifications: the buffer solution (**Table** 3.6) was supplemented with urea (0.5 g/l buffer, Stern and Hoover, unpublished) and the SDR and LDR were set at 2.5 and 5.0 %/h, respectively. There were two experimental periods of 10 days each.

Experimental treatments

Treatments were randomly assigned to one of four vessels. Two vessels were fed 22.5 g of grass or extensively preserved silage cell wall every 12 h. For each substrate the two vessels were supplemented with W_G or W_E at every feed on a fresh weight: dry matter basis. The final treatments were the isolated cell wall fraction of grass plus W_G, grass plus W_E, extensively preserved forage plus W_G and extensively preserved forage plus W_E.

Statistical analysis

Data were analysed using the statistical package of Genstat 5 (Lawes Agricultural Trust, 1990). The model used for non-periodic measurements was appropriate for a factorial analysis with terms for forage and W. For periodic measurements the model used was appropriate for a three-factor split-plot model with forage and W in the main plot and time in the sub-plot. Within significant interactions, means were compared using the LSD test (Steel and Torrie, 1960).

Chemical analysis

As described in Chapter 4

Calculation of the estimated rate of digestion (k_d)

Measured digestion coefficient = $[k_d / (k_d + k_p)]$, where k_d = rate of digestion and k_p = rate of passage = [SDR (/h)] (Schadt *et al.*, 1999)

Results and Discussion

Methodology

The herbage of the third harvest (detailed in Chapter 4) and the respective extensively fermented forage were chosen in this study as

- the biochemical composition of the ensiled forage was representative of that used in typical Irish production systems (Keating and O'Kiely, 1993, Steen *et al.*, 1997).
- the preservation of perennial ryegrass under conditions amenable to extensive but controlled fermentation gave the maximum biochemical alterations when compared with restrictive preservation (Chapter 4). The extensively preserved silage was used as the negative extreme to the pre-ensiled grass.

Chemical composition

In summary from Chapter 4, ensiling increased the forage DM ($p<0.01$) but did not affect forage CP or ash concentration (**Table** 6.7). Ensiling decreased the NDF concentration of the forage ($p<0.05$), with a subsequent increase in the ADIN content ($p<0.05$). There was no effect on the ADF content. These alterations did not affect the DMD or DOMD of the forage. The WSC fraction decreased during ensiling ($p<0.05$), with a concomitant increase in the VFA, lactic acid, ethanol, soluble and ammonia nitrogen concentration in the ensiled water-soluble fraction. During aqueous isolation of the cell wall fractions, 39.2 and 41.9 % of DM was lost from grass and silage respectively. The CP content of the grass cell wall was numerically higher than the preserved forage (**Table** 6.9). The ADF was also higher for the grass cell wall fraction, but there was little difference between NDF content of both isolated cell wall fractions.

Table 6.9 The chemical composition (g/kg DM (s.d.)) of isolated non-water soluble fraction.

Forage	[a]Grass	Extensively preserved silage
Composition of DM (g/kg)		
Crude protein	95.4 (3.25)	84.3 (2.12)
Neutral detergent fibre	842.0 (4.24)	839.5 (1.41)
Acid detergent fibre	518.0 (0.37)	506.0 (0.71)

[a]Perennial ryegrass (10 week regrowth) was ensiled after was ensiled under extensive (20 g sucrose/kg fresh weight) ensiling conditions.

In Chapter 3 and Chapter 4, the beneficial effects of nitrogen supplementation post-ensiling, were attributed to the replacement of peptide nitrogen lost by proteolytic degradation during ensiling.

Therefore under ammonia-excess conditions, the importance of replenishing the peptide nitrogen of the soluble component was examined in the current study. The soluble protein content of the W fractions was supplemented on a nitrogen DM basis as casein acid hydrolysate (0.93 and 0.5 g casein for W_G and W_E , respectively). Non-ammonia nitrogen (**NAN**) utilisation is influenced by the form, nature and rate of proteolysis in the rumen (Chen *et al.,* 1987, Broderick and Craig, 1989, Griswold *et al.,* 1995). Casein is highly soluble and rapidly hydrolysed *in vivo* (Cotta and Hespell, 1984). In the absence of any literature to the contrary, the assumption is made that there is a positive relationship between protein solubility and degradability for the water-soluble perennial ryegrass fraction. Therefore casein not only represents the nitrogen content of the water-soluble fraction, but also the biochemical nature of the inherent peptides and amino acids.

The water-soluble fraction, compiled from the chemical composition of the fresh herbages, was prepared before each feed. The carbohydrate composition of the water-soluble fraction was based on the work of McGrath (1988). Supplementation of the water-soluble fraction was on the fresh weight: DM content ratio where 134 ml of W_G and 117 ml of extensively fermented silage W_E were used to supplement 22.5 g fractionated cell wall DM.

The ammonia fraction was not supplemented but supplied through the buffer at a rate of 0.5 g urea/l. Satter and Slyter (1974) suggest that 50 mg ammonia-N/l is the minimum level for optimum cellulolytic activity. Assuming all urea nitrogen was released as ammonia this would supply 230 mg ammonia/l of buffer infused. The *in vitro* ammonia concentrations were, as a result of supplementation appreciably higher than the recommend limit. Ammonia nitrogen concentration *in vitro* will be influenced by pH (Shriver *et al.,* 1986), MP activity and LDR. The concentrations reported in this study were similar to other *in vitro* studies (206 mg/l, Merry *et al.,* 1987, 141 mg/l, Mansfield *et al.,* 1995). The system of Merry *et al.* (1987) had an LDR of 0.06 /h which is comparable to this current study.

Operational conditions of the RSC system

The pH control was not activated during the first 24 h so that the accuracy of pH readings by the internal probes could be assessed. One pH probe was replaced within this time and all probes differed from external readings by ± 0.3 pH. After 24 h, automatic pH control was imposed on all systems and probes were subsequently cleaned and re-calibrated every morning. Drifting between internal and external probe readings occurred at random. A pH drift from the real value occurred in V3 on day 4 and the system was overloaded with alkali, with an overnight pH of pH 11. At this point it was decided to remove automatic pH control and manually buffer the system. Based on the previous days, it was estimated that the buffer required to prevent a severe pH drop after W_E addition was 25 ml of 5 M NaOH. These additions were made after feeding and the recorded pH 1 h post feeding for W_E

supplemented vessels was 5.9 (sd. 0.12). The pH of all treatments remained above pH 6.2 after 2 h post feeding. The treatment of V3 was subsequently repeated.

The SDR was set at 2.5 /h, which is lower than the operational conditions of Merry *et al.* (0.03 /h, 1987) and Mansfield *et al.* (0.05 /h, 1995) but representative of *in vivo* conditions. An SDR of 0.025 /h is equivalent to a rumen turnover time of 40 h, which is similar to the *in vivo* findings of Bowman *et al.* (1991) who reported retention times of 40-50 h in heifers consuming vegetative and mature orchardgrass hay. As the DM fraction used in this study was the isolated cell wall fraction a lower SDR was chosen, as SRT can increase with cell wall content of the ingested feed (Bowman *et al.*, 1991, Bosch and Bruining, 1995). Bosch and Bruining. (1995) reported SDR of 0.025 to 0.04 /h for cows consuming silages differing in maturity, and an LDR of 0.06 to 0.1 /h. Huhtanen and Jaakola (1994) examining the *in sacco* digestibility of grasses differing in maturity assumed a passage rate of 0.02 /h, with measured *in vivo* values less than this reported by Rinne *et al.* (1997a).

The LDR did not differ between treatments (**Table** 6.10). Crawford *et al.* (1980a) examining the interactive effect of LDR and SDR, found that at 22 h retention time, up to an experimental maximum of 29 h, the liquid dilution rate no longer influenced the digestibility parameters of the study, which was dominated by the SDR. A lower LDR was therefore chosen to minimises the negative impact on the protozoal population *in vitro* (Abe and Kumeno, 1973, Hoover *et al.*, 1976a, Mansfield *et al.*, 1994). However rumen dynamics may differ *in vivo* between diets of grass and silage. Mambrini and Peyraud (1992) suggest that ensiling may decrease the rumen LDR and increase the retention time of rumen particles. Rinne *et al.* (1996) found no effect of silage maturity on the rumen LDR of 0.12 /h.

The SDR was higher for both silage cell wall treatments (2.3 vs. 2.0 %/h, $p<0.05$) and supplementation with W_E (2.4 vs. 1.9 %/h, $p<0.05$). This was equivalent to a minimum of 42 h to a maximum of 53 h retention time in the vessel interior. Studies have shown that the digestion coefficients of DM, NDF and ADF increased with increasing SRT (Hoover *et al.* 1982, Hoover *et al.* 1984, Shriver *et al.* 1986, Meng *et al.*, 1999, Schadt *et al.*, 1999), with experimental maxima of 30 h. However, in these studies DM input was decreased with increasing SRT to simulate *in vivo* situations. In the current study the substrate was a mature NDF isolate and the DM input was fixed. With digestibility limited by the degree of NDF lignification, little biological impact on digestibility parameters may be expected when SDR increases above 40 h. When reviewing the data, the inclusion of the SDR as a covariate during statistical analysis was not significant.

Table 6.10 Operational conditions for the rumen semi-continuous culture and the effect of forage (F[a]) and water soluble fraction (W[b]) on *in vitro* digestibility and microbial protein production.

	Grass		Silage		Significance [c]					
	W_G	W_E	W_G	W_E	s.e.d.	F	s.e.d.	W	s.e.d	FxW
Operational conditions										
LDR	5.2	5.4	5.5	5.4	0.17	ns	0.20	ns	0.26	ns
SDR	1.7	2.3	2.1	2.5	0.01	*	0.03	*	0.03	ns
Protozoa population (x 10^5)	1.5	1.2	0.9	0.9	1.65	ns	1.98	ns	2.57	ns
Digestibility (g/kg DM)										
Dry matter	609	580	569	566	33.1	ns	33.1	ns	46.8	ns
Neutral detergent fibre	777	759	771	793	36.4	ns	36.4	ns	51.5	ns
Acid detergent fibre	321	304	277	234	67.4	ns	67.4	ns	95.3	ns
Crude protein	598	614	561	651	43.5	ns	43.5	ns	61.5	ns
Estimated rate of digestion [d]	0.018	0.023	0.025	0.022	0.0016	ns	0.0016	ns	0.0023	ns
Microbial nitrogen (MN)										
g MN produced/ kg DM	8.00	9.70	8.75	8.75	0.74	ns	0.74	ns	1.04	ns
g MN produced/ kg DM digested	16.7	13.2	15.4	15.5	1.25	ns	1.25	ns	1.77	ns

[a]Perennial ryegrass (10 week regrowth) was ensiled after was ensiled under extensive (20 g sucrose/kg fresh weight) ensiling conditions. The F20 fraction of each was prepared as described in Section 2.2.

[b]Simulated water-soluble carbohydrate composition for Grass (W_G) and silage (W_E) (equivalent to 22.5 g forage DM (g/10ml distilled water))

[c] When digestibility results were re-analysed using SDR as a covariate there were no treatment effects

[d]As described by Schadt *et al.* (1999)

Table 6.11 The effect of Forage (F[a]) and simulated water-soluble carbohydrate fraction (W[b]) on the *in vitro* production of volatile fatty acid

	Forage (F)	Soluble (W)	Time (T) [c]										Significance						
			9	11	12	13	14	15	16	17	18	22		NGR	TVFA	C2	C3	C4	Tiso
Non glucogenic ratio (NGR)[d]	Grass	W_G	4.3	4.4	4.4	4.4	4.3	4.3	4.3	4.3	4.2	4.2							
		W_E	5.1	5.5	5.5	5.7	5.7	5.9	5.6	5.6	5.4	5.4	**F**	ns	*	ns	ns	ns	ns
	Silage	W_G	4.0	3.9	3.7	3.9	3.9	3.9	3.9	3.8	3.9	3.9	**s.e.d.**	0.60	0.116	0.023	0.016	0.030	0.007
		W_E	4.7	4.9	4.9	4.9	4.9	4.8	4.7	4.6	4.6	4.6							
													W	ns	ns	ns	ns	ns	ns
Total VFA *(Mmol /l, TVFA)*	Grass	W_G	93.8	98.2	98.0	99.1	97.4	97.6	103.5	98.9	95.2	90.4	**s.e.d.**	0.34	2.958	0.029	0.005	0.037	0.008
		W_E	108.0	118.6	121.1	119.6	121.2	120.5	120.7	115.9	113.5	107.8							
	Silage	W_G	90.8	93.0	99.2	97.3	97.9	96.8	98.0	97.4	95.8	91.0	**T**	ns	*	***	Ns	***	ns
		W_E	95.8	120.2	116.2	113.7	113.1	115.4	114.6	114.6	110.1	104.6	**s.e.d**	0.7	3.38	0.002	.003	0.003	0.002
mmol/mmol TVFA																			
Ethanoic (C2)	Grass	W_G	0.67	0.66	0.6	0.66	0.66	0.66	0.66	0.66	0.66	0.67	**FxW**	ns	ns	ns	ns	ns	ns
		W_E	0.63	0.61	0.58	0.59	0.59	0.61	0.61	0.61	0.62	0.62	**s.e.d**	0.69	2.960	0.037	0.016	0.058	0.10
	Silage	W_G	0.70	0.68	0.67	0.68	0.68	0.68	0.69	0.68	0.69	0.69							
		W_E	0.63	0.62	0.61	0.61	0.61	0.61	0.61	0.62	0.62	0.62	**FxT**	ns	ns	ns	ns	**	ns
													s.e.d	0.61	3.55	0.023	0.016	0.030	0.007
Propanoic (C3)	Grass	W_G	0.20	0.20	0.20	0.21	0.20	0.21	0.21	0.21	0.21	0.21							
		W_E	0.19	0.18	0.18	0.17	0.17	0.16	0.17	0.17	0.17	0.17	**WxT**	ns	ns	ns	ns	*	**
	Silage	W_G	0.21	0.22	0.23	0.22	0.22	0.22	0.22	0.22	0.22	0.22	**s.e.d**	0.36	4.82	0.029	0.007	0.037	0.008
		W_E	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20							
													FxWxT	ns	ns	ns	ns	ns	ns
Butyric (C4)	Grass	W_G	0.10	0.11	0.12	0.11	0.11	0.11	0.11	0.11	0.10	0.10	**s.e.d.**	0.71	5.06	0.037	0.018	0.048	0.010
		W_E	0.10	0.18	0.20	0.19	0.19	0.18	0.18	0.17	0.17	0.16							
	Silage	W_G	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08							
		W_E	0.14	0.15	0.16	0.16	0.16	0.15	0.15	0.15	0.14	0.14							
Total branched (Tiso)[e]	Grass	W_G	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02							
		W_E	0.03	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03							
	Silage	W_G	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02							
		W_E	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04							

[a]Perennial ryegrass (10 week regrowth) was ensiled after was ensiled under extensive (20 g sucrose/kg fresh weight) ensiling conditions. The F20 fraction of each was prepared as described in Section 2.2.
[b]Simulated water-soluble carbohydrate composition for Grass (W_G) and silage (W_E) (equivalent to 22.5 g forage DM (g/10ml distilled water))
[c] Real time, feeding was at 8am and 8 pm.
[d] NGR = [(acetate +2butyrate)/propionate)]
[e]Total iso = [iso-butyrate + iso-valerate]

Table 6.12 The effect of Forage (F [a]) and simulated water-soluble carbohydrate fraction (W [b]) on the *in vitro* concentration of ammonia and lactate

		Forage (F)	Soluble (W)	Time (T) [c]										Significance				
				9	11	12	13	14	15	16	17	18	22		*NH3*	s.e.d.	LA	s.e.d.
Ammonia	(NH_3, mg/l)	Grass	W_G	234.7	252.6	271.2	275.7	275.4	260.1	254.4	246.9	229.6	233.3	*F*	ns	15.96	ns	0.008
			W_E	235.4	252.5	252.7	262.1	257.4	249.8	245.7	238.2	229.0	220.0	W	ns	2.77	*	0.005
		Silage	W_G	252.3	273.9	286.4	290.2	288.8	277.1	267.8	263.2	248.5	250.1	T	***	5.70	***	0.007
			W_E	257.0	263.9	254.9	250.9	257.3	233.4	225.6	242.8	232.3	225.6	FxW	ns	16.20	ns	0.008
														FxT	*	17.11	***	0.012
Lactate	(LA, g/l)	Grass	W_G	0.07	0.07	0.08	0.07	0.07	0.07	0.06	0.07	0.06	0.06	WxT	ns	7.71	***	0.008
			W_E	0.06	0.30	0.09	0.07	0.07	0.07	0.08	0.06	0.07	0.06	FxWxT	*	18.0	***	0.014
		Silage	W_G	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.07	0.08					
			W_E	0.06	0.08	0.07	0.06	0.06	0.06	0.07	0.07	0.07	0.07					

[a]Perennial ryegrass (10 week regrowth) was ensiled after was ensiled under extensive (20 g sucrose/kg fresh weight) ensiling conditions. The F20 fraction of each was prepared as described in Section 2.2.
[b]Simulated water-soluble carbohydrate composition for Grass (W_G) and silage (W_E) (equivalent to 22.5 g forage DM (g/10ml distilled water))
[c] Real time, feeding was at 8am and 8 pm.

The effect of water-soluble carbohydrate supplementation on the in vitro fermentation of the isolated cell wall fractions pre- and post-ensiling

The biochemical alterations due to ensiling did not influence the cell wall DM, OM, NDF or ADF digestibility *in vitro* (**Table** 6.10). The findings of Chapter 3 and Chapter 4 and the *in vitro* estimates of digestibility (**Table** 6.7) support this. Keady *et al.* (1995, 1998) also found no effect of ensiling on *in vivo* apparent digestibility of DM, OM, NDF and ADF, while Cushnahan *et al.* (1995) and Cushnahan and Gordon (1995) found no effect on ADFD and NDFD respectively.

Soluble sugars were supplemented at 5 and 1 % DM for W_G and W_E respectively. Supplementation of the basal diet with carbohydrate sources can negatively affect fibre digestion *in vivo* (Dawson *et al.*, 1988, de Visser *et al.*, 1998) and *in vitro* (Mertens and Loften, 1980, Grant and Mertens, 1992c, Piwonka and Firkins, 1993). In this study the supplementation rate was substantially lower than that reported in the previous work, as the objective was to replace the nutrient fractions of the W component only. Supplementation therefore did not affect treatment or feed component digestion rates (**Table** 6.10). The SRT was assumed to be common for all feed fractions.

There was no effect of treatment on protozoal numbers or MP production (**Table** 6.10), though the MP production was numerically higher when supplemented with W_G, supporting the finding of section 6.2 that MP production from the water-soluble fraction was greater pre-ensiling. The ARC (1984) also reported that 1.43 and 0.71 g N was incorporated into microbial N/ MJ ME in diets based on grass and silage diets, respectively. These findings are supported by *in vivo* studies (Siddons *et al.*, 1985, Gill *et al.*, 1989).

There was a significant three-way interaction ($p<0.05$) for NH_3 concentration *in vitro*. Supplementation with W_G increased *in vitro* NH_3 concentration for the grass and silage cell wall fraction between 3 to 6 h and 4 h post-feeding respectively, which may reflect microbial utilisation of the supplemented peptide or the higher CP content of fractionated grass cell wall. Supplementation with W_E did not increase NH_3 concentration when compared with pre-feed values. No effect of treatment on MP production may be attributed to the availability of excess NH_3 nitrogen, the loss of which *in vivo* is partially attributed to reduced MP production (Chamberlain and Choung, 1995). In the present study the daily NH_3 available in each fermentation vessel (LDR 5.3 %/h, vol. 1.8 l) was 0.95 g. The overall mean concentration of NH_3 over the fermentation period was 253 mg NH_3/l. This value is higher than the minimum level suggested by Satter and Slyter (1974) and less than the upper limit of required NH_3 suggested by Ricke and Schaefer (1996).

The greatest rate of supplementation of peptide nitrogen in the current study was 4 % of the cell wall CP content (silage cell wall plus W_G). No significant response in MP production when peptide nitrogen was

replaced may suggest that the peptide content of the rumen degradable nitrogen was not limiting microbial activity. Czerkawski (1986) suggests that rumen fermentation can be optimised if the ingested feed supplies 25 g rumen degradable nitrogen /kg fermentable OM. The rumen degradable nitrogen and fermentable OM were calculated from **Table** 6.7 and **Table** 6.8. The rumen degradable nitrogen was defined in this study as the [(CP – ADIN) + supplemented AA-N], while the fermentable OM was defined as [(NDF –ADF) + supplemented carbohydrate]. The ratio was 44.6, 38.0, 38.3, 31.0 g rumen degradable nitrogen /kg fermentable OM for grass cell wall $+W_G$, grass cell wall + W_E, silage cell wall + W_G and silage cell wall + W_E, respectively. Though the proteolytic effect of ensiling is evident from the lower ratio for silage cell wall $+W_E$ all are above the recommended ratio. This ratio is dominated by the availability of structural fractions.

Keady and Murphy (1998) replaced the water-soluble carbohydrates and peptide nitrogen lost during ensiling (in the form of sucrose and fishmeal) such that the final crude, effective rumen degradable, undegradable dietary and digestible undegraded protein were comparable for fresh, ensiled and ensiled plus supplemented forages on a DM basis. Though there were improvements in animal production post-supplementation, they found no effect on rumen digestibility or nitrogen retention and concluded that ruminal digestion was not limited in AA or N supply to microbes. As the forage matures, the increasing lignification of the CW fraction may therefore be expected to have a greater consequence for ruminal nutrient availability than ensiling. The decrease in the soluble fraction is accompanied by a decrease in the CP content and the increase in ADIN (**Table** 4.3), thus restricting the available nitrogen source for microbial utilisation.

Increased MP production and thus concentration may not be a limiting factor for fibre digestion as Dehority and Tirabasso (1998) found that fibre digestion was not improved when the bacterial concentration was increased *in vivo*. However Hidaya *et al.* (1993) found that TVFA concentration and initial rate of fermentation *in vitro* increased with increasing bacterial concentration. The former result was attributed to the spatial saturation of fibre particles during attachement, which is necessary for effective cellulolytic enzyme activity. It follows that if the nitrogen requirements of this 'maximum' population are provided for, or if carbohydrate is limiting in the basal diet, further peptide supplementation may be of little advantage.

Total VFA concentration (**Table** 6.11) increased over time ($p<0.05$) but was lower for silage cell wall digestion ($p<0.05$). *In vivo* TVFA concentration for ensiled forages has been greater (Keady and Murphy, 1998) or not different (Cushnahan *et al.,* 1995) than the fresh herbage. Differences may be attributed to the composition of the soluble component as the ensiled forage in the latter had a lower concentration of fermented acids and DMI did not differ within studies. Reduced VFA production may be attributed to an increase in MP production (Blummel *et al.*, 1997) or a decrease in OM digestion. In

this study the numerically higher DMD for grass cell wall supported the greater TVFA concentration. There was no effect of W supplementation on TVFA in the current study. This supports the findings of section 6.2, which found no effect of ensiling on the proportions or concentrations of VFA.

The periodic increase in TVFA production was attributed to an increase in acetate ($p<0.001$) and butyrate ($p<0.001$) proportion over time. There was a significant forage x time interaction ($p<0.01$) and W x time interaction ($p<0.05$) for butyrate concentration. Both fractionated grass cell wall and supplementation with W_E supported a butyrate fermentation up to 4 h post-feeding, with levels decreasing to pre-feed levels after 8 h. Non-glucogenic precursors (acetate and butyrate) are normally associated with the fermentation of fibrous structural carbohydrates. The increased butyrate response for silage cell wall digestion may reflect the W_E supplementation, while the response to fractionated grass cell wall is not atypical as Moloney and O'Kiely (1994) and Syrjala (1972) reported a butyrate type fermentation when soluble sugars were metabolised in the rumen.

The diurnal variations in VFA concentrations did not affect the NGR, which is supported by Keady and Murphy (1998) but not by Cushanhan *et al.* (1995). This reflects the static nature of propionate concentration, which was not affected by treatment. Propionate production *in vivo* is associated with concentrate and lactate fermentation (Chamberlain *et al.,* 1983, Jaakola and Huhtanen, 1992). The lactate concentration during silage cell wall digestion in this study was 124 g LA/ kg DM, with a predicted immediate concentration *in vitro* post supplementation with W_E of 1.8 g/l. There was a significant three-way interaction ($p<0.001$) for LA concentration *in vitro* (**Table** 6.12). This was attributed to the transient increase in LA for grass cell wall plus W_E 1 h post feeding with a maximum level of 0.3 g/l. There was a common pre-feed minimum value of 0.06 g/l. The lactic content was rapidly metabolised for grass and silage cell wall fed cultures (2 and 1 h post-feeding respectively).

The rapid metabolism of lactate has previously been reported (Chamberlain *et al.,* 1983, Moloney and O'Kiely, 1993). Cushanhan *et al.* (1995) found an increase in propionate concentration post-feeding an extensively fermented silage of 111.0 g LA /kg DM, when compared with the fresh herbage. This was not supported by Keady and Murphy (1998) when an ensiled forage of 60 g LA/ kg DM was fed. Lactate did not support propionate fermentation in section 6.2. The discrepancies between *in vitro* and *in vivo* studies may be explained by the findings of Counette (1981) who suggests that the relative proportions of acetate and propionate production from lactate are influenced by pH, flow rate and lactate concentration in the rumen.

There was a significant W x time interaction ($p<0.01$) for branched chain fatty acids. The minimum and maximum concentration of total branched chain fatty acids were 0.9 mmol/l for silage cell wall plus W_G pre-feed and 4.0 mmol/l for silage cell wall plus W_E 6 h post-feed. Supplementation with W_E increased

the proportion over time, while W_G decreased the proportion of BCFA over time. Branched chain fatty acids arise from the fermentation of AA, which can occur due to peptide depletion or restrictions in carbohydrate availability (Baldwin and Allison, 1983). The greater BCFA for silage cell wall may therefore be attributed to the lower CP content (**Table** 6.9) of the structural fraction.

Conclusion

It is concluded that

- Ensiling did not affect the DM, NDF, ADF or CP digestibility of the aqueously extracted cell wall fraction of perennial ryegrass
- Ensiling did not influence the rate of digestion of forage components
- Supplementation of the cell wall fraction pre- and post-ensiling with the soluble carbohydrate/organic acids and protein fractions pre- and post-ensiling did not influence MP production or forage digestibility.

Implications

Ensiling under extensive conditions did not affect the *in vitro* digestibility of the structural fraction, which supports previous findings (Chapter 3 and Chapter 4). Ensiling decreased the nutritive value of the herbage by decreasing the MP production from the soluble carbohydrate fraction (Section 6.2). This effect may be expected to be more extreme *in vivo* if there is a reduction in required maintenance energy. Though the MP concentration was higher for supplementation with W_G fractions in the RSC study the difference was not significant. This may be attributed to the fractionated cell wall rumen degradable nitrogen :fermentable OM ratio, which was > 25 g/kg fermentable OM for every forage. It is therefore suggested that under good preservation conditions, forage maturity will have the greatest impact on the ruminal nutritive value, as unlike ensiling, it will decrease ruminal availability and digestibility of structural carbohydrate and nitrogen fractions.

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