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Identifying and improving the fibre-degrading activity of rumen microbe-derived fibrolytic bacteria

A thesis presented in partial fulfilment of the requirements for the degree of

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Renée Mary Louise Atua

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Abstract

The dairy industry is extremely important to the New Zealand (NZ) economy, and it accounts for approximately $16.6 billion in exports each year (1). In NZ, traditional feedstock for cattle, such as grass and hay, consists of cellulose-based fibrous material that have limited nutritional value due to their inherent resistance to degradation. As commercially available feed treatments that use fibrolytic enzymes (FEs) from aerobic fungi are not currently available in NZ, it is possible that pre-treatment of such foodstuffs with rumen microbe-derived FEs could enhance fibre degradation, boosting animal performance and productivity as such enzymes should be better suited to the anaerobic conditions of the rumen.

The main aim of this thesis was to identify effective fibre-degrading rumen bacteria and attempt to improve their fibre-degrading, or fibrolytic, activity using non-genetically modified methods. This was carried out by culturing 15 different rumen bacterial isolates on five separate fibrous substrates, which resulted in 46 strain/substrate combinations that were screened for fibrolytic activity.

The fibrolytic activity of each strain/substrate combination was assessed using two distinct biochemical assays: 1) degradation of oat spelt xylan (OSX) or filter paper (FP), and 2) degradation of *para*-nitrophenol-conjugated substrates that represent major biochemical linkages in the plant cell wall. Three candidate strains were chosen based on these results to improve fibrolytic activity further using mutagenesis and positive selection, and of these, two strains showed a statistically significant increase in fibrolytic activity after 31 subcultures on ryegrass (RG). The secretomes of these two strains was then investigated using proteomic methods, which included 1D SDS PAGE, in-gel trypsin digest and mass spectrometry.

The overall results from this research serve as a foundation for the development of a feed treatment to be used in NZ, which could provide financial benefit not only to dairy farmers, but the NZ economy as well.

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List of abbreviations

AmBic	Ammonium bicarbonate
AP	Apple pectin
APS	Ammonium persulphate
BSA	Bovine serum albumin
BTP	Bis-tris propane
CFUs	Colony forming units
CP	Cell pellet
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNS	3,5 dinitrosalicyclic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
FDR	False discovery rate
FE	Fibrolytic enzymes
Fig	Figure
FP	Filter paper
g	Gram
GenRFV	General rumen fluid with vitamins
GH	Glycoside hydrolase
GM	Genetic modification
GMO	Genetically modified organism
hr	Hour
ICRF	Incubated clarified rumen fluid
ID	Identification
kb	Kilobase
kD	Kilodalton
L	Litre
LOD	Limit of detection
LOQ	Limit of quantification
MES	2-ethanesulfonic acid
µL	Microlitre

mg	Milligram
mL	Millilitre
MQ	MilliQ water
MW	Molecular weight
NDF	Neutral detergent fibre
nm	Nanometres
NZ	New Zealand
OD	Optical density
OSX	Oat spelt xylan
PCR	Polymerase chain reaction
*p*NP	*Para*-nitrophenol
RG	Perennial ryegrass
RM02	Rumen Microbiology media series number 2
RPM	Revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	Room temperature
sec	Seconds
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SN	Supernatant
TAE	Tris-acetate EDTA buffer
TEMED	Tetramethylethylenediamine
UV	Ultraviolet
2SGenRFV	2x sugar and general rumen fluid with vitamin

Chapter One: Introduction

1.1 Introduction

1.1.1 Background

The dairy industry is New Zealand's (NZ) largest export earner, with annual exports in excess of $18.1 billion (1). With approximately 90% of milk produced in the country processed into dairy products and exported overseas (2), the significance of dairy farming to the NZ economy is apparent.

Nutrition is vital for maximising the productivity of dairy cows and optimal nutrition will result in larger quantities of better quality milk (3). Overseas, dairy cows are fed a diet high in starch and sugars that are readily digestible (4). In NZ, however, the ruminant diet is extremely high in fibre. Grasses, such as perennial ryegrass and Kikuyu, are the most common type of plant in NZ pasture (5). Supplemental feed, on the other hand, is used to bulk up feed when pasture is scarce and contains such forages as maize silage, hay or straw (5).

As feed costs represent approximately half of the total cost of milk production, enhancing fibre degradation and digestibility is predicted to provide financial benefit, not only to the country's economy, but to NZ dairy farmers as well (6). For a long time, forage breeding programmes and agronomic advances have been fundamental for improving forage cell wall digestibility (7), but research carried out overseas has explored the possibility of supplementing ruminant diets with fibre-degrading, or fibrolytic, enzyme treatments to improve feed utilization and overall animal performance (3).

When pasture was the predominant feed for dairy cows in NZ, improving fibre degradation by applying feed treatments to entire paddocks would have been extremely expensive and inefficient. However, NZ's booming dairy industry has seen an increase in the use of

supplemental feed, and this creates the realistic possibility of manipulating supplemental feed in order to improve fibre degradation and digestion for ruminants in NZ.

1.1.2 The rumen

Ruminants, which include dairy cows, as well as sheep, deer, llamas and buffalo for example, are classified as such based on the way they digest their food. The ruminant digestive tract consists of a mouth, esophagus, and a stomach that is made up of four compartments, as well as the small and large intestines (Fig 1.1). The rumen is the largest of the four compartments that make up the ruminant stomach, and it acts as a fermentation chamber by storing and mixing the feed, and allowing for the partial microbial degradation of consumed forage (8). The partially degraded forage, or cud, is then regurgitated and re-chewed at least once before it passes back through the digestive tract, where it is ultimately digested in the abomasum, or true stomach (8). The process of regurgitating and re-chewing, which is referred to as rumination, disrupts the plant tissue further, and this provides an opportunity for increased degradation and increased nutrient absorption.

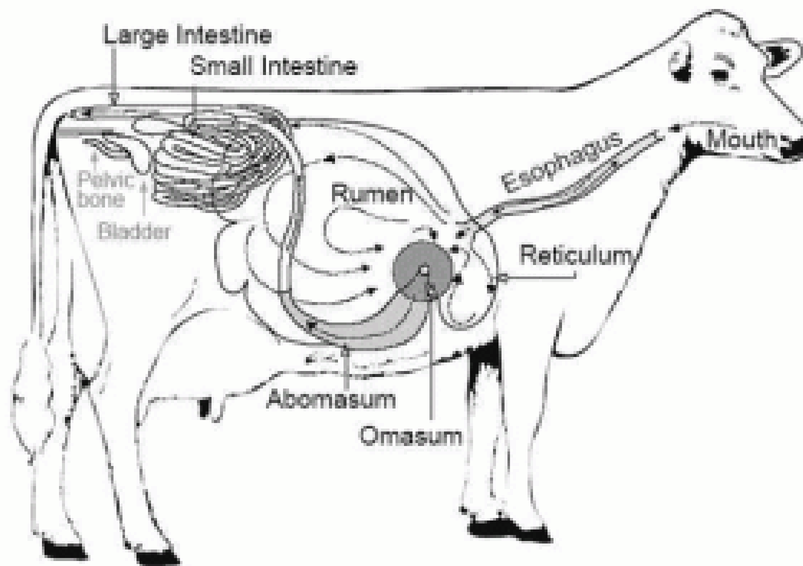

Fig 1.1. The gastrointestinal tract of the cow. The gastrointestinal tract consists of a mouth, esophagus, a stomach comprised of four compartments, and a small and large intestine. Figure taken from http://www.dairyvietnam.com/en/Structure-of-the-digestive-system/General-Anatomy-of-the-Ruminant-Digestive-System-282.html on 19th March, 2017.

As ruminants are unable to naturally produce the enzymes that can break down plant material themselves, they are reliant on the fibrolytic enzymes (FEs) produced by the large consortium of microbes that reside within the rumen (8). Survival of these microbes is reliant on their successful adaptation to the anaerobic conditions of the rumen, which is consistently at a pH of around 6.5 and a temperature of 39˚C (9). FEs hydrolyse cellulose and hemicellulose components into simple sugars, which are then fermented and utilised as energy by the host animal (10). Although many rumen microorganisms naturally produce FEs, ruminal plant cell wall degradation is still considered slow and incomplete (7).

1.1.2.1 Rumen microbes

The microorganisms present within the rumen are highly diverse, either strictly anaerobic or facultative anaerobes, and includes both prokaryotic and eukaryotic organisms. The ability of rumen microbes to ferment ruminant feed into absorbable volatile fatty acids accounts for approximately 70% of the energy that the animal obtains from forage (11).

There are various rumen microbes, particularly bacteria and fungi, that are considered the major players in fibre degradation within the rumen, and they can be classified based on their fibrolytic potential (8). Characterised cellulolytic fibre degraders include *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Neocallimastix* sp. (10-12). Characterised xylanolytic fibre degraders include *Butyrivibrio fibrisolvens*, *Pseudobutyrivibrio* sp., and *Prevotella* sp. (8, 11). Despite this, a large proportion of rumen microbes remain uncultured and unidentified, so it is difficult to say with certainty whether these species truly are the key players for fibre degradation in the rumen, or if better fibre degraders exist.

The Hungate 1000 project is an initiative with the aim of providing a reference set of rumen microbial genomes by sequencing the genomes of at least 1000 cultivated rumen microbes (www.rmgnetwork.org/hungate1000.htmL). It is hoped that the genomes made available by this project will support international efforts to mitigate greenhouse gases produced from livestock, initiate genome-based research that will help elucidate the mechanisms involved in rumen function and feed conversion efficiency, and also help understand plant cell wall degradation. Such information would be helpful when analysing the fibrolytic potential of

rumen microbes, and could aid in the identification of new and improved fibre degraders that reside in the rumen.

1.1.3 The plant cell wall

As already mentioned, fibre degradation in the rumen is considered slow and incomplete, and this is supported by the fact that fibre recovered from cow faeces is still fermentable (13). The major source of dietary fibre for ruminants is the forage plant cell wall (Fig 1.2). It is structurally complex, and consists of a large array of polysaccharides, proteins, lipids, lignin and phenolic acids, in tight association (3). Cellulose and hemicellulose are the major structural polysaccharides in the plant cell wall. Plant cellulose, a linear polymer of D-glucose units, is crystalline, strong and resistant to hydrolysis (3). Hemicellulose, on the other hand, has a weaker structure which is more susceptible to hydrolysis, and it contains a random mixture of different sugar polymers, including xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan (3).

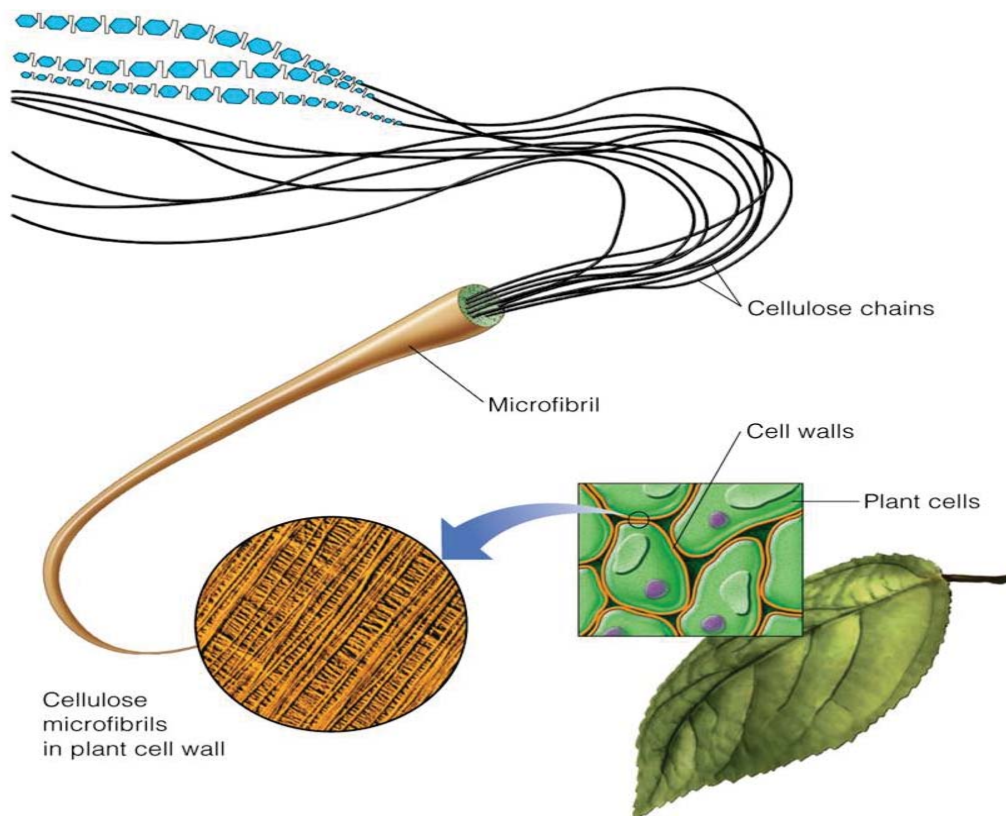

Fig 1.2. Organisation of the plant cell wall. Figure taken from Appling, et al. (14), Fig 9.20.

The carbohydrate polymers within the plant cell wall are unable to be digested by most animals, yet microbes within the rumen have become best adapted to hydrolyse and ferment forage material, resulting in the release of fatty acids, amino acids, sugars and protein in the form of microbial cells (13). Efficient degradation of plant tissues requires fibrolytic organisms to successfully adhere to and colonise plant material, as well as produce effective enzymatic systems to break down the polysaccharides in the plant material (11). Because cellulose and hemicellulose are insoluble, the initial degradation step has to take place outside of the cell, which may include the bacterial outer cell membrane (15). Although there are a few types of extracellular enzymatic systems that are capable of degrading the plant cell wall, the system of most interest to this research is the hydrolytic system, whereby secreted glycoside hydrolases (GHs) are responsible for the degradation of cellulose and hemicellulose (15).

1.1.4 Fibre degradation

1.1.4.1 Cellulose degradation

The linear polymer cellulose is composed of D-glucose units which are linked together by β-1,4 glycosidic bonds to form long chains (15), as shown in Fig 1.2. These chains associate with each other by hydrogen bonds and van der Waals forces that amplify the individual strength of the individual chains (15). There are two forms of cellulose within the plant cell wall; crystalline cellulose, which is difficult to break down due to the dense nature of the cellulose microfibrils packed tightly together, and amorphous cellulose, which is composed of disorganised cellulose chains and is therefore much easier to degrade (15). The microfibrils of cellulose are immersed within a complex matrix of hemicellulose and lignin.

Cellulases, which are enzymes that break down cellulose, generally hydrolyse the β-1,4 glycosidic linkages of cellulose, and can be divided into two classes: endoglucanases and exoglucanases (Fig 1.3) (15). Endoglucanases hydrolyse the internal bonds of the glucose chains within amorphous regions, and this generates new terminal ends (15). Exoglucanases, on the other hand, act on the existing, or endoglucanase-generated, chain ends (15). Both enzymes can degrade amorphous cellulose, but exoglucanases are the only enzymes that efficiently degrade crystalline cellulose, with some exceptions (15). Endoglucanases and exoglucanases both release cellobiose molecules, which are disaccharides of β-1,4-linked

glucose units, while another type of enzyme, β-glucosidase, breaks down the cellobiose into two glucose molecules (Fig 1.3) (15). The products from the degradation and fermentation of cellulose can be used as carbon and energy sources for rumen microorganisms and the host animal (15).

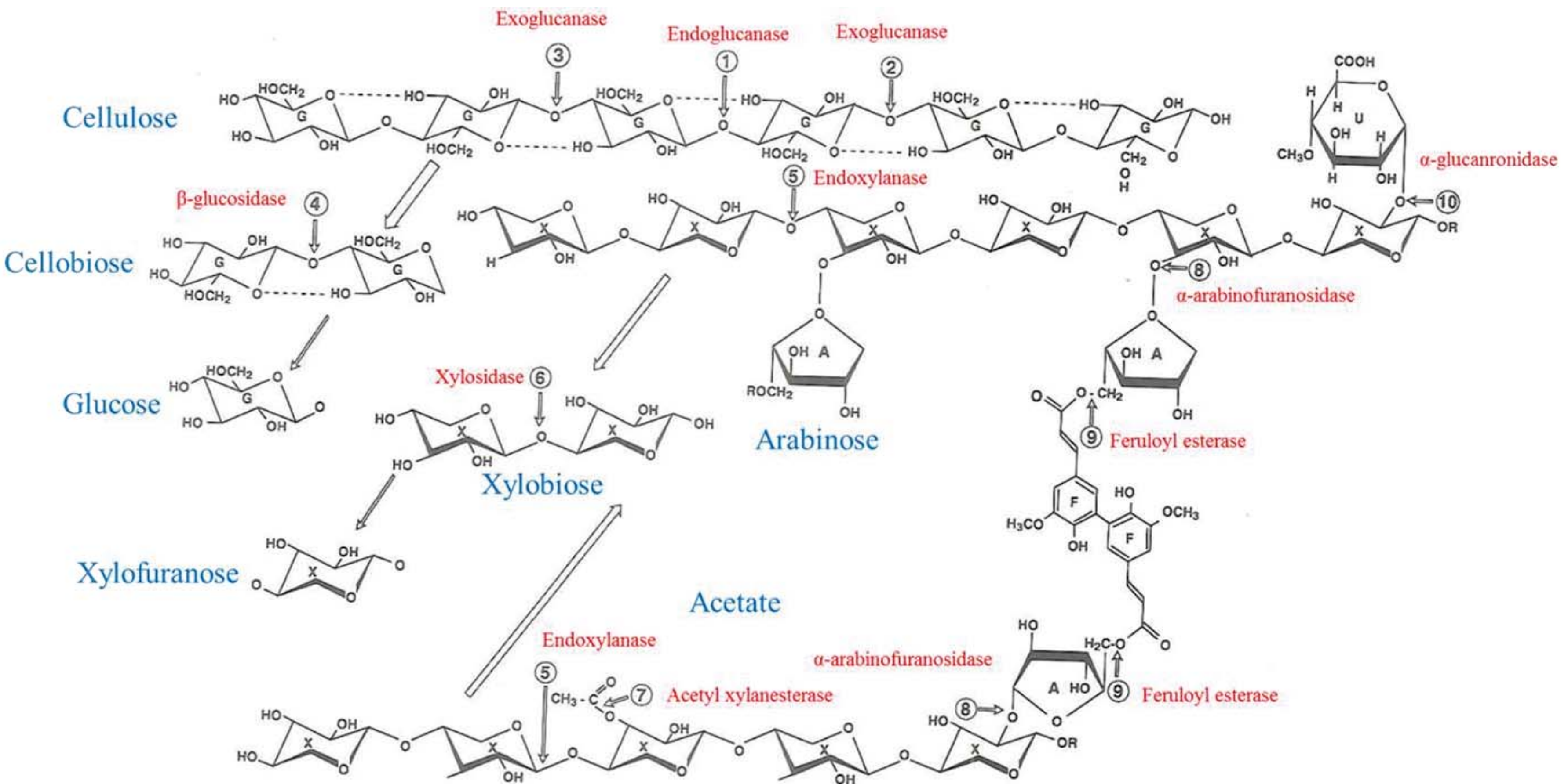

Fig 1.3 Schematic diagram illustrating the major linkages in the plant cell wall that are hydrolysed by rumen microorganisms. Sugars are labelled in blue and enzymes are labelled in red. Figure taken and modified from Hobson and Stewart (16), Fig 8.6.

Cellulose degradation differs between species (17). One process of cellulose degradation involves the individual secretion of a combination of endoglucanases and exoglucanases which act synergistically to break down the glycosidic bonds within the cellulose (17). Another mode, which is commonly employed by certain anaerobic microorganisms such as *Clostridium thermocellum* and *Ruminococcus flavefaciens*, relies on large, extracellular multienzyme complexes, called cellulosomes, to hydrolyse cellulose (15, 18). The organisation of FEs into cellulosomes is beneficial as it concentrates the enzymes in close proximity so that the catalytic units can act synergistically (15). It is also ideal for the extracellular degradation of cellulose as the enzymes are located at the interface between the cell and the insoluble substrate (15).

1.1.4.2 Hemicellulose degradation

Hemicellulose is a complex carbohydrate polymer that has a random, amorphous structure with little strength, and because of this, it is slightly easier to degrade than cellulose. The constituents that make up hemicellulose vary between different species of plants, however, xylan is the main carbohydrate found in hemicellulose (15). Hemicellulose is composed of a backbone of xylose monomers that can be substituted with an assortment of carbohydrates, including D-mannose, D-galactose, D-glucose, and L-arabinose, as well as 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids, which are linked together by β-1,4-glycosidic bonds, or occasionally β-1,3-glycosidic bonds (15, 19). Particular side groups can be esterified and subsequently crosslinked to other xylan chains or lignin (19). The array of covalent linkages within hemicellulose, and also between hemicellulose and other plant components such as pectin or lignin, makes hemicellulose difficult to degrade (19).

Hemicelluloses are generally broken down to monomeric sugars and acetic acid, and similarly to cellulose degradation, this process requires a variety of hydrolytic enzymes that include GHs, carbohydrate esterases, or polysaccharide lyases (15, 19). Endoxylanases, such as endo-β-1,4 xylanase, cleave internal linkages within the xylan backbone generating xylooligosaccharides (polymers of xylose), and β-xylosidases such as xylan-1,4-β-xylosidase hydrolyse the xylooligosaccharides, releasing xylose (Fig 1.3). Hemicellulose degradation also requires accessory enzymes, such as arabinases, arabinofuranosidases, esterases and glucuronidases that act synergistically to efficiently debranch the xylan backbone, and this promotes access to the xylan backbone by the endoxylanases (Fig 1.3) (19).

1.1.5 FEs as ruminant feed additives

Overseas, there is growing interest in the application of commercially available FE preparations to ruminant feed in an effort to enhance fibre degradation and improve animal performance and productivity. Exogenous enzymes extracted from the fibre hydrolysing system of the aerobic fungus *Trichoderma* are generally used in FE products commercially available overseas, as well as enzymes from *Aspergillus niger* and other aerobic fibrolytic fungi (7).

Cellulases and xylanases, the generic terms used for enzymes that can convert cellulose and xylan plant cell wall polysaccharides to soluble sugars, constitute many commercially available enzyme products where they are present in varying proportions and have variable activities (7). Commercial enzyme preparations also include secondary enzyme activities such as amylases, proteases and pectinases (7). However, despite consisting of a large array of activities, they are only standardised for their capacity to degrade cellulose or xylan (20).

Feed treatments are thought to enhance the hydrolytic capacity of the rumen by increasing bacterial attachment to fibre, stimulating rumen microbial populations and producing synergistic effects with rumen hydrolases (21, 22). Foreign studies have shown that the addition of commercial FE preparations to ruminant feed enhances the *in vitro* degradation of a) plant cell wall component substrates, such as cellulose or xylan (3, 22), and b) complex forage materials, including maize silage or alfalfa (21). In addition, FE additives have also been shown to enhance *in vivo* fibre degradation and digestion, which resulted in improved animal productivity, although results are quite variable and inconsistent (23, 24).

1.1.5.1 FEs and fibre degradation *in vitro*

Commercially available FEs have been repeatedly shown to enhance the *in vitro* degradation of simple, insoluble plant cell wall components, such as cellulose or xylan (25). *In vitro* degradation of a variety of forages, including alfalfa, corn stalks, maize silage, and other indigenous grasses has also been investigated, with promising results (7, 26-29).

Two separate experiments examined the effect of two similar commercial enzyme products on the *in vitro* degradation of either alfalfa or maize silage (30, 31). Liquicell 2500, which is

derived from *Trichoderma reesei,* was used along with Depol 40, a commercial product consisting of a mixture of enzyme activities from the *Trichoderma* spp. and *Aspergillus niger (31)*. In one experiment, both products were shown to increase the initial rates of organic matter degradation, as well as the degree of alfalfa leaf digestion when incubated at pH 5.5 and 39˚C (31). The second experiment demonstrated that regardless of ensiling temperature, Depol 40 and Liquicell 2500 decreased the starch and fibre content of maize silage, respectively, due to increased degradation (30).

Taking such results at face value would suggest that the addition of commercial enzyme products to forage *in vitro* enhances degradation, potentially improving fibre digestion and animal productivity. However, further characterization of Depol 40 and Liquicell 2500 revealed that although these products were expected to have very similar activities, they were, in fact, complex mixtures of proteins with distinct arrays of activities, likely due to the different species of origin (31).

Enzyme variability for similar commercial products is one of many factors that can affect the overall efficacy of enzyme treatments. The reporting of different activity values for similar commercial enzyme products appears to be common (24, 26). Discrepancies between experiments could also result from variations in assay conditions, including the type of forage used or moisture content, as well as variations in procedures between research groups, such as the length of pre-treatment enzyme incubation (26, 32).

Such issues highlight the need for a standardization of methodologies used to determine enzyme activities (3). Colombatto and Beauchemin (33) reported attempts to standardize fibrolytic activities using specific methodologies and arbitrary enzyme activity units. In saying this, it is unclear how much of an impact this has had on the field, as only a handful of articles published after 2003 have adopted their recommendations.

1.1.5.2 FEs and fibre degradation *in vivo*

Research investigating the effect of commercially available FEs on *in vivo* fibre degradation has increased in recent years. Although literature on the whole seems to conclude that FE additives enhance fibre degradation and increase animal productivity, such results are variable and inconsistent (23, 24).

Holtshausen, et al. (34) hypothesized that pre-treating the forage fed to early lactating cows with the commercial fibrolytic product, Econase, would increase milk production. Early lactating cows are in a negative energy balance and therefore require optimal nutrition in order to maintain milk production (34). Their experiment investigated the hypothesis using *in vitro, in situ* and *in vivo* methodologies, and concluded that supplementing feed with a high level of Econase resulted in better nutrient utilisation, increasing milk production by 10.7%.

In contrast, the earlier research carried out by Vicini, et al. (6) did not support an increase in fibre digestion and animal productivity through the addition of supplemental exogenous enzymes. Vicini, et al. (6) found that the addition of supplemental FEs to feed had no effect on milk production or milk composition in early lactating cows. The direct fed mode of application was a major difference of the study, and it was therefore hypothesized that the FE treatment may have been degraded by proteases upon entry into the rumen (6). This, and other papers that found no change or a decrease in fibre digestion and animal performance, were important for highlighting the variation of treatments and results found within the field.

The inconsistency of responses to supplementary FEs could be due to a number of factors, including differences in diet composition, type of enzyme used, enzyme level provided, enzyme stability and the method of application (7). This, along with differences between animals, such as rumen pH, microbial populations and energy balances for instance, contribute to the variability of results, making it difficult to compare experiments or draw any overarching conclusions (34). Moreover, experiments have been conducted with a large variety of forage and animal species, most of which are not relevant to NZ dairy farming. As overseas dairy farming systems differ, the applicability of such treatments and technologies based on foreign grasses and animals would need to be optimised in order to be applicable to dairy farming in NZ.

Overall, the current literature on experiments conducted both *in vitro* and *in vivo* could be interpreted as support for the hypothesis that FE additives enhance fibre degradation and, in turn, the amount of milk produced by the ruminants. However, this interpretation should be made with caution and an awareness of the variability and inconsistency present in the literature. The efficacy of the products is likely to be more consistent once the variables that have the largest effects on enzyme performance have been identified, and additional research focussed on this would be of great value. Furthermore, any new research should attempt to

incorporate some of the recommendations by Colombatto and Beauchemin (33) in order to standardise treatments and aid in valid comparisons.

One factor that has the potential to improve the efficacy of FE treatments is the biological source of enzymes. Most commercially available enzymes are derived from saprophytic aerobic fungi, and have optimal activity at a pH of approximately 5 to 6 and an optimal temperature of 50˚C (6). The rumen, however, has a pH ranging from 6 to 6.7 and a temperature of approximately 39˚C (6). With that in mind, it is possible that the aerobic fungal-derived FEs are not as effective as their rumen microbe-derived counterparts for use as feed additives, and thus rumen microbe-derived enzymes might represent a promising new avenue to explore as alternative FE treatments of supplemental feeds.

1.1.6 FE feed treatments in NZ

Currently, there are no commercially available forage treatment products available in NZ, which is surprising considering the huge influence that the dairy industry has on the economy. The lack of FE products available in NZ generates the potential to develop effective FE preparations that can be optimised for NZ dairy farming practices. As enzymes derived from rumen microbes are considered better suited to the anaerobic conditions of the rumen, the possibility of using rumen microbe-derived FEs to enhance fibre degradation and digestion should be explored.

1.1.7 The global use of rumen microbe-derived FEs

As already mentioned, the rumen acts as both a fermentation chamber and mixer, and provides the necessary conditions for rumen microorganisms to degrade forage (10, 13). Specific rumen microorganisms can break down the cellulose and hemicellulose components of the plant cell wall into simple sugars, and then ferment these into compounds that can be utilised as an energy source by the host animal (10). It is important to note that a single rumen microorganism strain can encode numerous activities, with *R. flavefaciens* for instance, possessing a wide range of activities, including cellulases, xylanases, pectinases and esterases (35). A large variety of bacteria, fungi and protozoa reside in the rumen, although the microbial population of the rumen is greatly influenced by diet (10).

Previous studies have characterized the fibrolytic potential of enzymes isolated from rumen microorganisms, including the fungal species *Piromyces* spp., *Orpinomyces* spp., and the bacterial species *Ruminococcus* spp., with promising results (36-38). It has been demonstrated that seven out of ten *Piromyces* spp. strains isolated from cattle improved the *in vitro* digestibility of wheat straw, with one isolate in particular, CF1, significantly improving digestibility by 53.4% (38). Another study showed enhanced growth of, and fibre degradation by, *R. flavefaciens* strain 17 after selecting for natural variants that produced increased levels of FEs following 23 serial subcultures on a perennial ryegrass based culture medium (35). Such research has demonstrated that the rumen microbiome possesses potent fibrolytic activity, and these activities can be enhanced without using genetic modification (GM) methodologies, which is an important consideration in the development of FE treatments for use in NZ where the use of GM is highly restricted.

Rumen fungi are regarded as the most potent fibre degraders, but they grow much slower and require a lot more finesse with media preparation and subculturing compared to rumen bacteria. This, along with the increased availability of a variety of rumen bacterial strains in culture, specifically through the Hungate 1000 project, and the potential for improving fibrolytic activity by non-recombinant means, makes rumen bacteria ideal to use as a foundation for the development of a rumen microbe-derived FE feed additive.

1.1.8 Improving fibrolytic activity

Although rumen bacterial strains naturally possess potent fibre-degrading enzymes, for use as a supplemental feed treatment, FE production would have to be significantly enhanced. Two potential methods to enhance fibre degradation by rumen microbe-derived FEs are a) express individual enzymes in *Escherichia coli,* which will allow the up-scaled production of enzymes, or b) isolate native organisms with exceptional degradation abilities and express the FEs from the native strain (39). However, based on NZ's reluctance to use genetically modified organisms (GMOs), an appropriate method to enhance fibre degradation using rumen microbe-derived FEs would involve identifying native organisms with exceptional degradation abilities and improving these abilities via positive selection and/or directed evolution.

As the rate at which spontaneous mutations occur in a microbe's genome is low, directed evolution is a tool that can be used in the laboratory to alter the activities of individual genes and gene products in a practical timescale (40). The study conducted by Saluzzi, et al. (35) attempted to increase the FE production of *R. flavefaciens* by selecting for natural variants with improved levels after adaptation to growth on a variety of fibrous substrates. After 23 serial subcultures on a perennial ryegrass based culture medium, there was increased bacterial growth and a significant increase in fibre degradation by 60% in 24 hour (hr) incubations (35). Other avenues to explore for improving production would include altering media composition, which has been shown to alter FE production (41), or the use of random mutagenesis to increase the proportion of variants that can produce higher quantities of FEs.

1.1.8.1 Random mutagenesis

The use of random mutagenesis can increase the chances of obtaining cells with desired mutations, and this requires the use of chemical and physical agents that randomly damage deoxyribonucleic acid (DNA), resulting in point mutations, insertions, and deletions in the genomic strands which can lead to phenotypic changes (40). Physical mutagens include ionizing radiation such as x-rays or gamma rays, and ultraviolet (UV) radiation (40, 42). Chemical mutagenic agents include intercalating agents such as ethidium bromide, alkylating compounds such as ethyl methanesulfonate (EMS) and ethylnitrosourea, and deaminating compounds such as nitrous acid (40, 42). Although chemical mutagenesis can result in the random deactivation of genes, it is used for directed evolution less often because of biases in the mutation spectrum (40). It is easier to handle and safer to use when used correctly, however. Non-chemical methods that also randomly mutate genes, as well as enhance the rate of errors during DNA replication, are used more routinely, but also have biases in the mutation spectrum (40). Therefore, in order to increase the variety of mutation types, and further increase the likelihood of generating mutations of interest, the best approach would ideally utilise a combination of chemical and non-chemical mutagenic agents.

1.1.8.2 Random mutagenesis using ultraviolet (UV) radiation

UV radiation is one of the most effective physical mutagenic agents, and has been used extensively to enhance the production of cellulases and xylanases in various microbes (43-46). UV radiation induces adjacent pyrimidines on the same DNA strand to bind together, resulting in the formation of pyrimidine dimers (45). These pyrimidine dimers inhibit the

correct base pairing of DNA, and the altered DNA structure prevents replication (45). Pyrimidine dimers can be repaired by two DNA-repair mechanisms, photoreactivation or nucleotide excision repair, but unrepaired pyrimidine dimers can result in mutations in the DNA sequence through a cytosine (C) to thymine (T) transition (45).

1.1.8.3 Random mutagenesis using EMS

As with UV radiation, EMS has also been used as a mutagenic agent to improve the production of cellulases and xylanases in various microbes (45-48). EMS mutates DNA by alkylating at the O^6 or N^7 position of guanine (G) which can impair normal base pairing and result in G/C to adenine (A)/T transitions during DNA repair as polymerases tend to base pair the chemically modified G with T (42, 48). Subsequent rounds of replication result in the original G/C pair being replaced with A/T. Treatment of DNA with EMS can also generate transversions, such as G/C to C/G, or G/C to T/A, but this occurs at a much lower frequency (48). Along with random point mutations, EMS can also produce a low level of chromosomal breaks and lethal effects (42).

As both UV radiation and EMS generate different types of mutations in DNA, combining both treatments could induce a much wider range of genetic variation. Using these mutagenic agents in combination with positive selection in the form of long-term serial subculturing on a fibrous substrate could therefore increase the likelihood of rapidly developing non-recombinant rumen bacterial strains with improved FE production and activity. Generating improved strains in this way is considered non-recombinant as DNA from another organism is not introduced to, or combined with, the DNA of the rumen bacterial strains, and this should therefore be acceptable for use in NZ. Such rumen microbe-derived FEs could prove more effective than the commercial preparations currently available overseas.

1.1.9 Proteomics

Proteomics is the large-scale study of proteins present in a tissue or organism, and it often relies on biochemical methods to analyse proteomes of interest (49). As the development of new sequencing technologies has provided researchers with copious amounts of genomic information, it has become apparent that biological properties are not determined by genetic sequence alone. Proteomic methods can therefore be employed to identify or quantify

proteins within a sample of interest, as well as elucidate post-translational modifications and protein interactions (49).

In regards to the rumen, proteomic research has helped to establish the proteome of several rumen microbes, such as *Mannheimia succiniciproducens*, *B. proteoclasticus* B316 and *R. albus* 007 (18, 19, 50). Proteomic methods have also been used to elucidate differences in the protein expression of *B. fibrisolvens* grown on different substrates (39), and to identify cellulose-binding proteins that are present in the sheep rumen (51), or produced by *R. albus* 20 (52).

As there is much that is unknown about fibre degradation in the rumen, proteomic information that can add to a deeper understanding of the mechanisms that underlie the process will prove valuable and help to ascertain if current knowledge and theories are accurate. Therefore, it would be interesting to identify the secreted proteins, or secretome, of any rumen bacteria with exceptional fibre-degrading abilities in order to add to the current literature on fibre degradation.

1.1.10 Significance

The rumen bacterial strains and FEs identified through this project could form the basis for feed pre-treatment products to be developed for use as supplemental feed additives in NZ. These products should enhance the digestion of supplemental feeds, thereby improving animal performance and milk solids production. This project will also provide an enhanced understanding of both *in vitro* FE production from native rumen bacteria, as well as methods for improving enzyme production.

1.2 Project aims

This project aims to:

1. Screen a diverse range of plant-adherent rumen bacterial strains that are predicted to be fibrolytic for fibrolytic activities *in vitro*, and identify the top ranking strains.

2. Improve the fibrolytic activity of three rumen bacterial strains using a combination of mutagenesis and artificial selection techniques.
3. Identify the suite of FEs that are expressed from strains with improved fibrolytic activity using proteomic methods, including SDS PAGE and mass spectrometry.

Chapter Two: Material and Methods

2.1 Materials

2.1.1 Bacterial strains

Bacterial strains used in this study are listed in Table 2.1.

Table 2.1. Rumen bacterial strains used in this study.

Hungate ID	Species	Strain	Reference
HUN171	*Bacteroides* sp.	Ga6A1	Ronimus, et al., unpublished
HUN051	*Butyrivibrio fibrisolvens*	MD2001	Noel (53)
n/a	*Butyrivibrio proteoclasticus*	B316	Attwood, et al. (54)
HUN072	*Butyrivibrio* sp. 2	FC2001	Noel (53)
HUN012	Clostridiales bacterium	NK3B98	Kenters, et al. (55)
HUN122	*Lachnospira multipara*	LB2003	Noel (53)
HUN002	Lachnospiraceae genus 6	NK4A136	Kenters, et al. (55)
HUN011	Lachnospiraceae genus 7	NK4A144	Kenters, et al. (48)
HUN030	*Oribacterium* sp.	NK2B42	Kenters, et al. (48)
HUN167	*Prevotella brevis*	P6B11	n/a
n/a	*Prevotella ruminicola*	23	Bryant, et al. (56)
n/a	*Pseudobutyrivibrio* sp.	MA3014	Noel (53)
HUN093	*Ruminococcus albus*	AD2013	Noel (53)
HUN082	*Ruminococcus flavefaciens*	AE3010	Noel (53)
n/a	*Ruminococcus flavefaciens*	FD1	n/a

The bacterial strains used in this study were pre-selected based on their predicted functional activities that were derived from their genome sequences.

2.1.2 Oligonucleotides

The primers used for 16S ribosomal ribonucleic acid (rRNA) gene amplification were made by Integrated DNA Technologies (Custom Science). The fD1 primer (AGA GTT TGA TCC

TGG CTC AG) and the rD1 primer (AAG GAG GTG ATC CAG CC) were resuspended in MilliQ water (MQH_2O) to a concentration of 100 µM and stored at -20˚C. For use in polymerase chain reactions (PCR), the primers were diluted to a working concentration of 10 µM using MQH_2O, and stored at -20˚C.

2.1.3 Laboratory chemicals and enzymes

General laboratory chemicals were manufactured by Sigma-Aldrich, Becton-Dickinson (USA), Thermo Fisher Scientific (USA), Merck (USA) and Life Technologies (USA). BD Bacto Agar was manufactured by Becton-Dickinson and bacteriological agar was manufactured by Oxoid (UK). All chemicals were analytical grade, unless stated otherwise. Analytical grade 100% ethanol, 96% ethanol, isopropanol and methanol were supplied by VWR International Ltd. (USA). Gas mixtures and liquid nitrogen were supplied by BOC Gases (Auckland, NZ).

2.1.4 Buffers and solutions

Standard buffers and solutions were prepared as described in Sambrook, et al. (57). Sterilisation was preformed either by autoclaving at 121ºC for 20 min at 15 pounds per square inch (psi), or by filtration through a sterile Millex®-GP Filter Unit containing a Millipore Express PES membrane with a 0.22 µm pore size (Millipore). Buffers and solutions were stored at room temperature (RT) unless stated otherwise.

2.1.4.1 Trace element solution SL10

The components of the trace element solution SL10 (58) were added and dissolved in the order listed in Table 2.2.

Table 2.2. Trace element solution SL10 components.

Components	Quantity
25% (v/v) HCl	10 millilitres (mL)
$FeCl_2 \cdot 4H_2O$	1.5 grams (g)

$CoCl_2 \cdot 6H_2O$	190 milligrams (mg)
$MnCl_2 \cdot 4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
Distilled water (dH_2O)	To 1 litre (L)

The trace element solution was sterilised in 25 mL aliquots within 50 mL screw-capped bottles by autoclave.

2.1.4.2 Selenite/Tungstate solution

The components of the Selenite/Tungstate solution (58) were added and dissolved in the order listed in Table 2.3.

Table 2.3. Selenite/Tungstate solution components.

Components	Quantity
NaOH	0.5 g
$Na_2SeO_3 \cdot 5H_2O$	3 mg
$Na_2WO_4 \cdot 2H_2O$	4 mg
dH_2O	To 1 L

The Selenite/Tungstate solution was sterilised in 50 mL aliquots within 100 mL screw-capped bottles by autoclave.

2.1.4.3 0.1% (w/v) resazurin solution

Resazurin was dissolved in dH_2O to give a final concentration of 0.1% (w/v) resazurin.

2.1.4.4 Vitamin 10 concentrate

The components listed in Table 2.4 were added together and filter sterilized into sterile 100% N_2-filled serum bottles. The bottles were then wrapped in aluminium foil to protect against light, and stored at 4°C.

Table 2.4. Vitamin 10 concentrate components.

Components	Quantity
4-aminobenzoate	40 mg
d-(+)-biotin	10 mg
Nicotinic acid	100 mg
Hemicalcium D-(+)-pantothenate	50 mg
Pyridoxamine hydrochloride	150 mg
Thiamine chloride hydrochloride	100 mg
Cyanocobalamin	50 mg
D,L-6,8-thioctic acid	30 mg
Riboflavin	30 mg
Folic acid	10 mg
dH_2O	To 1 L

2.1.4.5 Mineral 1

Mineral 1 was made by dissolving K_2HPO_4 in dH_2O to a final concentration of 0.6%, and then sterilised by autoclaving.

2.1.4.6 Mineral 2

Mineral 2 was made by mixing all of the components in Table 2.5, and sterilised by autoclaving.

Table 2.5. Mineral 2 components.

Components	Quantity
dH_2O	100 mL
KH_2PO_4	0.6 g
$(NH_4)_2SO_4$	0.6 g
NaCl	1.2 g
$MgSO_4 \cdot 7H_2O$	0.255 g
$CaCl_2 \cdot 2H_2O$	0.169 g

2.1.4.7 40% (v/v) anaerobic buffered glycerol

All of the components listed in Table 2.6, except L-cysteine HCl H_2O, were added to an Erlenmeyer flask, mixed thoroughly and brought to the boil in the microwave. It was immediately gassed with 100% CO_2 and simultaneously cooled in an ice bath. Once the solution cooled, L-cysteine HCl H_2O was added. The solution was dispensed in 75 mL aliquots into serum bottles, crimp-sealed with a rubber bung and aluminium seal, and autoclaved. This solution was aseptically added to cultures to give a final glycerol concentration of 10% (v/v).

Table 2.6. 40% (v/v) anaerobic buffered glycerol components.

Components	Quantity
Salt solution A	85 mL
Salt solution 2B	85 mL
dH_2O	130 mL
Glycerol	200 mL
$NaHCO_3$	2.5 g
0.1% Resazurin solution	2 drops
L-cysteine HCl H_2O	250 mg

2.1.4.8 Salt solution A

The components in Table 2.7 were added and mixed together. The solution was then filter sterilised into a sterile Schott bottle.

Table 2.7. Salt solution A components.

Components	Quantity
NaCl	15 g
KH_2PO_4	7.5 g
$(NH_4)_2SO_4$	3.75 g
$CaCl_2.2H_2O$	1.98 g
$MgSO_4.7H_2O$	3.0 g
dH_2O	To 2.5 L

2.1.4.9 Salt solution 2B

To make salt solution 2B, 15 g of K_2HPO_4 was dissolved in 2.5 L of dH_2O and the solution was then filter sterilised into a sterile Schott bottle.

2.1.4.10 Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (TAE buffer)

A stock solution of 50x TAE buffer was made up of 2 M Tris base, 1 M acetic acid and 50 mM EDTA, pH 7.0. A working solution of 1x TAE buffer was prepared using a 50-fold dilution of 50 x TAE buffer in dH_2O to obtain the following final concentrations: 40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 7.0. The 1x TAE buffer was used as is.

2.1.4.11 50 mM phosphate buffer, pH 6.5

To make 50 mM phosphate buffer, pH 6.5, 4.82 g of $NaPO_4$ H_2O and 4.04 g of Na_2PO_4 $7H_2O$ were added separately to 1 L of dH_2O (Table 2.10). The pH was adjusted to 6.5 using phosphoric acid, and the buffer was filter sterilised into a sterile Schott bottle.

2.1.4.12 50 mM 2-ethanesulfonic acid (MES) buffer, pH 6.5

To make 50 mM MES buffer, pH 6.5, 9.8 g of MES monohydrate was dissolved in 1 L of dH_2O, and the pH was adjusted to 6.5 using 10 M NaOH. The solution was then autoclaved.

2.1.4.13 Bradford reagent

The Coomassie Brilliant Blue G-250 was dissolved in 95% ethanol, and then 85% (w/v) phosphoric acid was added (Table 2.12). Once the dye had completely dissolved, the solution was made up to 1 L with dH_2O. The reagent was then filtered through Whatman #1 filter paper (FP, GE Healthcare) and stored at 4˚C.

Table 2.8. Bradford reagent components.

Components	Quantity
Coomassie Brilliant Blue G-250	100 mg
95% ethanol	50 mL
85% (w/v) phosphoric acid	100 mL
dH_2O	To 1 L

2.1.4.14 Biuret reagent

To make the Biuret reagent (59), $CuSO_4$ $5H_2O$ and $NaKC_4H_4O_6$ $4H_2O$ were dissolved in 500 mL of dH_2O (Table 2.13). To this, 10% NaOH (w/v) and potassium iodide (KI) were added, and the solution was made up to 1 L with dH_2O (Table 2.13). The reagent was stored in a plastic container, protected from light.

Table 2.9. Biuret reagent components.

Components	Quantity
$CuSO_4$ $5H_2O$	1.5 g
$NaKC_4H_4O_6$ $4H_2O$	6.0 g
10% (w/v) NaOH	300 mL
KI	2 g
dH_2O	To 1 L

2.1.4.15 3,5 Dinitrosalicylic acid (DNS) reagent

As listed in Table 2.10, the DNS reagent was dissolved in 300 mL of dH_2O. With continuous stirring, the NaOH was added gradually and left to dissolve. The potassium tartrate tetrahydrate was added in small portions and dH_2O was added to a final volume of 500 mL. The reagent was stored in a glass bottle, protected from the light.

Table 2.10. DNS reagent components.

Components	Quantity
DNS	5 g
NaOH	8 g
Potassium tartrate tetrahydrate	150 g
dH_2O	To 500 mL

2.1.4.16 1% (w/v) oat spelt xylan (OSX) solution

To make a solution of 1% (w/v) OSX, 1 g of OSX was added to 100 mL of 50 mM phosphate buffer, pH 6.5, and the solution was heated in the microwave until it began to boil. It was then cooled overnight with stirring. The solution was stored at 4˚C for up to a week.

2.1.4.17 1% (w/v) Sigmacell cellulose solution

To make a solution of 1% (w/v) Sigmacell cellulose, 1 g of Sigmacell cellulose was added to 100 mL of dH_2O and mixed with stirring. The solution was stored at 4˚C for up to a week.

2.1.4.18 100 mM bis-tris propane (BTP) buffer, pH 7.3

To make 100 mM BTP buffer, pH 7.3, 1.41 g of BTP was added to 50 mL of dH_2O. The pH was then adjusted to 7.3 with 5M HCl at RT to give a pH of 7.0 at 37˚C, the assay temperature. The buffer was stored at 4˚C.

2.1.4.19 1.5 M Tris-HCl, pH 8.8

To make 1.5 M Tris-HCl, pH 8.8, 18.15 g of Tris-HCl was dissolved in 100 mL of dH_2O. The pH was adjusted to 8.8, and the solution was then autoclaved.

2.1.4.20 0.5 M Tris-HCl, pH 6.8

To make 0.5 M Tris-HCl, pH 6.8, 6 g of Tris-HCl was dissolved in 100 mL of dH_2O. The pH was adjusted to 6.8, and the solution was then autoclaved.

2.1.4.21 10% (w/v) sodium dodecyl sulfate (SDS)

SDS was dissolved in dH_2O with gentle stirring to give a final concentration of 10% (w/v) SDS. The solution was then stored at RT.

2.1.4.22 10% (w/v) ammonium persulphate (APS)

APS was dissolved in dH_2O to give a final concentration of 10% (w/v), and either used immediately or made up in a larger volume and stored in 100 microlitre (µL) aliquots at -20˚C.

2.1.4.23 10x running electrode buffer, pH 8.3

All components listed in Table 2.11 were mixed with stirring. To use when running SDS-PAGE gels, the 10x running electrode buffer was diluted to a 1x working solution using dH_2O to obtain the following final concentrations: 1.44% glycine, 25 mM Tris base and 0.1% SDS.

Table 2.11. 10x running electrode buffer, pH 8.3, components.

Components	Quantity
Glycine	144 g
Tris base	30 g
SDS	10 g
dH_2O	To 1 L

2.1.4.24 5x SDS PAGE sample loading buffer

All of the components listed in Table 2.12 were mixed together and stored at -20˚C in 50 µL aliquots.

Table 2.12. SDS-PAGE 5x sample loading buffer components.

Components	Quantity
SDS	1 g
Glycerol	5 mL
DTT	0.154 g
0.25 M Tris-HCl, pH 6.8	5 mL
Bromophenol blue	0.005g

2.1.4.25 SDS PAGE colloidal Coomassie fixing solution

To make the colloidal Coomassie fixing solution, 100 mL of acetic acid and 400 mL of ethanol were added to 500 mL of MQH_2O in a fume hood. The solution was stored at RT.

2.1.4.26 SDS PAGE colloidal Coomassie stock solution

The components listed in Table 2.13 were mixed together.

Table 2.13. Stock solution components.

Components	Quantity
10% ammonium sulphate	100 g
1% phosphoric acid	12 mL
0.1% Coomassie blue G-250 stock	1 g
MQH_2O	To 1 L

2.1.4.27 SDS-PAGE Colloidal Coomassie working solution

To make the colloidal Coomassie working solution, 800 mL of stock solution (Section 2.1.4.26) was added to 200 mL of methanol. The solution was stored at RT.

2.1.4.28 1x ammonium bicarbonate (AmBic)

To make 1x AmBic, 0.8g of AmBic was dissolved in 100 mL of MQH_2O.

2.1.4.29 Reducing solution

The reducing solution consisted of 10 mM dithiothreitol (DTT) made up in 1x AmBic (Section 2.1.4.28). The reducing solution was prepared immediately before use.

2.1.4.30 Alkylating solution

The alkylating solution consisted of 20 mM iodoacetamide made up in 1x AmBic (Section 2.1.4.28). The alkylating solution was prepared immediately before use.

2.1.4.31 Trypsin digestion solution

The trypsin digestion solution consisted of 20 ng/µL of trypsin and 1 mM $CaCl_2$ made up in 1x AmBic (Section 2.1.4.28). The trypsin digestion solution was prepared immediately before use.

2.1.5 Media

Standard media were prepared as described in Sambrook, et al. (57). Sterilisation was performed by autoclaving at 121ºC for 20 min at 15 psi and all media were stored at RT unless stated otherwise.

Anaerobic media was always prepared in Hungate tubes or Schott bottles, and bottles were sealed with butyl rubber stoppers and plastic stoppers in order to keep the media anaerobic. Any additives added to the anaerobic media after sterilisation were done so aseptically and anaerobically under the flame from a Bunsen burner using 100% CO_2 on a gassing bench. All pouring of anaerobic solid media into petri dishes was carried out under anaerobic conditions in an anaerobic glove box (Coy Laboratory Products Inc).

2.1.5.1 Rumen Microbiology media series number 2 (RM02)

The basic medium used for this study was RM02 (Table 2.14) (55). All of the components were mixed, except $NaHCO_3$ and L-cysteine HCl H_2O, and brought to boil in the microwave. The media was cooled in an ice bath as it was bubbled with 100% CO_2. Once cooled, $NaHCO_3$ and L-cysteine HCl H_2O were added, and the media was anaerobically dispensed into appropriate vessels as it continued to be gassed with 100% CO_2. The media was autoclaved, and then stored in the dark for at least 24 hours before use.

Table 2.14. RM02 media components.

Component	Quantity
KH_2PO_4	1.4 g
$(NH_4)_2SO_4$	0.6 g
KCl	1.5 g
Trace element solution SL10	1 mL
Selenite/Tungstate solution	1 mL
0.1% (w/v) resazurin solution	4 drops
dH_2O	To 950 mL
$NaHCO_3$	4.2 g
L-cysteine HCl H_2O	0.5 g

2.1.5.2 RM02 salts

RM02 salts were prepared in the same manner as RM02 media, except that trace element solution SL10 and Selenite/ Tungstate solution were omitted.

2.1.5.3 RM02 containing fibrous substrates

Fibrous substrates were prepared by weighing out 50 mg of either dried perennial ryegrass (RG), neutral detergent fibre (NDF), oat spelt xylan (OSX), apple pectin (AP) or Whatman #1 filter paper (FP) into clean Hungate tubes. RM02 media was prepared as described in Section 2.1.5.1, and dispensed in 8.75 mL aliquots into each pre-gassed tube containing the fibrous substrate. Hungate tubes were then sealed with a rubber bung and plastic lid, and autoclaved.

2.1.5.4 Preparing anaerobic RM02 + 2SGenRFV agar plates

Bacto agar was added into a Schott bottle to a final concentration of 1.5% and gassed with 100% CO_2. RM02 media (Section 2.1.5.1) was prepared and added to a gassed Schott bottle containing agar powder before the bottle was sealed with a rubber bung and plastic lid. The media was autoclaved and then transferred to a pre-warmed water bath immediately, where it was left to equilibrate to 55˚C. 2SGenRFV (Section 2.1.6.2) was added aseptically through a 0.22 μm PES filter to a final concentration of 5%. The media was then transferred into the anaerobic chamber and approximately 25 mL was carefully poured into each Petri dish. The plates were allowed to set in the chamber covered and upright overnight. Before use, the plates were dried inverted over paper towels for at least 1 hr. Plates were stored inverted within the anaerobic chamber at RT and used within two weeks.

2.1.6 Rumen fluid

2.1.6.1 Rumen fluid collection

Rumen fluid was collected from fistulated cows at the Ulyatt Reid Facility (animal ethics application 12754), Grasslands, AgResearch. The cows were fed hay for the four days prior to collection. Up to 10 L of rumen contents per animal was carefully removed from the rumen and squeezed through two layers of cheesecloth into a plastic bottle. Some of the

rumen content was returned to the rumen of the cow. The rumen fluid was then kept at 4˚C until centrifugation within 2 days, or frozen at -20˚C until required.

2.1.6.2 2x sugar and general rumen fluid with vitamins (2SGenRFV)

2SGenRFV was based on Kenters, et al. (55). The rumen fluid was thawed (if applicable) and centrifuged three times at 10, 000 xg for 20 min at 4˚C. The rumen fluid was then bubbled with 100% N_2 for 20 min, sealed and autoclaved. Per 100 mL of rumen fluid preparation, 1.63 g of magnesium chloride and 1.18 g of calcium chloride was added with stirring. The heavy precipitate that formed was removed by centrifuging the rumen fluid preparation at 10,000 xg for 20 min at 4˚C. The supernatant (SN), or clarified rumen fluid, was then tipped off into a clean beaker, and the additives listed in Table 2.15 were added with stirring.

Table 2.15. 2SGenRFV components.

Component	Quantity
Rumen fluid	100 mL
D-glucose	0.36 g
Cellobiose	0.36 g
Xylose	0.3 g
Arabinose	0.3 g
N-lactate syrup	0.88 mL
Yeast extract	2 g
Casamino acids	2 g
Tryptone	2 g

The clarified rumen fluid was gassed with 100% N_2 for 20 min, and then filter sterilised into a sealed, sterile bottle filled with 100% N_2. The 2SGenRFV mixture was stored at 4˚C. Before use, 2 mL of vitamin 10 concentrate (Section 2.1.4.4) was aseptically and anaerobically added per 100 mL of rumen fluid.

2.1.6.3 General rumen fluid with vitamins (GenRFV)

GenRFV was prepared in the same manner as 2SGenRFV (Section 2.1.6.2), except that no sugars were added.

2.1.6.4 Incubated clarified rumen fluid (ICRF) collection

Rumen fluid was collected from pasture fed cows (animal ethics application 12754). Rumen contents were squeezed through two layers of cheesecloth to fill half of a pre-warmed thermos flask. The thermos flask was then filled with whole rumen contents and quickly transported to the laboratory.

2.1.6.5 Incubated clarified rumen fluid (ICRF) incubation

The following protocol was based on the methodology outlined by Leedle and Hespell (60). Rumen contents were immediately transferred to a blender and blended for 1 min as it was vigorously gassed with 100% CO_2. The blended contents were then squeezed through four layers of cheesecloth and the fluid collected into a clean, 100% CO_2 filled Schott bottle partly submerged in a bucket full of warm water to keep the rumen fluid warm. To 300 mL of rumen fluid, 35 mL of mineral 1 (Section 2.1.4.5), 35 mL of mineral 2 (Section 2.1.4.6) and 300 mL of dH_2O were added under 100% CO_2. The rumen fluid mixture was gassed with 100% CO_2 for 15 min, and 2 mL of 2.5% L-cysteine HCl was added before the rumen fluid mixture was gassed for a further 5 min. The pH was adjusted to 6.8 with 100% CO_2 equilibrated 1 M Na_2CO_3. The Schott bottle was then sealed with a rubber bung stopper and plastic lid, and the mixture was incubated for 3 days at 39˚C shaking at 100 RPM. The pH was checked periodically and readjusted to 6.8 as required, and the pressure build up within the bottle was carefully released daily. After 3 days, the rumen fluid mixture was centrifuged at 13,000 xg for 30 min at 4 ˚C. The SN was carefully tipped off into clean bottles, and the resulting ICRF was kept at 4˚C until use, or stored long term at -20˚C.

2.1.6.6 ICRF GenRFV for the evolution experiment

The ICRF GenRFV was prepared in the same manner as GenRFV (Section 2.1.6.3), except that ICRF (Section 2.1.6.4 and Section 2.1.6.5) was used instead of undiluted rumen fluid, and close attention was paid to ensure the amount of additives added were relative to the amount of rumen fluid in the diluted ICRF preparation.

2.2 Methods

2.2.1 Subculturing of rumen bacteria

Anaerobic media used for subculturing rumen bacteria consisted of 9 mL of RM02 media (Section 2.1.5.1) prepared in Hungate tubes, 0.5 mL of 2S GenRFV (Section 2.1.6.2), and 0.5 mL of inoculum. Tubes were incubated at 39˚C for one to three days in the dark, shaking horizontally at 100 revolutions per min (RPM). To subculture the rumen bacteria, this process was repeated using 0.5 mL of culture from strains that had been incubated for one to three days as the inoculum.

2.2.2 Freezing and reviving rumen bacterial cultures

2.2.2.1 Freezing/preserving bacterial cultures

Anaerobic 40% (v/v) glycerol (Section 2.1.4.7) was added separately to each culture in order to give a final glycerol concentration of 10%. Cultures were stored at -20˚C for 24 hr and then transferred to -85˚C for long term storage.

2.2.2.2 Reviving bacterial cultures from frozen

Cultures to be resuscitated from frozen stocks were removed from the -85˚C freezer and placed on ice so that only the top of the frozen culture began to thaw. To 9 mL of RM02 (Section 2.1.5.1), 0.5 mL of 2SGenRFV (Section 2.1.6.2) and 0.5 mL of thawed culture was added. Frozen culture stocks were immediately returned to -85˚C, and resuscitated bacteria were then incubated at 39˚C for 2 days shaking at 100 RPM. The growth of resuscitated cultures was checked by measuring the optical density (OD) at 600 nanometres (nm) daily and purity was analysed by Gram stain (Section 2.2.4).

2.2.3 Growth of bacterial strains on fibrous substrates

2.2.3.1 Initial screening for fibrolytic activities

To RM02 tubes containing fibrous substrate (Section 2.1.5.3), 0.5 mL of GenRFV (Section 2.1.6.3) and 0.5 mL of inoculum from cultures grown on RM02 and 2SGenRFV for three days (Section 2.2.1) were added. All tubes were then incubated at 39 ˚C for up to 3 days in the dark, shaking horizontally at 100 RPM. The OD of each tube was measured at 600 nm every 24 hr using appropriate blanks.

2.2.3.2 Evolution experiment: long term sub culturing

To give a final rumen fluid concentration of 5%, 1.25 mL of ICRF GenRFV (Section 2.1.6.6) was added into RM02 tubes containing fibrous substrate (Section 2.1.5.3). To each tube, 0.5 mL of inoculum from cultures grown on RM02 and 2SGenRFV for three days (Section 2.2.1) was added, and tubes were then incubated horizontally at 39˚C in the dark for up to three days, shaking at 100 RPM. The OD of each tube was measured at 600 nm every 24 hr using appropriate blanks. Tubes were subcultured using fresh media each day, as described above, and when transferring the inoculum from the previous subculture on fibrous substrates into new tubes, it was imperative to ensure pieces of the fibrous substrate were included in the transfer.

2.2.4 Gram staining rumen bacteria

2.2.4.1 Gram staining from broth cultures

Approximately 100 µL of culture was removed from bacterial cultures, spread over a clean microscope slide and heat fixed. Samples were stained with 10% (w/v) crystal violet for 1 min followed by fixation of the stain with 0.3% (w/v) iodine and 0.7% (w/v) potassium iodine for 1 min. The cells were then de-colourised with 50% (v/v) acetone, and counterstained with 2.5% (w/v) safranin for 1 min. Slides were gently rinsed with tap water between each step, and left to dry overnight. Each slide was viewed using a Leica DM2500 microscope (Bio–Strategy).

2.2.4.2 Gram staining from colonies

Single colonies obtained from anaerobic agar plates were smeared onto a microscope slide using a sterile 10 µL inoculating loop, either within the anaerobic chamber or on a laboratory bench. Slides were then heat fixed and gram staining was carried out as per Section 2.2.4.1.

2.2.5 Gel electrophoresis and PCRs

2.2.5.1 Agarose gel electrophoresis

Agarose gels were prepared using 1% (w/v) agarose in 1× TAE buffer (Section 2.1.4.10) with 1× SYBR safe nucleic acid dye (Life Technologies). Gels were cast in trays manufactured by Bio-Rad and submerged in a running tank with 1× TAE buffer. A 1 kilobase (kb) Plus DNA ladder (100 to 12,000 base pairs, Life Technologies) was loaded in to the first lane of the gel, and 10 µL of each sample was mixed with 2 µL of 10× Blue Juice loading dye (Life Technologies) and loaded separately into wells. Gels were run at 100 V for 30 min and visualised using UV trans-illumination. Gels were photographed using a Nikon D700 camera with Kodak Gel Logic 200 Imaging System (Eastman Kodak Company).

2.2.5.2 Preparation of cell pellets (CPs) from broth cultures for PCR

One millilitre of culture was placed into a sterile Eppendorf tube and centrifuged at 14,000 xg for 1 min at RT. The SN was removed and discarded. The CPs were then resuspended in 50 µL of sterilised dH_2O and stored at -20˚C until required.

2.2.5.3 PCR

Per 50 µL PCR reaction, 1 µL of CP preparation (Section 2.2.5.2) was used, along with 1 µL of bacterial 16S forward primer fD1 (Section 2.1.2), 1 µL of bacterial 16S reverse primer rD1 (Section 2.1.2), and 47 µL of Supermix (Thermo Fisher). Reactions were carefully mixed and run on the proS Mastercycler PCR machine (Eppendorf) using the following protocol supplied by Thermo Fisher: initial denaturation at 94˚C for 15 seconds (sec), followed by 35 cycles of 94˚C for 15 sec, 54˚C for 30 sec, 72˚C for 1 min. The final elongation step was 72˚C for 10 min. Reactions were kept at 4˚C till removed from the proS Mastercycler machine for analysis.

2.2.6 SDS polyacrylamide gel electrophoresis (SDS PAGE)

2.2.6.1 SDS PAGE gel preparation

SDS PAGE gels were prepared by combining all separating gel components listed in Table 2.16 in order, except that 10% (w/v) APS and tetramethylethylenediamine (TEMED) were added immediately after the solution was degassed for 15 min. The solution was then pipetted into the casting plates of the Mini Protean III system (Bio-Rad), carefully overlaid with MQH_2O, and left to set at RT for 30 min.

Table 2.16. SDS PAGE separating gel components for 2 gels.

Components	7.5% (Separating)
MQH_2O	5.4 mL
1.5 M Tris-HCl pH 8.8	2.5 mL
10% (w/v) SDS	100 µL
Acrylamide/Bis (40%) (19:1)	1.9 mL
10% (w/v) APS	100 µL
TEMED	10 µL

All stacking gel components listed in Table 2.17 were prepared in a similar manner to the separating gel solution, and once the MQH_2O was removed from the gel casts, the stacking gel solution was carefully overlaid, followed by a 10 or 15 well comb. Gels were then allowed to set at RT for 30 min before they were stored in a MQH_2O-saturated paper towel at 4˚C for at least 24 hr and no longer than a month.

Table 2.17. SDS PAGE stacking gel components for 4 gels.

Components	4% (Stacking)
MQH_2O	6.4 mL
0.5 M Tris-HCl pH 6.8	2.5 mL
10% (w/v) SDS	100 µL
Acrylamide/Bis (40%) (19:1)	1 mL
10% (w/v) APS	100 µL
TEMED	10 µL

2.2.6.2 SDS PAGE gel electrophoresis

One part SDS PAGE sample loading buffer (Section 2.1.4.24) was mixed with 4 parts culture SN in a clean Eppendorf tube. To prepare the protein ladder, 1 µL of Low Range Protein Ladder (Bio-Rad) was added to 11 µL of dH_2O and 3 µL of sample loading buffer in a clean Eppendorf tube, and mixed. The samples and ladder were then boiled for 5 min, and loaded into the wells of a SDS PAGE gel (Section 2.2.6.1) set up in the Mini Protean III system within 350 mL 1x Running Electrode buffer, pH 8.3 (Section 2.1.4.23). Gels were run at 200 V until the dye front reached the bottom of the gel (approximately 45 min). Gels were then stained by submersion in 30 mL of the colloidal Coomassie fixing solution (Section 2.1.4.25) with shaking at 30 RPM for 15-30 min. The fixing solution was then replaced with 30 mL of colloidal Coomassie working solution (Section 2.1.4.27) and left shaking at 30 RPM overnight. The next day, the working solution was replaced with MQH_2O to destain until bands were visible.

2.2.7 Mutagenesis experiments

2.2.7.1 Serial dilutions for colony forming unit (CFU) analysis

One millilitre of suspension from each strain was used for serial dilutions in order to anaerobically plate 10 µL of cells at 10^{-4}, 10^{-5}, 10^{-6} dilutions on RM02 agar plates (Section 2.1.5.4) supplemented with 5% 2SGenRFV (Section 2.1.6.2). Typically, a 1/10 dilution factor was used as 100 µL of sample was serially diluted with 900 µL of RM02 salts (Section 2.1.5.2) in a 96 2-mL deep well plate (Medi'Ray) within the anaerobic chamber. Each sample was mixed before transfer by carefully pipetting the solution up and down at least three times. The agar plates with the desired dilutions plated out were inverted and incubated at 39˚C in anaerobic conditions until visible colonies could be counted.

2.2.7.2 EMS mutagenesis optimisation

Rumen bacteria to be tested were resuscitated (Section 2.2.3.2) and subcultured twice (Section 2.2.1). For each strain, 5 mL of resuscitated culture was inoculated into 100 mL of RM02 (Section 2.1.5.1) with 5% 2SGenRFV (Section 2.1.6.2). Cultures were incubated for 48 hr at 39˚C shaking at 100 RPM. After incubation, the OD of each culture at 600 nm was recorded, and cultures were pelleted anaerobically at 4000 xg for 6 min at 4˚C in sterile 15 mL tubes (Falcon). The cells were washed twice by anaerobically removing the SN, re-

suspending CPs in 10 mL of sterile RM02 salts (Section 2.1.5.2), and centrifuging at 4,000 xg for 6 min at 4˚C. Serial dilutions to assess time 0 CFUs (Section 2.2.7.1) were then carried out before the CPs were split equally into treatments. Each CP was resuspended in 5 mL of RM02 salts containing EMS which ranged in concentration (v/v) from 0%- 5%. The suspensions were incubated at 39˚C for times that ranged from 10 min to 1 hr with gentle shaking at 30 RPM. To each treatment, 5 mL of 10% (w/v) sodium thiosulphate was anaerobically added before centrifugation at 4,000 xg for 6 min at 4 ˚C. CPs were then washed twice in the same manner mentioned previously. Cells were resuspended in 10 mL of RM02 containing 5% 2SGenRFV and incubated at 39˚C for 1 hr gently shaking at 30 RPM. Serial dilutions were then used to assess CFUs after treatment, and these were compared to the time 0 CFUs in order to determine the optimal EMS concentration to give approximately 50% lethality.

2.2.7.3 EMS mutagenesis for evolution experiment

Rumen bacterial strains were resuscitated and subjected to EMS mutagenesis as described in Section 2.2.7.2, except that strains to be mutagenised were resuspended in RM02 salts containing 0.5% EMS and incubated at 39˚C for 15 min.

2.2.7.4 UV radiation mutagenesis optimisation

Rumen bacteria to be tested were resuscitated (Section 2.2.3.2) and subcultured twice (Section 2.2.1). For each strain, 0.5 mL of resuscitated culture was inoculated into 10 mL of RM02 containing RG (Section 2.1.5.3) with 1.25 mL of ICRF GenRFV (Section 2.1.6.6). Cultures were incubated at 39˚C for 24 hrs with shaking at 100 RPM. Strains were subcultured in the same manner twice, with additional tubes for each treatment in duplicate inoculated during the second subculture. The OD of each strain was measured, and then serial dilutions (Section 2.2.7.1) to assess time 0 CFUs were carried out for each duplicate treatment. Each culture was then inverted two times and placed vertically on the UV trans-illuminator (Spectroline, model TVD-1000), and subjected to UV exposure at 312 nm for an amount of time that ranged from 0 min to 2 hr in a dark room. After UV radiation, each culture was covered with tin foil and incubated at 39˚C for 1 hr, gently shaking at 20 RPM. Serial dilutions were then used to assess CFUs after treatment, and these were compared to the time 0 CFUs in order to determine the optimal EMS concentration to give approximately 95% lethality.

2.2.7.5 UV radiation mutagenesis for evolution experiment

Rumen bacterial strains from the evolution experiment were subjected to UV mutagenesis as described in Section 2.2.7.4, except that strains were exposed to the UV (312 nm) trans-illuminator for 40 min in the dark at RT and rotated every 15 min.

2.2.8 Measuring protein concentrations

2.2.8.1 Processing culture SN for protein and enzyme activity assays

Rumen bacterial cultures were centrifuged at 3,000 xg for 10 min at 4˚C. The SN was carefully removed and concentrated using Vivaspin^{TM} columns (20 mL, 10,000 MWCO, Life Technologies) centrifuged at 7,000 xg for 15 min at 4˚C in a fixed angle rotor. The SN was washed twice with two volumes of 50 mM MES, pH 6.5 (Section 2.1.4.12), and then concentrated to approximately 5 mL for strains grown on AP or FP, or approximately 10 mL for strains grown on RG or OSX (due to clogging of the filter). Sterile glycerol was added to the SN to give a final glycerol concentration of 20% (v/v), and enzyme preparations were stored at -80˚C.

2.2.8.2 Processing culture CPs for protein and enzyme activity assays

The CPs from rumen bacterial cultures centrifuged at 3,000 xg for 10 min at 4˚C were kept at -80˚C until assayed. CPs were then defrosted on ice, and resuspended in 1 mL of 50 mM phosphate buffer, pH 6.5 (Section 2.1.4.11).

2.2.8.3 Bradford assay

A standard curve was constructed, as detailed in Table 2.18, using a stock solution of 2 mg/mL bovine serum albumin (BSA) and dH_2O in a final volume of 100 µL.

Table 2.18. BSA standard curve volumes for the Bradford assay.

Standards (final [BSA] (mg/mL) in 100 µL)	Volume of BSA (2 mg/mL) to add (µL)	Volume of dH_2O to add (µL)
0.00	0	100
0.05	2.5	98

0.10	5	95
0.15	7.5	93
0.20	10	90
0.25	12.5	88
0.30	15	85

To each 100 µL standard, 1 mL of Bradford reagent (Section 2.1.4.13) was added. Standards were mixed and left at RT for 10 min before the ODs were measured at 595 nm. A standard curve was then constructed using Microsoft Excel. Enzyme samples were diluted in a final volume of 100 µL to a protein concentration that gave an OD that fit within the BSA standard curve. To each 100 µL enzyme sample, 1 mL of Bradford reagent was added. Samples were mixed and left at RT for 10 min before the ODs were measured at 595 nm. The amount of protein in each 100 µL sample was determined from the standard curve, and used to calculate the concentration of protein in the original (undiluted) sample. See Section 2.2.12.1 for calculations.

2.2.8.4 Biuret assay

A BSA standard curve was constructed, as detailed in Table 2.19, using a stock solution of 10 mg/mL BSA and 50 mM phosphate buffer, pH 6.5 (Section 2.1.4.11) to a final volume of 500 µL.

Table 2.19. BSA standard curve volumes for Biuret assay.

Standards (final [BSA] (mg/mL) in 500 µL)	Volume of BSA (10 mg/mL) to add (µL)	Volume of 50 mM phosphate buffer, pH 6.5 to add (µL)
0.0	0	500
0.5	25	475
1.0	50	450
1.5	75	425
2.0	100	400
2.5	125	375
3.0	150	350
4.0	200	300
5.0	250	250

6.0	300	200
8.0	400	100
10.0	500	0

To each 500 µL standard, 2 mL of Biuret reagent (Section 2.1.4.14) was added. The standards were mixed and left at RT for 25 min before the ODs were measured at 540 nm. A standard curve was constructed from the standards using Microsoft Excel. Samples were diluted (typically 400 µL of sample and 100 µL of 50 mM phosphate buffer, pH 6.5) to a protein concentration that gave an OD that fit within the BSA standard curve. To each 500 µL sample, 2 mL of Biuret reagent was added. Samples were mixed and left at RT for 25 min before measuring the absorbance at 540 nm. The amount of protein in each sample was determined from the standard curve and used to calculate the concentration of protein in the original (undiluted) sample. See Section 2.2.12.2 for calculations.

2.2.9 Cellulolytic and xylanolytic assays for initial screening of rumen bacterial strains

The SN and CP of each sample were incubated separately with either FP or OSX, and then assayed for cellulolytic or xylanolytic activity using DNS. The protocol used was based on the methodology outlined by Colombatto and Beauchemin (33).

2.2.9.1 Incubation of enzyme preparations with FP

Slits were cut into the skirt of a 96 2-mL deep well plate to allow even water contact to all wells of the plate. Into each inner well, 8 mg of FP was added. Freshly made glucose standards were prepared in dH_2O, with glucose concentrations of 0 mg/mL, 0.15 mg/mL, 0.3 mg/mL and 0.6 mg/mL, and 100 µL of each standard was added to the wells using a randomised set-up. Plates were put on ice and 100 µL of freshly prepared 5 mg/mL cellulase to be used as the enzyme control was added in quadruplicate to relevant wells. Finally, 100 µL of each enzyme preparation from either the culture SN (Section 2.2.8.1) or CP (Section 2.2.8.2) was similarly added in quadruplicate to relevant wells. To each well, 300 µL of 50 mM phosphate buffer, pH 6.5 (Section 2.1.4.11) was added, the plate was sealed with a breathable plate seal, and it was immediately incubated in a 39˚C water bath for three hours. The plate was then placed on ice until analysis using DNS was carried out.

2.2.9.2 Incubation of enzyme preparations with OSX

The incubation of enzyme preparations from either the culture SN or CP were carried out as described in Section 2.2.9.1, except that 300 µL of freshly made 1% OSX (Section 2.1.4.16) was used instead of FP and 50 mM phosphate buffer, pH 6.5, 100 µL of 5 mg/mL xylanase was used as the enzyme control, and xylose standards were prepared in 50 mM phosphate buffer, pH6.5 (Section 2.1.4.11), with xylose concentrations of 0 mg/mL, 0.2 mg/mL, 0.45 mg/mL and 0.9 mg/mL.

2.2.9.3 DNS assay to measure cellulolytic and xylanolytic activity

From each well of the plates used for the incubation of enzyme preparations with either FP or OSX, a 150 µL sample was removed and transferred into a new 2 mL deep well plate with slits cut into the skirt, followed by 120 µL of DNS reagent (Section 2.1.4.15). Plates were then sealed with a breathable seal and heated in a boiling water bath for exactly 5 min. Once cooled, plates were centrifuged (Sigma) at 600 xg for 5 min at RT, and then 100 µL of sample was removed and transferred into a new 96 flat-bottom well plate (Eppendorf). The absorbance was read at 540 nm, and the amount of sugar released in each treatment was calculated using the glucose or xylose standard curve.

2.2.9.4 Cellulolytic and xylanolytic assays for evolved strains

Assaying the enzyme activity of select evolved rumen bacterial strains was carried out as described in Section 2.2.9, except that a 1% suspension of Sigmacell cellulose (Section 2.1.4.17) was used instead of FP.

2.2.10 *Para*-nitrophenol (*p*NP)-conjugated substrate assays

The SN of each enzyme sample was incubated separately with *p*NP-conjugated artificial substrates that represent major biochemical linkages in the plant cell wall. Fibrolytic activity was analysed by measuring absorbance at 405 nm every 2 min using a plate reader overnight.

2.2.10.1 Standard preparation for the *p*NP-conjugated substrate assay

Standards were prepared by diluting 10 mM *p*NP with 100 mM BTP buffer, pH 7.3 (Section 2.1.4.18), as listed in Table 2.20.

Table 2.20. *p*NP standards for the *p*NP-conjugated substrate assay.

Standard	Standard concentration (mM)	Volume of 10 mM pNP to add (µL)	Volume of 100 mM BTP buffer, pH 7.3 to add (µL)
S0	0.0	0	1000
S10	0.1	10	990
S20	0.2	20	980
S30	0.3	30	970
S40	0.4	40	960
S50	0.5	50	950

With reference to the plate layout (Figure 2.1), 150 µL of each freshly made standard was aliquoted in duplicate into the appropriate wells of a 96-well flat bottom plate.

	Standards		Enzyme 1				Enzyme 2					
	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S0	Sub1	Sub1	Sub9	Sub9	Sub1	Sub1	Sub9	Sub9		
B	S10	S10	Sub2	Sub2	Sub10	Sub10	Sub2	Sub2	Sub10	Sub10		
C	S20	S20	Sub3	Sub3	Sub11	Sub11	Sub3	Sub3	Sub11	Sub11		
D	S30	S30	Sub4	Sub4	Sub12	Sub12	Sub4	Sub4	Sub12	Sub12		
E	S40	S40	Sub5	Sub5	Sub13	Sub13	Sub5	Sub5	Sub13	Sub13		
F	S50	S50	Sub6	Sub6	Sub14	Sub14	Sub6	Sub6	Sub14	Sub14		
G			Sub7	Sub7	Sub15	Sub15	Sub7	Sub7	Sub15	Sub15		
H			Sub8	Sub8	Sub16	Sub16	Sub8	Sub8	Sub16	Sub16		

Fig 2.1. Plate layout for *p*NP-conjugated substrate assays.

2.2.10.2 Substrate preparation for the *p*NP-conjugated substrate assays

*p*NP-conjugated substrates (Table 2.21) were diluted in 100 mM BTP buffer, pH7.3 (Section 2.1.4.18), to a final concentration of 15 mM, and stored at -20˚C in 1 mL aliquots. For use in the assay, substrates were thawed at RT on ice and mixed gently. Substrate wells contained 25 µL of 100 mM BTP buffer, pH 7.3, 25 µL of 15 mM *p*NP-conjugated substrate in duplicate and 100 µL of culture SN or CP from samples to be tested. All plates were kept on ice, and the culture SN or CP was added to the plate immediately before starting the assay. Absorbance was measured at 405 nm every two min over 16 hr at 37˚C.

Table 2.21. List of *p*NP-conjugated substrates tested.

Substrate	Substrate ID	Molecular weight	Manufacturer
*p*NP α-D-mannopyranoside	Sub1	301.25	Sigma Aldrich
*p*NP ß-D-fucopyranoside	Sub2	285.26	Sigma Aldrich
*p*NP α-D-galactopyranoside	Sub3	301.25	Sigma Aldrich
*p*NP β-D-glucopyranoside	Sub4	301.25	Sigma Aldrich
*p*NP α-D-glucopyranoside	Sub5	301.25	Sigma Aldrich
*p*NP β-D-mannopyranoside	Sub6	301.25	Sigma Aldrich
*p*NP β-D-glucuronide	Sub7	315.23	Sigma Aldrich
*p*NP α-D-maltoside	Sub8	463.4	Merck
*p*NP α-L arabinofuranoside	Sub9	271.22	Sigma Aldrich
*p*NP α-L-rhamnopyranoside	Sub10	285.25	Sigma Aldrich
*p*NP α-L-arabinopyranoside	Sub11	271.22	Sigma Aldrich
*p*NP acetate	Sub12	181.15	Sigma Aldrich
*p*NP xylopyranoside	Sub13	271.22	Sigma Aldrich
*p*NP β-D Cellobioside	Sub14	463.39	Sigma Aldrich
*p*NP α-L fucopyranoside	Sub15	285.25	Sigma Aldrich

2.2.11 Proteomic methods

2.2.11.1 In-gel digestion with trypsin

The SDS PAGE gel to be used for the in-gel trypsin digest was kept at 4˚C, submerged in MQH_2O until use. Bands to be digested were separately cut out using a clean scalpel and placed into clean 2 mL Eppendorf tubes. Gel pieces were treated with 300 µL of fresh 1x AmBic (Section 2.1.4.28)/ 50% acetonitrile at 45˚C in a heating block until they became colourless. They were then dehydrated using 300 µL of 80% acetonitrile for 1 min. Gel pieces were reduced using 100 µL of freshly prepared reducing solution (Section 2.1.4.29) for 1 hr at 42˚C in a heating block, and tubes were inverted every 15 min. The gel pieces were washed by adding 100 µL of 1x AmBic and left to incubate at RT for 5 min. Tubes were then centrifuged at 14,000 RPM for 1 min, and the SN discarded. The gel pieces were subsequently dehydrated by adding 100 µL of 80% acetonitrile and left to incubate for 1 min, before being centrifuged at 14,000 RPM for 1 min to remove the SN. Tubes were dried by spinning in the Speedvac on medium heating for 10 min. Next, 100 µL of alkylation solution

(Section 2.1.4.30) was added to each tube and gel pieces were incubated in the dark at RT for 20 min. Again, the gel pieces were washed by adding 100 µL of 1x AmBic and left to incubate at RT for 5 min. Tubes were then centrifuged at 14,000 RPM for 1 min, and the SN discarded. The gel pieces were subsequently dehydrated by adding 100 µL of 80% acetonitrile and left to incubate for 1 min, before being centrifuged at 14,000 RPM for 1 min to remove the SN. The gel pieces were then washed again, following the exact same process. The tubes were again dried in the Speedvac for 10 min on medium heating. The gel pieces were digested with 30 µL of trypsin solution (Section 2.1.4.31) and incubated on ice for 30 min. The excess trypsin solution was then removed and 30 µL of 1x AmBic was added. Tubes were then incubated at 37˚C overnight. The next day, tubes were centrifuged at 14,000 RPM for 1 min, and then sonicated for 2 min. The SN was collected into a new tube, and pooled with the SN from the following washes that were carried out twice: a) 60 µL of 5% formic acid in 20% acetonitrile was added to gel pieces, and then tubes were sonicated, and b) 60 µL of 0.1% formic acid/ 80% acetonitrile was added to gel pieces, and then tubes were sonicated for 2 min. The volume of each tube was reduced using the Speedvac on high heating. The top half of the SN (~20 µL) was subsequently transferred to sample vials and run on the mass spectrometer by a trained technician.

2.2.11.2 Liquid chromatography mass spectrometry (LC-MS analysis)

The digested samples were separated by on-line reversed-phase chromatography for each run using a nano liquid chromatography (LC) system with a reversed-phase peptide trap and a reversed-phase analytical column. The liquid chromatography system was coupled on-line with the Q Exactive Plus mass spectrometer equipped with a higher-energy collision-induced dissociation (HCD) collision cell, an Orbitrap mass analyser and a Nano Flex ion source (ThermoFisher Scientific, Waltham, MA USA). Details for the chromatographic and mass spectrometric settings are listed in Table 2.22 and 2.23.

Table 2.22. Instrument configuration of LC-MS

nanoLC system	Dionex Ultimate 3000 Binary RSLC nano LC system (ThermoFisher Scientific)
Mass spectrometer	QExactive Plus (ThermoFisher Scientific)
Ionization source	Nano Flex (ThermoFisher Scientific)

Trapping column	PepMap100 C18, 3µm particle size, 75 µm inner diameter, 2 cm length (ThermoFisher Scientific)
Analytical column	PepMap C18, 2 µm particle size, 75 µm inner diameter, 50 cm length (ThermoFisher Scientific)
Flow rates	Trap: 25 µL/min Analytical: 300 nL/min
Column oven temperature	50°C
Gradient	3-40% acetonitrile in 0.1% formic acid over 60 minutes

Table 2.23. Method parameter settings.

Capillary temperature	250°C
S-Lens RF level	50%
Source voltage [kV]	1.6
Maximum injection times	Full MS 150 ms, MS*n* 110 ms (HCD)
Full MS mass range	375 - 1600 [*m/z*]
AGC target	Full MS 3e6, MS*n* (HCD) 1e5
Resolution settings	Full MS 70,000, MS*n* (HCD) 17,500
Number of microscans	Full MS 1
Isolation width [*m/z*]	1.4
Loop count (TopN)	10
MSX count	1
Collision energies	25, 30, 35
Charge Exclusion	Unassigned, 1, 6-8, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic Exclusion [s]	20
Detector for MS*n* spectra	Orbitrap

Data-dependent tandem MS acquisition method was used. In all experiments, Full MS1 scans were acquired over a mass range of 375-1,600 *m/z* with detection in the Orbitrap mass analyser at a resolution setting of 70,000. Fragment ion spectra produced via HCD was acquired with a resolution setting of 17,500. For data-dependent acquisition of HCD spectra, the top ten most intense ions were selected for fragmentation in each scan cycle and Full MS as well as fragment ion spectra were detected in the Orbitrap mass analyser. Exclusion

conditions were optimized according to observed chromatographic peak width (typically 10-30 sec).

2.2.11.3 Database Search

The raw data files were searched using Proteome Discoverer™ v. 2.1 search engine (ThermoFisher Scientific, Waltham, MA USA). The search parameters applied in the database searches are listed in Table 2.24. The decoy database search was enabled by default, and all data were filtered to satisfy a false discovery rate (FDR) of 1% or better.

Table 2.24. Data analysis parameter

Search engine	Proteome Discoverer v 2.1 SP1
Database	TrEMBL 2016-10-05
Taxonomy	Bacteroides (Tax ID=816) or Ruminococcus albus (Tax ID=1264)
Enzyme	Trypsin
Max # of missed cleavages	2
Min peptide length	6
Precursor mass tolerance	10 ppm
Fragment mass tolerance	0.02 Da
# 13C	1
Peptide charge	2+ to 6+
Protein mass	Unrestricted
Decoy database search	Enabled
Significance threshold	0.01%
Instrument type	Q-Exactive-General
Static modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M), N-terminal acetylation
Experimental mass values	Monoisotopic
False Discovery Rate (FDR)	Fixed to 1%

2.2.12 Calculations

Due to the large amount of data to be processed, all calculations were carried out using Microsoft Excel in an attempt to reduce errors. One example has been provided for each type of calculation.

2.2.12.1 Protein concentration calculations using the Bradford assay

Firstly, a standard curve was generated using BSA standards (Table 2.25).

Table 2.25. BSA absorbances used to generate the standard curve for the Bradford assay.

Final BSA concentration (mg/mL)	Average absorbance @ 595 nm
0	0.008
0.05	0.161
0.1	0.433
0.15	0.559
0.2	0.661
0.25	0.783
0.3	1.02

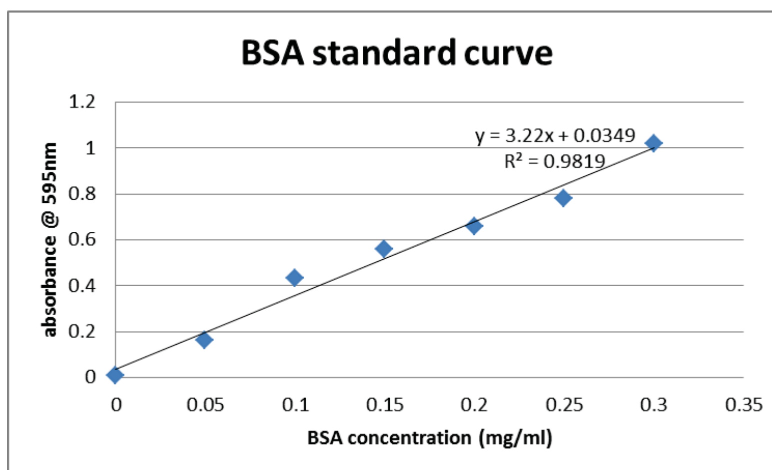

Fig 2.2. An example of the BSA standard curve used to calculate protein concentrations for the Bradford assay.

To work out the working concentration of SN samples, the trendline equation from the standard curve was used (Table 2.26). To determine the final protein concentration in mg/mL, the total amount of sample used in the reaction was taken into account.

Table 2.26. Example of calculations used to determine the protein concentration of SN samples using the Bradford assay.

Strain/substrate combination	Amount of sample used (µL)	Amount of dH_2O used (µL)	Average absorbance @ 595 nm	Working concentration	Protein concentration (mg/mL)
Bacteroides sp. Ga6A1 on FP	100	0	0.171	0.042267	0.042267

2.2.12.2 Protein concentration calculations using Biuret reagent

Protein concentrations for CPs were calculated in a similar manner to the Bradford assay. Firstly, a standard curve was generated using BSA standards (Table 2.27).

Table 2.27. BSA absorbances used to generate the standard curve for the Biuret assay.

Final BSA concentration (mg/mL)	Average absorbance @ 540 nm
0	0.000
0.5	0.035
1.0	0.047
1.5	0.087
2.0	0.123
2.5	0.135
3.0	0.168
4.0	0.216
5.0	0.247
6.0	0.329
8.0	0.402
10.0	0.503

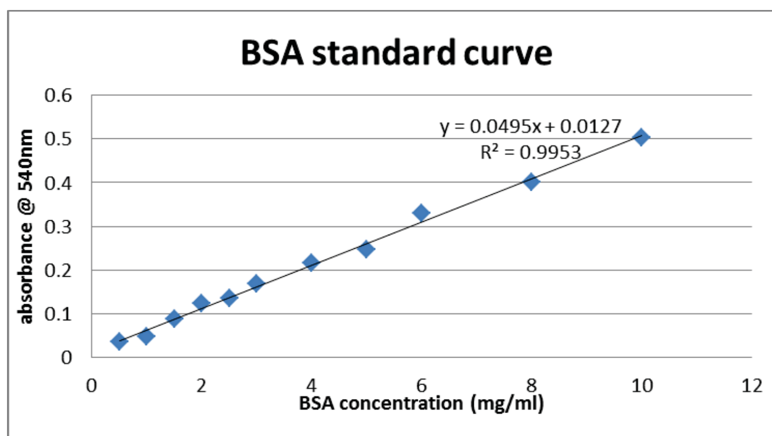

Fig 2.3. An example of the BSA standard curve used to calculate protein concentrations using the Biuret assay.

To work out the working concentration of CPs, the trendline equation from the standard curve was used (Table 2.28). To determine the final protein concentration in mg/mL, the total amount of sample used in the reaction was taken into account.

Table 2.28. Example of calculations used to determine the protein concentration of CP samples using the Biuret assay.

Strain/substrate combination	Amount of sample used (µL)	Amount of dH_2O used (µL)	Average absorbance @ 595 nm	Working concentration	Protein concentration (mg/mL)
Bacteroides sp. Ga6A1 on FP	200	50	0.104	1.844444	2.3055556

2.2.12.3 Calculations to determine the amount of reducing sugars released using DNS

To calculate cellulolytic (or xylanolytic) activity, shown here, a standard curve was generated using the glucose (or xylose) standards that were included in the incubation and DNS assay (Table 2.29). As the buffer blank (0 mg/mL of glucose or xylose) average was subtracted from all values, it was not plotted.

Table 2.29. Glucose absorbances used to generate the standard curve for the DNS assay in order to determine cellulolytic activity.

Glucose concentration (mg/mL)	Average absorbance @ 540 nm
0.2	0.060225
0.2	0.054425
0.2	0.070225
0.2	0.054025
0.25	0.088925
0.25	0.083525
0.25	0.088525
0.25	0.073325
0.5	0.240425
0.5	0.244225
0.5	0.194925
0.5	0.220625
1.0	0.537025
1.0	0.552625
1.0	0.552125
1.0	0.566625

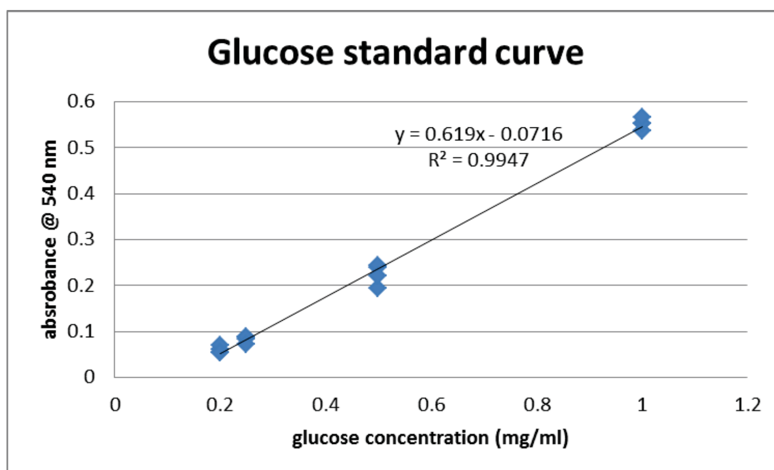

Fig 2.4. An example of the glucose standard curve used to calculate the amount of glucose equivalents released in order to determine cellulolytic activity.

Next, the trendline from the standard curve was used to work out the mean concentration of glucose (or xylose) equivalents from each strain/substrate combination (Table 2.30). Standard errors were calculated using the individual OD readings from each of the four replicates using the Microsoft Excel standard error formula.

Table 2.30. Example of calculations used to determine the amount of sugar released using absorbances from the DNS assay.

Strain/substrate combination	Average absorbance	Mean conc glucose equivalents (g/L)	Standard Error	Standard error Percentage
Bacteroides sp. Ga6A1 on FP	0.305175	0.608683	0.018429	6.0%

The concentration of glucose (or xylose) equivalents released from the entire volume of SN sample was normalised (Table 2.31), which involved multiplying the amount of glucose equivalents calculated in the 100 µL of SN by the total volume of SN left after concentrating.

Table 2.31. Example of calculations used to determine the amount of sugar released using absorbances from the DNS assay.

Strain/substrate combination	Mean conc glucose equivalents (g/L)	Total volume of SN after concentrating (mL)	Normalised conc glucose equivalents (g/L)
Bacteroides sp. Ga6A1 on FP	0.608683	9.0	5.478150242

2.2.12.4 Specific activities calculations

The specific activity was calculated by dividing the mean concentration of glucose (or xylose) equivalents (Section 2.2.12.3) by the MW of glucose (or xylose), and then multiplying this value by 10^6 in order to convert the units into µmol/mL. This value was then divided by the time of incubation (180 min) and then divided again by the protein concentration which gave values with specific activity units of µmol min^{-1} mg^{-1}.

The p value specified for each specific activity was calculated using the "T.TEST" formula in Microsoft Excel, with individual absorbance values for each replicate used for the 1 tailed, type 2 calculation.

2.2.12.5 Limit of detection (LOD) calculations

The LOD was calculated using the formula below in Microsoft Excel, where x = the mean concentration of glucose (or xylose) equivalents for each strain/substrate, and y= the standard deviation of the mean concentration of glucose (or xylose) equivalents for the water blank multiplied by the mean concentration of glucose (or xylose) equivalents for the water blank + 3.

=IF(x>y,"OK","below LOD")

2.2.12.6 Limit of quantification calculations

The LOQ was calculated using the formula below in Microsoft Excel, where x = the mean concentration of glucose (or xylose) equivalents for each strain/substrate, and y= the standard deviation of the mean concentration of glucose (or xylose) equivalents for the water blank multiplied by the mean concentration of glucose (or xylose) equivalents for the water blank + 10.

=IF(x>y,"OK","below LOQ")

2.2.12.7 Kill rate calculations for EMS mutagenesis

The kill rates for each strain/substrate combination subjected to mutagenesis with EMS were calculated as in Table 2.32. CFUs/mL were estimated by multiplying the number of colonies observed by the dilution factor, and this value was then divided by the amount of culture plated (0.01 mL). Kill rates were then determined by dividing the CFUs/mL for strain/substrate combinations after 31 subcultures by CFUs/mL after 1 subculture and multiplying by 100. For each strain and/or lineage, the standard error was calculated using the individual OD readings for each of the four replicates, as opposed to using the average OD reading calculated from the four replicates.

Table 2.32. Example of calculations used to determine the kill rates for strain/substrate combinations subjected to mutagenesis with EMS.

Strain/substrate combination	Dilution factor	Average CFUs	Average CFUs/mL	Kill rate
Bacteroides sp. Ga6A1 on RG, 1st subculture	10^5	39	3.90E+08	-
Bacteroides sp. Ga6A1 on RG, 31st subculture	10^5	19.3	1.93E+08	49.57%

2.2.12.8 Kill rates and standard error calculations for UV radiation mutagenesis

Both the kill rates for each strain/substrate combination subjected to mutagenesis using UV radiation and the standard errors were calculated in the exact same manner as in Section 2.2.12.7.

Chapter Three: *In vitro* assessment of fibrolytic activities of fibre-degrading rumen bacteria

3.1 Introduction

To investigate rumen bacterial strains that had the highest fibre-degrading activities *in vitro*, 12 strains that were isolated from NZ ruminants were selected based on their availability, *in silico* analyses of their genome sequences that indicated the presence of a considerable abundance of genes for fibre degradation, and the ease with which they could be cultured in the laboratory. The ability of the strains to express FEs in culture was unknown, thus a wide range of species were screened in parallel, alongside control rumen bacterial strains that had known fibrolytic activity: *Butyrivibrio proteoclasticus* B316 (xylanolytic) (19), *Ruminococcus flavefaciens* FD1 (cellulolytic) (61), and *Prevotella ruminicola* 23 (xylanolytic) (62). Table 3.1 lists the bacterial strains used for this study and their main predicted and known fibrolytic function.

Table 3.1. Rumen bacterial strains and their predicted and known primary fibrolytic function.

Species	Strain	Predicted primary fibrolytic function
Ruminococcus flavefaciens	AE3010	Cellulolytic
Ruminococcus albus	AD2013	Cellulolytic
Ruminococcus flavefaciens [a]	FD1	Cellulolytic
Lachnospira multipara	LB2003	Pectinolytic
Lachnospiraceae genus 6	NK4A136	Unknown
Lachnospiraceae genus 7	NK4A144	Unknown
Clostridiales bacterium	NK3B98	Unknown
Oribacterium sp.	NK2B42	Unknown
Butyrivibrio proteoclasticus [a]	B316	Xylanolytic
Prevotella ruminicola [a]	23	Xylanolytic
Butyrivibrio fibrisolvens	MD2001	Xylanolytic/pectinolytic
Butyrivibrio sp. 2	FC2001	Xylanolytic/pectinolytic
Prevotella brevis	P6B11	Xylanolytic/pectinolytic

Bacteroides sp.	Ga6A1	Xylanolytic/pectinolytic
Pseudobutyrivibrio sp.	MA3014	Xylanolytic/pectinolytic

[a] Control strains with known fibrolytic activity

In order to assess fibrolytic activity, the rumen bacterial strains were grown on five different fibrous substrates as the sole carbohydrate source to stimulate the production of FEs. Secreted (SN) and cell-associated (CP) proteins were harvested and concentrated, and the activity of these enzymes was measured using two separate biochemical assays.

3.2 Results and discussion

3.2.1 Growth and degradation of fibrous substrates by rumen bacterial strains

To induce FE production from a selection of 15 rumen bacterial strains predicted to express fibrolytic activities (Table 3.1), strains were resuscitated from frozen stocks (Section 2.2.3.2) and then grown on five fibrous substrates: RG, NDF, OSX, AP and cellulose in the form of FP, in RM02 growth medium (Section 2.1.5.3), with GenRFV (Section 2.1.6.3) at 37˚C for up to three days.

Several bacterial strains were able to successfully grow on, and in some cases visibly degrade, specific substrates, with examples of growth on each substrate shown in Fig 3.1. Despite this, it was difficult to quantify bacterial growth through OD readings because of the presence of the degraded fibrous substrate in the culture medium. The majority of strains grew on RG, OSX and AP, while just below half of the strains grew in media with FP (Table 3.2). NDF did not appear to support growth for any of the strains tested (Table 3.2). In total, 46 strain/substrate combinations displayed growth, from which protein preparations were made to quantify fibrolytic activity (Table. 3.2).

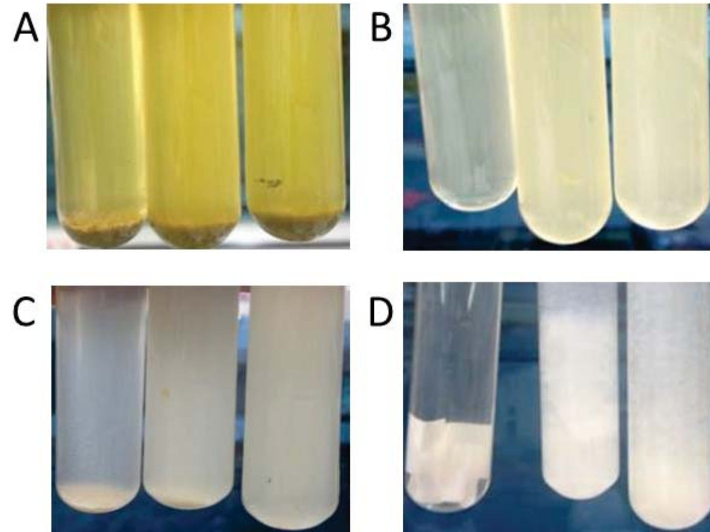

Fig 3.1. Examples of rumen bacterial growth on fibrous substrates. In each image, the left tube is the negative control containing substrate only. The remaining two tubes were inoculated with culture and incubated for three days. (A) *Ruminococcus albus* AD2013 grown on RG. (B) *Lachnospira multipara* LB2003 grown on AP (C) *Ruminococcus flavefaciens* FD1 grown on OSX. (D) *Ruminococcus albus* AD2013 grown on FP.

Table 3.2. Growth[a] of 15 rumen bacterial strains on fibrous substrates.

Species	Strain	Primary predicted fibrolytic function	RG	OSX	AP	FP	NDF
Bacteroides sp.	Ga6A1	Xylanolytic/pectinolytic	+	+	+	+	-
Butyrivibrio fibrisolvens	MD2001	Xylanolytic/pectinolytic	+	+	+	+	-
Butyrivibrio proteoclasticus	B316	Xylanolytic	+	+	+	-	-
Butyrivibrio sp. 2	FC2001	Xylanolytic/pectinolytic	+	+	+	+	-
Clostridiales bacterium	NK3B98	Unknown	+	+	+	-	-
Lachnospira multipara	LB2003	Pectinolytic	+	-	+	-	-
Lachnospiraceae genus 6	NK4A136	Unknown	+	+	+	-	-
Lachnospiraceae genus 7	NK4A144	Unknown	+	+	+	-	-
Oribacterium sp.	NK2B42	Unknown	+	+	-	-	-
Prevotella brevis	P6B11	Xylanolytic/pectinolytic	+	+	+	-	-
Prevotella ruminicola	23	Xylanolytic	+	+	+	-	-
Pseudobutyrivibrio sp.	MA3014	Xylanolytic/pectinolytic	+	-	-	+	-
Ruminococcus albus	AD2013	Cellulolytic	+	+	+	+	-
Ruminococcus flavefaciens	FD1	Cellulolytic	-	+	+	+	-
Ruminococcus flavefaciens	AE3010	Cellulolytic	+	-	+	+	-

[a] Strains that grew on and/or visibly degraded a fibrous substrate are indicated with "+", and those that did not are indicated with "-".

The substrates that supported the growth of each bacterial strain could be generally correlated with their main predicted functions. For example, the ruminococci were predicted to possess cellulolytic activities, and they were able to grow on the culture medium containing FP (Table 3.2). Likewise, the putative xylanolytic bacteria, *Bacteroides* sp. Ga6A1, *Prevotella brevis* P6B11 and the *Butyrivibrio* strains were able to grow on medium containing OSX, and the putative pectinolytic bacterium *Lachnospira multipara* LB2003 was able to grow on medium containing AP (Table 3.2). In addition, the bacterial strains predicted to have both xylanolytic and pectinolytic activities grew on both OSX and AP. The only exception was *Pseudobutyrivibrio* sp. MA3014, which was predicted to possess both xylanolytic and pectinolytic activity, but did not appear to grow on AP.

Conversely, a number of strains appeared to grow on additional fibrous substrates that differed from their main predicted fibrolytic function. For instance, the ruminococci were predicted to be cellulolytic, but they were observed to grow on FP, as well as OSX and AP. Likewise, four of the putative xylanolytic/pectinolytic strains, *Bacteroides* sp. Ga6A1, *Pseudobutyrivibrio* sp. MA3014 and two *Butyrivibrio* strains, grew on FP as well OSX and AP. Although such observations could have been due to the incorrect annotation of putative fibre-degrading enzyme encoding genes, or technical issues associated with growing the organism on the fibrous substrate, this was unlikely as it has already been established that a single fibrolytic bacterial strain can encode a variety of fibrolytic activities (35). This seems highly plausible when considering the complex nature of ruminant feed and the eco-system within the rumen. These observations demonstrate the complexity associated with the expression and regulation of FEs, and highlights limitations associated with relying solely on predicted functional activities from *in silico* analyses of genome sequences.

Lastly, of the bacterial strains tested, none appeared to grow on NDF. NDF was prepared by stripping the RG of components such as plant pectins, proteins, sugars and lipids so that only the fibrous structural components (cellulose, hemicellulose and lignin) of the plant cell wall remained. The fact that NDF did not support growth suggested that these bacterial strains required additional important factors for growth that were no longer present after processing and were not provided for in the base medium used, or that substances which may be inhibitory to bacterial growth were co-extracted during the preparation.

3.2.2 FE preparations

Strains that were able to grown on fibrous substrates were immediately separated into CP and SN fractions by centrifugation. The SN was then concentrated using ultrafiltration (Vivaspin 20, 10,000 MW cut-off) while the CPs were stored at -80˚C. SN fractions were concentrated to varying amounts relative to the fibrous substrate strains were grown on. This was because particular substrates, namely RG and OSX, clogged the filter of the Vivaspin columns which made it difficult to concentrate to volumes smaller than 9 mL. Conversely, strains grown on FP and AP were much easier to concentrate to volumes as low as 1 mL. Therefore, there were varying amounts of SN volumes, ranging from 1 mL to 15 mL. After concentrating, sterile glycerol was added into the SN fractions to a final concentration of 20% (v/v) before they were stored at -80°C. CP and SN fractions were thawed at RT immediately before use.

3.2.2.1 Measuring the protein concentration of rumen bacterial culture SNs

The protein concentration for the concentrated culture SN of each of the 46 strain/substrate combinations to be assayed was measured using the Bradford assay (Section 2.2.8.3; Table 3.3) as this reagent is widely used to measure protein concentrations. Unfortunately, standard errors were not calculated as the individual ODs could not be found. This was because the ODs were recorded on a separate piece of paper, and although the average of the ODs was transferred to an Excel spreadsheet, the individual values were not. As the original piece of paper with all of the data could not be located and was likely discarded, it was impossible to calculate standard errors. This made it difficult to comment on the degree of variability for each concentration. See Section 2.2.12.1 for calculations.

Table 3.3. Protein concentrations of the culture SN preparations using the Bradford assay.

Species	Strain	Substrate	Protein concentration (mg/mL)[a]
Bacteroides sp.	Ga6A1	FP	0.042
Bacteroides sp.	Ga6A1	OSX	0.198
Bacteroides sp.	Ga6A1	AP	0.048
Bacteroides sp.	Ga6A1	RG	0.096
Butyrivibrio fibrisolvens	MD2001	FP	0.026
Butyrivibrio fibrisolvens	MD2001	OSX	0.112

Butyrivibrio fibrisolvens	MD2001	AP	0.033
Butyrivibrio fibrisolvens	MD2001	RG	0.139
Butyrivibrio proteoclasticus	B316	OSX	0.06
Butyrivibrio proteoclasticus	B316	AP	0.13
Butyrivibrio proteoclasticus	B316	RG	0.119
Butyrivibrio sp. 2	FC2001	FP	0.027
Butyrivibrio sp. 2	FC2001	OSX	0.072
Butyrivibrio sp. 2	FC2001	AP	0.063
Butyrivibrio sp. 2	FC2001	RG	0.14
Clostridiales bacterium	NK3B98	OSX	0.048
Clostridiales bacterium	NK3B98	AP	0.075
Clostridiales bacterium	NK3B98	RG	0.183
Lachnospira multipara	LB2003	AP	0.07
Lachnospira multipara	LB2003	RG	0.116
Lachnospiraceae genus 6	NK4A136	OSX	0.036
Lachnospiraceae genus 6	NK4A136	AP	0.044
Lachnospiraceae genus 6	NK4A136	RG	0.12
Lachnospiraceae genus 7	NK4A144	OSX	0.094
Lachnospiraceae genus 7	NK4A144	AP	0.042
Lachnospiraceae genus 7	NK4A144	RG	0.105
Oribacterium sp.	NK2B42	OSX	0.057
Oribacterium sp.	NK2B42	RG	0.108
Prevotella brevis	P6B11	OSX	0.058
Prevotella brevis	P6B11	AP	0.1
Prevotella brevis	P6B11	RG	0.237
Prevotella ruminicola	23	FP	0.004
Prevotella ruminicola	23	OSX	0.029
Prevotella ruminicola	23	AP	0.031
Pseudobutyrivibrio sp.	MA3014	FP	0.013
Pseudobutyrivibrio sp.	MA3014	RG	0.122
Ruminococcus albus	AD2013	FP	0.066
Ruminococcus albus	AD2013	OSX	0.117
Ruminococcus albus	AD2013	AP	0.035
Ruminococcus albus	AD2013	RG	0.222
Ruminococcus flavefaciens	AE3010	FP	0.038
Ruminococcus flavefaciens	AE3010	AP	0.051

Ruminococcus flavefaciens	AE3010	RG	0.134
Ruminococcus flavefaciens	FD1	FP	0.039
Ruminococcus flavefaciens	FD1	OSX	0.176
Ruminococcus flavefaciens	FD1	AP	0.031

[a] Protein concentrations calculated using four technical replicates (see Section 2.2.13 for calculations).

3.2.2.2 Bacterial CP protein concentrations

The protein concentration for each of the CPs from the 46 strain/substrate combinations was measured using the Biuret assay (Section 2.2.8.4; Table 3.4) as it was thought that the NaOH within the Biuret reagent would help break down the CPs and give a more accurate reading of protein concentration than the Bradford assay. Only 43 strain/substrate CPs were measured as the pellets of Lachnospiraceae genus 6 NK4A136 grown on AP, Lachnospiraceae genus 7 NK4A144 grown on OSX and *Prevotella brevis* P6B11 grown on RG were misplaced and could not be located. See Section 2.2.12.2 for calculations. As with the SN protein concentration, standard errors were not calculated and the individual ODs could not be found, so again this made it difficult to comment on the degree of variability for each concentration.

Table 3.4. Protein concentrations of bacterial CP preparations measured using the Biuret assay.

Species	Strain	Substrate	Protein concentration (mg/mL)[a]
Bacteroides sp.	Ga6A1	FP	2.31
Bacteroides sp.	Ga6A1	AP	9.86
Bacteroides sp.	Ga6A1	RG	9.83
Bacteroides sp.	Ga6A1	OSX	62.81 [b]
Butyrivibrio fibrisolvens	MD2001	FP	2.63
Butyrivibrio fibrisolvens	MD2001	AP	21.07 [b]
Butyrivibrio fibrisolvens	MD2001	RG	6.32
Butyrivibrio fibrisolvens	MD2001	OSX	1.88
Butyrivibrio proteoclasticus	B316	AP	11.27
Butyrivibrio proteoclasticus	B316	RG	7.05
Butyrivibrio proteoclasticus	B316	OSX	25.69 [b]
Butyrivibrio sp. 2	FC2001	FP	2.38
Butyrivibrio sp. 2	FC2001	AP	4.73
Butyrivibrio sp. 2	FC2001	RG	6.04

Butyrivibrio sp. 2	FC2001	OSX	1.88
Clostridiales bacterium	NK3B98	AP	12.31
Clostridiales bacterium	NK3B98	RG	8.42
Clostridiales bacterium	NK3B98	OSX	4.73
Lachnospira multipara	LB2003	AP	20.56 [b]
Lachnospira multipara	LB2003	RG	6.70
Lachnospiraceae genus 6	NK4A136	RG	3.04
Lachnospiraceae genus 6	NK4A136	OSX	6.78
Lachnospiraceae genus 7	NK4A144	AP	11.04
Lachnospiraceae genus 7	NK4A144	RG	2.41
Oribacterium sp.	NK2B42	RG	2.79
Oribacterium sp.	NK2B42	OSX	2.00
Prevotella brevis	P6B11	AP	6.12
Prevotella brevis	P6B11	OSX	14.41
Prevotella ruminicola	23	FP	1.04
Prevotella ruminicola	23	AP	3.72
Prevotella ruminicola	23	OSX	4.63
Pseudobutyrivibrio sp.	MA3014	FP	2.28
Pseudobutyrivibrio sp.	MA3014	RG	6.78
Ruminococcus albus	AD2013	FP	5.54
Ruminococcus albus	AD2013	AP	8.80
Ruminococcus albus	AD2013	RG	5.29
Ruminococcus albus	AD2013	OSX	1.88
Ruminococcus flavefaciens	AE3010	FP	1.42
Ruminococcus flavefaciens	AE3010	AP	9.86
Ruminococcus flavefaciens	AE3010	RG	7.61
Ruminococcus flavefaciens	FD1	FP	1.70
Ruminococcus flavefaciens	FD1	AP	4.81
Ruminococcus flavefaciens	FD1	OSX	24.6 [b]

[a] Protein concentrations calculated using four technical replicates.

[b] Unexpectedly high absorbance readings were likely due to precipitate present in the samples.

A few CPs had a large amount of precipitate or growth substrate present which interfered with the Biuret assay and gave inaccurately high OD readings. Attempts were made to remove the precipitate, including decanting, centrifugation and filtering, but each method proved unsuccessful. As it was difficult to remove the growth substrate from the samples

when re-assayed, the affected samples could not be accurately quantified, and this was taken into consideration when the subsequent assay results were interpreted.

Overall, the protein concentrations for the CPs were approximately an order of magnitude higher than those for the SNs, although the use of two different assays made accurate comparison difficult. In order to make direct comparisons between the separate fractions, all samples would need to be re-tested using one assay, however, as the CP fraction was of less interest and would not be used for further analysis, the results were left as is. Therefore, it is likely that the observed differences between the CP and SN samples could be due to the use of the two separate assays. Other explanations to consider include the presence of the growth substrate which was difficult to remove from the CP fraction, or that the CPs have a higher protein content than the secreted enzymes within the SN simply due to the fact that the bacterial strains were fibre adherent.

3.2.3 Determining the fibrolytic activity of the rumen bacterial strains

The CP and SN preparations from each of the strain/substrate combinations were incubated separately with FP (Section 2.2.9.1) and OSX (Section 2.2.9.2). The reducing sugars released were measured using the DNS assay (Section 2.2.9.3) to give an indication of cellulolytic and xylanolytic activity. Strain/substrate combinations were ranked based on the highest mean concentration of glucose/xylose equivalents (g/L) from the SN fractions. The normalised concentration of glucose/xylose equivalents (g/L) was also estimated for SN fractions only, and this took into account the varying volumes of concentrated SN for each sample. The normalised concentration of glucose/xylose equivalents (g/L) was not calculated for CP fractions as the final CP volumes were the same for each sample (1 mL). Additionally, the specific activity (μmol $min^{-1}mg^{-1}$) for both SN and CP fractions was calculated (Table 3.5 and Table 3.6). Due to the large number of strain/substrate combinations tested and difficulties in accurately quantifying protein concentrations in the preparations, these initial biochemical screens were used to give a general estimate of the more active fibre degraders.

3.2.3.1 Cellulolytic activities of bacterial SN and CP preparations

The results from the incubation of the CPs and SNs from each of the strain/substrate combinations on FP are shown in Table 3.5 (see Section 2.2.13.3 and Section 2.2.13.4 for

calculations). The SN fractions resulted in higher activity compared to the CP fractions, thus strain/substrate combinations were ranked based on the highest mean concentration of glucose equivalents (g/L) released from the SNs only. In addition, it was difficult to know what proportion of the reducing sugars released from the CP fraction were due to the activity of FEs within the sample versus carry over from the culture or CP itself as these were not filtered. By assaying the CP and SN fractions separately, however, any enzymatic synergies that may occur between each fraction was excluded.

Table 3.5. Relative cellulolytic activity of SN and CP fractions for all strain/substrate combinations ranked in order of highest mean concentration of glucose equivalents (g/L) from SN fractions.

			SN				CP		
Species	Strain	Growth substrate	Mean conc glucose equivalents (g/L) [a]	Standard error [b]	Specific activity (µmol min^{-1} mg^{-1}) [c]	Normalised conc glucose equivalents (g/L) [d]	Mean conc glucose equivalents (g/L) [a]	Standard error [b]	Specific activity (µmol min^{-1} mg^{-1}) [c]
Bacteroides sp.	Ga6A1	AP	0.61	6%	395	5.48	0.08	10%	0.26
Bacteroides sp.	Ga6A1	OSX	0.55	60%	85.2	3.01	0.1	31%	1.15
Ruminococcus flavefaciens	FD1	AP	0.37	13%	372	3.34	0.06	8%	0.39
Ruminococcus flavefaciens	FD1	OSX	0.26	15%	45	1.93	0.24	25%	2.67
Lachnospiraceae genus 6	NK4A136	AP	0.21	4%	150	1.59	0.05	12%	0.5
Ruminococcus albus	AD2013	RG	0.2	18%	27.8	2.61	0.07	17%	0.4
Lachnospira multipara	LB2003	AP	0.2	5%	87.6	1.48	0.05	6%	0.89
Clostridiales bacterium	NK3B98	RG	0.18	16%	33.7	1.75	0.04	23%	0.16
Clostridiales bacterium	NK3B98	AP	0.17	8%	70.8	1.63	0.06	8%	0.14
Lachnospiraceae genus 7	NK4A144	RG	0.17	5%	49.3	1.26	0.05	19%	0.65
Butyrivibrio proteoclasticus	B316	RG	0.16	19%	41.3	1.91	0.07	20%	0.3
Oribacterium sp.	NK2B42	RG	0.16	22%	44.9	1.1	0.05	11%	0.82
Ruminococcus albus	AD2013	OSX	0.16	12%	41.2	1.1	0.37	24%	6.05
Butyrivibrio proteoclasticus	B316	AP	0.12	19%	28.8	0.61	0.07	2%	0.2
Bacteroides sp.	Ga6A1	RG	0.11	17%	33.8	1.26	0.07	26%	0.22

Lachnospira multipara	LB2003	RG	0.09	13%	24.6	0.74	0.07	4%	0.31
Ruminococcus flavefaciens	AE3010	AP	0.08	3%	51.2	0.59	0.08	5%	0.25
Ruminococcus flavefaciens	AE3010	RG	0.05	9%	12.2	0.5	0.07	18%	0.27
Lachnospiraceae genus 6	NK4A136	RG	0.04	1%	11.4	0.33	0.07	9%	0.31
Lachnospiraceae genus 6	NK4A144	AP	0.03	14%	25.2	0.24	n/a	n/a	n/a
Prevotella brevis	P6B11	AP	0.03	18%	9.2	0.64	0.1	30%	0.53
Butyrivibrio sp. 2	FC2001	AP	0.02	16%	12	0.26	0.06	12%	0.41
Lachnospiraceae genus 6	NK4A136	OSX	0.02	9%	23.7	0.34	0.01	34%	0.27
Lachnospiraceae genus 7	NK4A144	AP	0.01	21%	10	0.1	0.03	11%	0.08
Butyrivibrio fibrisolvens	MD2001	RG	0.01	140%	3.2	0.16	0.02	82%	0.08
Ruminococcus flavefaciens	FD1	FP	0.01	9%	9.9	0.03	0.05	99%	0.85
Prevotella brevis	P6B11	OSX	0.01	25%	8	0.23	0.04	39%	2.45
Prevotella ruminicola	23	AP	0.01	109%	10.6	0.06	0.02	6%	0.16
Butyrivibrio proteoclasticus	B316	OSX	0.01	21%	5.5	0.08	0.24	22%	3.93
Ruminococcus flavefaciens	FD1	RG	0.01	19%	2.7	0.07	n/a	n/a	n/a
Pseudobutyrivibrio sp.	MA3014	RG	0.01	177%	2.7	0.12	0.02	9%	0.09
Oribacterium sp.	NK2B42	OSX	0.01	67%	6.9	0.19	0.01	40%	0.11
Ruminococcus albus	AD2013	FP	0.01	21%	4.4	0.01	0.04	581%	0.23
Clostridiales bacterium	NK3B98	OSX	0.01	19%	7.7	0.08	0.05	21%	0.34
Pseudobutyrivibrio sp.	MA3014	FP	0.01	31%	-140.1	0.04	0.04	242%	0.57
Butyrivibrio fibrisolvens	MD2001	FP	0.01	20%	18.4	0.05	0.05	28%	0.64
Butyrivibrio sp. 2	FC2001	OSX	0.01	3%	3.7	0.05	0.07	22%	1.22
Butyrivibrio fibrisolvens	MD2001	AP	0.01	50%	11.4	0.05	0.06	42%	0.6

Prevotella ruminicola	23	OSX	0.01	88%	7.3	0.03	0.04	862%	0.28
Lachnospiraceae genus 7	NK4A144	OSX	0.01	53%	2.5	0.1	n/a	n/a	n/a
Bacteroides sp.	Ga6A1	FP	0.01	40%	4.6	0.04	0.05	142%	0.65
Butyrivibrio sp. 2	FC2001	FP	0.01	12%	14.7	0.04	0.05	83%	0.64
Ruminococcus albus	AD2013	AP	0.01	46%	5.5	0.04	0.05	4%	0.16
Prevotella ruminicola	23	FP	0	16%	34.8	0.02	0.05	171%	1.42
Prevotella brevis	P6B11	RG	-0.01	-18%	-0.8	-0.06	n/a	n/a	n/a
Butyrivibrio fibrisolvens	MD2001	OSX	-0.01	-51%	-3.3	-0.09	0.02	32%	0.4
Butyrivibrio sp. 2	FC2001	RG	-0.01	-6%	-3	-0.11	0.02	22%	0.11
Ruminococcus flavefaciens	AE3010	FP	-0.01	-2%	-10.1	-0.07	0.02	37%	0.47

[a] Mean concentration of glucose equivalents released during the DNS assay

[b] Standard error calculated using four replicates

c Concentration of glucose equivalents released per minute per mg of protein

[d] Mean glucose concentration estimated in total concentrated SN volume

n/a denotes lost pellets

It was interesting to note that the top ranked strain/substrate combination for cellulolytic activity in this assay (*Bacteroides* sp. Ga6A1) was grown on AP, and amongst the other top ranking strain/substrate combinations tested for cellulolytic activity, none included growth on FP. The highest ranked strain/substrate combination that was grown on FP was *R. flavefaciens* FD1, which was ranked 26th. It was assumed that the cellulose present in the medium containing FP would induce the expression of FEs that specifically degraded the cellulose, and that these strain/substrate combinations would be most active when incubated on cellulose, however, this did not appear to be the case.

There were a few scenarios that could have explained this observation. Firstly, it was possible that growth on AP, or a broader range of fibrous substrates, could have induced the global expression of FEs which were not substrate specific. One study was able to show that *Clostridium thermocellum* B8 produced carboxymethylcellulases, xylanases, mannases and pectinases during growth in the presence of crystalline cellulose (63). This would suggest that cellulase, hemicellulase and pectinase-encoding gene expression is co-regulated using common control mechanisms (64). Another explanation could be that the effective degradation of cellulose required FEs that were present in the CP fraction and/or required the synergy of activities found in both the CP and SN fractions. It was also possible that the FEs were attached to the growth substrate in the CP fraction, and thus, they were not freely transferred to the new assay substrate.

The top ranked strain/substrate combination for cellulolytic activity in this assay (*Bacteroides* sp. Ga6A1) was not predicted to be largely cellulolytic, yet it appeared to rank higher than *R. flavefaciens* FD1, which was included as a model cellulolytic strain for reference (61). Of the other top ranked strain/substrate combinations, a small proportion were predicted to have cellulolytic activity (*R. flavefaciens* FD1 and *R. albus* AD2013), while the rest were predicted to have xylanolytic/pectinolytic activity (*Bacteroides* sp. Ga6A1), pectinolytic activity (*L. multipara* LB2003), or unknown activity (Lachnospiraceae genus 6 NK4A136, Lachnospiraceae genus 7 NK4A144, and Clostridiales bacterium NK3B98). This was surprising, and could emphasize how unreliable it can be assigning functions to microorganisms based on genetic sequence alone.

When comparing the specific activity or mean concentration of glucose equivalents from the CPs and SN samples incubated on FP (Table 3.5), it was clear that the majority of the activity

came from exogenous FEs that were present in the SN. This was interesting as the rumen bacteria used in this study were plant-associated and it was thought that cell-associated FEs may be of importance for fibre degradation, or at least play a bigger role than they appeared to in this assay. It was also curious to note that the protein concentrations for the CPs appeared to be a magnitude higher than the protein concentrations for the SNs, and this could have suggested that the amount of protein present in the CP was grossly overestimated or that there was very little fibrolytic activity associated with CP fractions.

The absence of fibrolytic activity from the CPs could have been due to the lack of active sites of the FP, as the SNs incubated on FP had low values from the DNS assay too, or it could have been due to the way the CPs were processed which prevented the release of cell-associated FEs. Originally, CPs were to be sonicated to break up the rumen bacterial cells which would have released endogenous enzymes, but it was thought to be unlikely that the fibre-degrading enzymes of interest would have remained within the cell. Therefore, the CPs were merely resuspended in buffer and assayed in order to test for the activity of any cell membrane-associated FEs, but lysing the cells using sonication, for instance, may have resulted in better activity.

The results from this assay using FP were consistent with the extensive literature which describes ruminococci as dominant cellulolytic bacterial strains within the rumen (17, 18, 35, 65, 66). However, the high ranking of *Bacteroides* sp. Ga6A1 and the Lachnospiraceae strains was surprising as there is currently only a small amount of literature concerning the cellulolytic activity of the genera *Bacteroides* and Lachnospiraceae, which are considered average fibre degraders at best (67, 68). Therefore, the fact that *Bacteroides* sp. Ga6A1 appeared to perform better than the benchmark strain (*R. flavefaciens* FD1), as well as the other prominently cellulolytic ruminococci strains, was exciting, and revealed the potential for efficient fibre degradation of these strains from lesser characterised groups.

Based on the available literature, and despite the observations mentioned above, the top ranked strain/substrate combination (*Bacteroides* sp Ga6A1 grown on AP) appeared to be the best, and most interesting, candidate strain to be considered for improving fibrolytic activity. This was because it had the highest overall performance when assay results were measured directly and normalised for SN volume or protein concentration (Table 3.5).

3.2.3.2 Overall xylanolytic activities of SN and CPs ranked in order of highest activity

The results from the incubation of the CPs and SNs on OSX (Section 2.2.9.2 and Section 2.2.9.3) were calculated in the same way that samples incubated on FP (Table 3.5) were calculated (Table 3.6**)**. Similarly, this excluded any synergies that may occur between the CP and SN fractions. As with the results from the assay using FP (Table 3.5), the results from this assay were ranked in order of highest mean concentration of xylose equivalents (g/L) from SN samples. This allowed for the direct comparison of results from the assay with FP (Table 3.5). See Section 2.2.12.3 and Section 2.2.12.4 for calculations.

Table 3.6. Relative xylanolytic activity of SN and CP fractions for all strain/substrate combinations ranked in order of highest mean concentration of xylose equivalents (g/L) from SN fractions.

			SN				CP		
Species	Strain	Growth substrate	Mean conc xylose equivalents (g/L) [a]	Standard error [b]	Specific activity (μmol min^{-1} mg^{-1}) [c]	Normalised conc xylose equivalents (g/L) [d]	Mean conc xylose equivalents (g/L) [a]	Standard error [b]	Specific activity (μmol min^{-1} mg^{-1}) [c]
Ruminococcus flavefaciens	FD1	OSX	4.77	3%	1000	35.76	1.19	3%	15.79
Ruminococcus albus	AD2013	RG	4.53	4%	754	58.87	1.12	4%	7.85
Bacteroides sp.	Ga6A1	OSX	4.39	4%	820	24.15	1.07	3%	14.24
Ruminococcus albus	AD2013	OSX	3.94	4%	1242	27.6	1.41	1%	27.85
Ruminococcus flavefaciens	AE3010	RG	3.86	4%	1068	36.7	0.82	3%	3.99
Bacteroides sp.	Ga6A1	RG	3.22	3%	1243	38.59	1.21	4%	4.57
Butyrivibrio proteoclasticus	B316	RG	2.2	8%	685	26.37	0.35	7%	1.84
Butyrivibrio proteoclasticus	B316	AP	1.36	9%	387	6.8	0.32	6%	1.06
Bacteroides sp.	Ga6A1	AP	1.08	6%	843	9.75	0.26	9%	0.97
Ruminococcus flavefaciens	FD1	AP	0.69	14%	827	6.19	0.14	9%	1.05
Ruminococcus flavefaciens	AE3010	FP	0.66	3%	640	3.96	1.05	2%	27.39
Ruminococcus flavefaciens	AE3010	AP	0.61	6%	447	4.29	1.06	5%	3.99
Butyrivibrio proteoclasticus	B316	OSX	0.57	6%	355	4.3	1.39	4%	27.43
Lachnospiraceae genus 6	NK4A136	AP	0.52	7%	443	3.91	0.1	9%	0.39
Oribacterium sp.	NK2B42	RG	0.52	13%	177	3.61	0.1	14%	1.29

Lachnospira multipara	LB2003	AP	0.49	10%	263	3.71	0.08	5%	1.59
Ruminococcus albus	AD2013	FP	0.47	11%	261	0.7	0.57	13%	3.83
Clostridiales bacterium	NK3B98	AP	0.46	9%	228	4.36	0.15	12%	0.46
Lachnospiraceae genus 6	NK4A136	RG	0.46	4%	140	3.41	0.13	6%	1.53
Lachnospira multipara	LB2003	RG	0.42	8%	136	3.4	0.13	11%	0.73
Lachnospiraceae genus 7	NK4A144	RG	0.42	17%	148	3.15	0.09	10%	1.42
Clostridiales bacterium	NK3B98	RG	0.39	16%	86.3	3.73	0.09	10%	0.4
Ruminococcus albus	AD2013	AP	0.37	3%	394	2.62	0.73	3%	3.09
Pseudobutyrivibrio sp.	MA3014	RG	0.26	2%	87.7	3.2	0.38	8%	2.1
Lachnospiraceae genus 7	NK4A144	OSX	0.19	23%	88.6	2.88	n/a	n/a	n/a
Butyrivibrio sp. 2	FC2001	AP	0.19	7%	143	2.61	0.19	5%	1.52
Lachnospiraceae genus 6	NK4A144	AP	0.16	4%	142	1.13	n/a	n/a	n/a
Butyrivibrio sp. 2	FC2001	FP	0.15	11%	429	0.91	0.25	51%	3.84
Prevotella ruminicola	23	FP	0.15	27%	1361	0.75	0.11	6%	3.85
Ruminococcus flavefaciens	FD1	FP	0.15	8%	141	0.37	0.18	41%	3.94
Butyrivibrio sp. 2	FC2001	OSX	0.15	15%	94	1.09	0.26	5%	5.08
Butyrivibrio fibrisolvens	MD2001	FP	0.14	24%	452	1.08	0.21	48%	2.95
Clostridiales bacterium	NK3B98	OSX	0.14	15%	156	1.41	0.17	6%	1.35
Lachnospiraceae genus 6	NK4A136	OSX	0.14	1%	230	2.75	0.05	11%	0.28
Prevotella brevis	P6B11	AP	0.14	23%	59.3	3.43	0.36	14%	2.15
Butyrivibrio fibrisolvens	MD2001	OSX	0.14	6%	52.1	1.16	0.48	21%	9.43
Lachnospiraceae genus 7	NK4A144	AP	0.12	9%	102	0.82	0.21	21%	0.71
Bacteroides sp.	Ga6A1	FP	0.12	5%	101	0.69	0.14	9%	2.23

Prevotella ruminicola	23	AP	0.09	11%	112	0.51	0.08	31%	0.79
Butyrivibrio fibrisolvens	MD2001	RG	0.09	6%	27.5	1.11	0.18	15%	1.05
Prevotella brevis	P6B11	RG	0.09	5%	15.1	0.95	n/a	n/a	n/a
Butyrivibrio sp. 2	FC2001	RG	0.09	8%	26.8	0.82	0.15	42%	0.93
Prevotella brevis	P6B11	OSX	0.09	31%	72.6	1.74	0.19	47%	12.8
Oribacterium sp.	NK2B42	OSX	0.08	59%	72.2	1.65	0.05	10%	0.91
Prevotella ruminicola	23	OSX	0.07	15%	93.8	0.33	0.19	10%	1.54
Butyrivibrio fibrisolvens	MD2001	AP	0.06	12%	129	0.45	0.24	10%	2.83
Ruminococcus flavefaciens	FD1	RG	0.06	12%	21	0.43	n/a	n/a	n/a
Pseudobutyrivibrio sp.	MA3014	FP	0.05	14%	-1176	0.27	0.2	41%	3.29

[a] Mean concentration of xylose equivalents released during the DNS assay

[b] Standard error calculated using four replicates

c Concentration of xylose equivalents released per minute per mg of protein

[d] Mean xylose concentration estimated in total concentrated SN volume

n/a denotes lost pellets

The top ranked strain/substrate combination for xylanolytic activity was *R. flavefaciens* FD1 grown on OSX, as it resulted in the highest average concentration of reducing sugars released as xylose equivalents from the DNS assay (4.77 g/L, Table 3.6). This strain/substrate combination, along with the other two top ranking strains, *R. albus* AD2013 and *Bacteroides* sp. Ga6A1 grown on various substrates, ranked higher in this assay than the benchmark strains *B. proteoclasticus* B316 and *P. ruminicola* 23 that were included due to their known hemicellulolytic activities (19, 62).

As with the FP incubations (Table 3.5), it was clear that the majority of the activity came from exogenous FEs that were present in the SN. This could have been due to either a lack of cell-associated FEs or because the CPs were resuspended in buffer instead of using sonication to lyse the cells. Therefore, based on the results from this assay, the top ranked strain/substrate combination for highest mean concentration of xylose equivalents released from the incubation of SN fractions on OSX was *R. flavefaciens* FD1 grown on OSX. Although, three different strains (including *R. flavefaciens* FD1, as well as *Bacteroides* sp. Ga6A1 and *R. albus* AD2013) grown on OSX ranked within the top four strain/substrate combinations for xylanolytic activity, the remaining top ranked strain/substrate combinations were grown on RG and AP. This observation supported the notion that the expression of FEs was not specific to the substrate present in the growth medium, as discussed in Section 3.2.3.1.

As were common themes in the assay that used FP (Table 3.5), the top ranked strain/substrate combination for xylanolytic activity (*R. flavefaciens* FD1) was not predicted to be largely xylanolytic. Furthermore, the ruminococci strains were dominant amongst the top ranked strains for xylanolytic activity, which was surprising as these strains were classified as cellulolytic. Although *Bacteroides* sp. Ga6A1 and *B. proteoclasticus* B316 were expected to be xylanolytic and appear amongst the top ranked strains, the results from this assay emphasize the caution needed when relying on genome sequences alone to predict function.

An alternative idea to explain the dominance of the ruminococci strains was that the cellulases expressed by ruminococci could be multifunctional. Pettipher and Latham (65) suggested that *R. flavefaciens* can degrade the cellulose, hemicellulose and pectin present within the plant cell wall, and further suggested that the cellulase and xylanase activities of *R. flavefaciens* originates from the same protein complex but uses different active sites. This

hypothesis was later substantiated by Doerner and White (66). Although it seemed intriguing and unexpected that the ruminococci strains were active on both FP and OSX, it appeared that this was actually unsurprising as multifunctional cellulases with xylanase activity have been isolated from *R. albus* SY3 and *R. flavefaciens* FD1 (66). Moreover, *R. flavefaciens* has already been shown to possess a variety of fibrolytic activities (35). It was worth noting that the xylanolytic potential of the ruminococci strains in this assay dominated the other rumen bacterial strains tested, especially the strains with predicted xylanolytic activity, and this made the ruminococci strains strong contenders that to be considered for improving fibrolytic activity.

It has been well established that ruminococci and *Butyrivibrio* strains are capable of extensively degrading and fermenting xylan (69-71), so it is of no surprise that strains belonging to these genera appeared extensively throughout the top of Table 3.6. To date, literature that confirms the efficient xylanolytic potential of *Bacteroides* strains is scarce, although it is clear that *Bacteroides* strains, such as *B. intestinalis*, *B. xylanisolvens* and *B. ovatus* for example, are significant xylan degraders that are found within the human colonic microbial ecosystem (70). Therefore, any of these strains would have also been considered as good candidates for further work based on their xylanolytic activity.

Although the top ranked strain/substrate combination (*R. flavefaciens* FD1) had the highest concentration of xylose equivalents in the DNS assay (4.77 g/L, Table 3.6), *R. albus* AD2013 grown on OSX had a higher SN specific activity (1242 µmol min-1 mg-1, Table 3.6) and *R. albus* AD2013 grown on RG had the normalised concentration of xylose equivalents from SN fractions (58.9 g/L, Table 3.6). Therefore, these results made *R. albus* AD2013 a better candidate for improving fibrolytic activity further. Despite *Bacteroides* sp. Ga6A1 grown on OSX ranking highly, it was chosen for further work based on its cellulolytic activity (Section 3.2.3.1).

3.2.3.4 Determining fibrolytic activity profiles using artificial substrates that represented major linkages in the plant cell wall (*p*NP-conjugated assays)

To determine the activities of the strain/substrate preparations on bonds that were likely to represent the biochemical linkages between side groups and major structural polysaccharides, fibrolytic activity profiles were generated for the SN preparations only (Table 3.7, Section

2.2.10) since little activity was detected from the CP fractions (Table 3.5 and Table 3.6). The *p*NP-conjugated substrates used (Table 2.21) represented some of the major bonds found within the polysaccharides within the plant cell wall, and results from this assay indicated the types of activities each strain may possess. The *p*NP-substrates were chosen based on previous studies (72), and also by what was commercially available.

Activities were determined by a significant increase in OD due to catalysis of the *p*NP-conjugated substrate and release of the yellow dye, *p*NP. To quantify activities, the maximum OD reached during the length of the assay was recorded. However, some strain/substrate combinations resulted in very minor, but detectable, activity that was recorded as a + or ++. Samples were ranked in order of highest number of activities (Table 3.7).

Table 3.7. Rumen bacterial strain/substrate combinations incubated with *p*NP-conjugated substrates that represented major biochemical linkages in the plant cell wall.

Species	Strain	Substrate	Sub1: *p*NP α-D mannopyranoside	Sub2: *p*NP β-D fucopyranoside	Sub3: *p*NP α-D galactopyranoside	Sub4: *p*NP β-D glucopyranoside	Sub5: *p*NP α-D glucopyranoside	Sub6: *p*NP β-D mannopyranoside	Sub7: *p*NP β-D glucuronide	Sub8: *p*NP α-D maltoside	Sub9: *p*NP α-L arabinofuranoside	SubC10: *p*NP α-L rhamnopyranoside	Sub11: *p*NP α-L arabinopyranoside	Sub12: *p*NP acetate	Sub13: *p*NP β-D xylopyranoside	Sub14: *p*NP β-D cellobioside	Sub15:*p*NP- α-L fucopyranoside
Butyrivibrio fibrisolvens	MD2001	RG	++	++	4.0	1.4	1.2	+	++	+	1.9	2.4	++	+	3.3	+	+
Butyrivibrio proteoclasticus	B316	AP		1.1	3.2	1.5	+	1.1		+	2.2			2.3	+		+
Butyrivibrio sp. 2	FC2001	FP		0.5	0.8	1.4	+			+	0.6				1.7		0.6
Prevotella brevis	P6B11	RG	+	++	4.0	1.5	2.1	++	++	++	2.7	++	++	++	1.6	++	++
Oribacterium sp.	NK2B42	RG	+	0.9	1.2	0.9	1.1	+	+	0.6	+	+	+	+	+	+	+
Ruminococcus albus	AD2013	RG	+	+	1.3	0.9	+	+	+	+	2.7	+	+	4.0	+	1.5	+
Prevotella brevis	P6B11	AP			2.9		1.5			+	2.6						
Butyrivibrio sp. 2	FC2001	RG	+	++	++	1.5	+	+	+	+	++	+	+	+	1.6	+	1.0
Ruminococcus flavefaciens	AE3010	FP		+	+	+	++				0.9				++	1.3	

Ruminococcus flavefaciens	AE3010	RG	+	+	2.8	++	1.2	+	+	+	1.3	+	+	+	+	+	+
Bacteroides sp.	Ga6A1	RG	+	+	+	1.0	0.8	+	+	+	1.5	+	+	+	+	+	+
Ruminococcus flavefaciens	FD1	OSX		+	+	+	+				1.0		+		+	1.1	
Clostridiales bacterium	NK3B98	OSX			1.0	+	0.9		+		+			4.0	+		+
Bacteroides sp.	Ga6A1	OSX		+	+	1.9					+		+		3.9	1.2	
Butyrivibrio sp. 2	FC2001	OSX		+	0.5	0.5	+							4.0	+		
Butyrivibrio fibrisolvens	MD2001	OSX			0.8		+				1.2				1.1		
Lachnospiraceae genus 7	NK4A144	RG	+	+	3.3	+	+	+	++	+	2.0	+	+	+	+	+	+
Clostridiales bacterium	NK3B98	RG	+	+	1.1	1.2	+	+	+	+	+	+	+	+	+	+	+
Butyrivibrio proteoclasticus	B316	RG		+	1.0	+	+	+		+	1.1	+		+	+		+
Butyrivibrio fibrisolvens	MD2001	FP			+	+								4.0	++		+
Lachnospiraceae genus 6	NK4A136	RG	+	+	+	+	2.3	+	+	+	+	+	+	+	+	+	+
Pseudobutyrivibrio sp.	MA3014	RG	+	+	1.2	+	+	+	+	+	4.0	+	+	+	+	+	+
Prevotella ruminicola	23	AP			+	+	+		+		3.6				+	+	
Bacteroides sp.	Ga6A1	FP					+			+	+	+					
Lachnospiraceae genus 7	NK4A144	AP			+						0.8	+			+		
Prevotella ruminicola	23	FP				+	+				1.1				+		
Ruminococcus albus	AD2013	OSX					+			+	+				1.7		
Bacteroides sp.	Ga6A1	AP					+				+	+					
Prevotella brevis	P6B11	OSX			+		+										
Ruminococcus flavefaciens	AE3010	AP			+		0.4										
Pseudobutyrivibrio sp.	MA3014	FP			+						0.9						
Lachnospira multipara	LB2003	AP												0.8			

Prevotella ruminicola	23	OSX									0.3						
Lachnospira multipara	LB2003	RG	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lachnospiraceae genus 7	NK4A144	OSX			+		+			+	+			+	+		+
Butyrivibrio proteoclasticus	B316	OSX		+	+	+				+	+				+		
Ruminococcus albus	AD2013	FP		+		+	+										
Butyrivibrio sp. 2	FC2001	AP									+				+		
Clostridiales bacterium	NK3B98	AP				+					+						
Ruminococcus albus	AD2013	AP									+						
Ruminococcus flavefaciens	FD1	FP					+										
Butyrivibrio fibrisolvens	MD2001	AP			+						+						
Lachnospiraceae genus 6	NK4A136	OSX															
Lachnospiraceae genus 6	NK4A136	AP															
Oribacterium sp.	NK2B42	OSX															
Ruminococcus flavefaciens	FD1	AP															

Activity was recorded as an absorbance value at 405 nm. Minor activity was recorded as a + or ++. Samples were ranked in order of highest number of activities.

The three top ranked strains (*Butyrivibrio fibrisolvens* MD2001, *Butyrivibrio proteoclasticus* B316 and *Butyrivibrio* sp. 2 FC2001) were active on a total of six substrates each and varied in the number of substrates where they scored ++ and +. Interestingly, the top ranked strains for this assay contrasted greatly to those for cellulolytic and xylanolytic activity (Table 3.5 and 3.6), and this highlighted the need to use complementary biochemical assays to comprehensively measure fibrolytic activity in order to encapsulate the wide range of activities required for effective fibre degradation.

The activities of the three top ranked strains varied, with activity common on certain substrates (*p*NP β-D fucopyranoside, *p*NP α-D galactopyranoside, *p*NP β-D glucopyranoside, *p*NP α-D glucopyranoside, *p*NP α-L arabinofuranoside and *p*NP β-D xylopyranoside), and activity differing on a variety of other substrates (*p*NP β-D mannopyranoside, *p*NP α-D maltoside, *p*NP α-L rhamnopyranoside, and *p*NP acetate) (Table 3.7). This was to be expected when assaying a variety of strains with a range of fibrolytic activities and it was important to consider when narrowing down which strains should be used as candidates for improving fibrolytic activity.

Strains grown on RG featured predominately in the top ranked strain/substrate combinations tested, and this was likely because when ranking samples in order of highest number of activities, the number of activities that were too small to be considered as an obvious reaction were also taken into account. SNs from strains grown on RG appear to possess activity, albeit very low in some cases, on all *p*NP-conjugated substrates in the assay. It was likely that this occurred because, as RG is the most complex growth substrate, it contained all of the bonds represented by the *p*NP-conjugated substrates, and hence induced their activities in culture.

It was difficult to compare how these results related to the available literature as little research on specific plant cell wall linkages that are targeted by rumen-derived FEs was available. Current literature in the field is focused on identifying putative FEs using genome sequences (8, 11, 17, 73), or improving ruminal colonization and degradation of fresh fibrous substances (20, 74-76).

Based on the results from this assay, *B. fibrisolvens* MD2001 grown on RG appeared to be a good candidate strain to consider for improving fibrolytic activity. This was because it showed considerable activity on a variety of *p*NP-conjugated substrates and it grew within a

similar time frame to the other two top ranked strains for cellulolytic activity (Table 3.5) and xylanolytic activity (Table 3.6).

3.2.4 Selection of candidate strains for improving fibrolytic activity using directed evolution and mutagenesis

As cellulose and hemicellulose are the major structural polysaccharides found in the plant cell wall, it was essential that candidate bacterial isolates for enzyme production exhibited strong cellulolytic and xylanolytic activities. *Bacteroides* sp. Ga6A1 and *R. albus* AD2013 appeared to possess high fibrolytic activity on FP and OSX, respectively (Table 3.5 and 3.6), and were therefore good choices to use as a candidate strains for improving fibre degradation.

In addition to selecting strains that possessed high cellulolytic and xylanolytic activity, a strain that was proficient in degrading a variety of plant cell wall linkages was preferred in order to improve the chances of obtaining a strain with improved fibrolytic activity. *B. fibrisolvens* MD2001 was chosen from the top three ranked strains in the *p*NP-conjugated substrate assay (Table 3.7) as it had a broad range of activities, including substantial activity on those that are more commonly found in major plant cell wall polysaccharides. MD2001 also grew well within a similar timeframe to *Bacteroides* sp. Ga6A1 and *R. albus* AD2013.

Given that the expression of suites of FEs did not appear to be specific to the fibrous substrate used for growth, based on the results from the two biochemical assays used thus far, it was decided that all candidate strains would be grown on RG as it was a complex substrate, and it appeared to enable the expression of activities that were effective on all *p*NP-conjugated substrates tested in this project. This was also consistent with the expectation that RG is among the main forages that any pre-treatments arising from this work may be developed from.

3.3 Chapter summary

The screening of FEs present in the SN and CP fractions from rumen bacterial strains grown on various fibrous substrates showed that a) *Bacteroides* sp. Ga6A1 possessed the highest cellulolytic activity and was of considerable interest, b) *R. albus* AD2013 also possessed high

xylanolytic activity, and c) *B. fibrisolvens* MD2001 stood out as it had activity on substrates that were of more relevance to major biochemical linkages in the plant cell wall (Table 3.7). These three strains were therefore selected as ideal candidate strains to take forward for improving FE production using evolutionary adaptation and mutagenesis.

Chapter Four: Enhancing fibre-degrading activity of rumen bacteria *via* mutagenesis and selection

4.1 Introduction

The second aim of this thesis was to improve the fibrolytic activities of candidate bacterial strains using mutagenesis and extended positive selection for fibre-utilisation by subculturing on plant substrates. This strategy followed that undertaken by Saluzzi et al. (35) but with the addition of mutagenesis which was hypothesised to increase the chances of generating mutations that positively impacted expression, or potency of, FEs and their related activities in relation to fibre degradation (Fig 4.1).

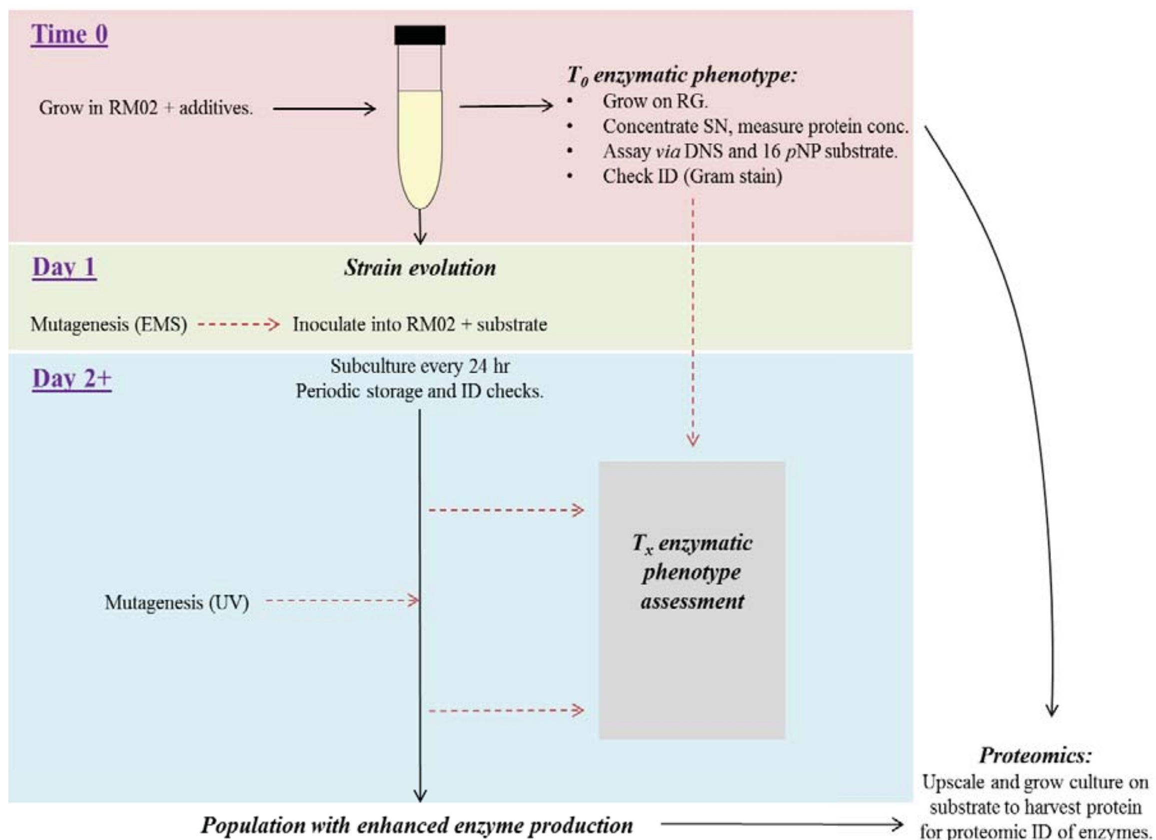

Fig 4.1. Schematic diagram of mutagenesis and serial sub-culturing experiment.

The three candidate strains, *Bacteroides* sp. Ga6A1, *R. albus* AD2013 and *B. fibrisolvens* MD2001, selected based on pre-screening for high cellulolytic and xylanolytic activities (Chapter 3), were chosen for improvement. EMS and UV radiation were chosen as mutagenic agents as each agent induced a different spectrum of mutations (42, 45). As the susceptibility of each strain to the mutagenic agents was likely to differ from published protocols for other microbes (43, 45-48), preliminary experiments to establish optimal mutagenesis conditions were required.

The aim of these preliminary experiments was to establish the necessary conditions that would give a desired kill rate of approximately 50% and 95% for EMS and UV radiation respectively. A desired kill rate of 50% using EMS was chosen so that a good proportion of cells survived mutagenesis and the cultures could continue to be subcultured. A higher desired kill rate using UV radiation was chosen, however, to increase the likelihood of generating mutants as it was thought that the original desired kill rate of 50% may have been too low. The desired kill rates were achieved by adjusting the amount of time cultures were exposed to both mutagenic agents, as well as adjusting the concentration of EMS.

In order to select for variants with enhanced fibrolytic activity, each strain was grown in two independent parallel lineages under positive selective pressure in growth medium with RG as the sole carbon source (Fig 4.2). An additional two lineages for each strain that were not mutagenised were grown in parallel, which served as negative controls to determine any effect of mutagenesis on fibrolytic activity (Fig 4.2).

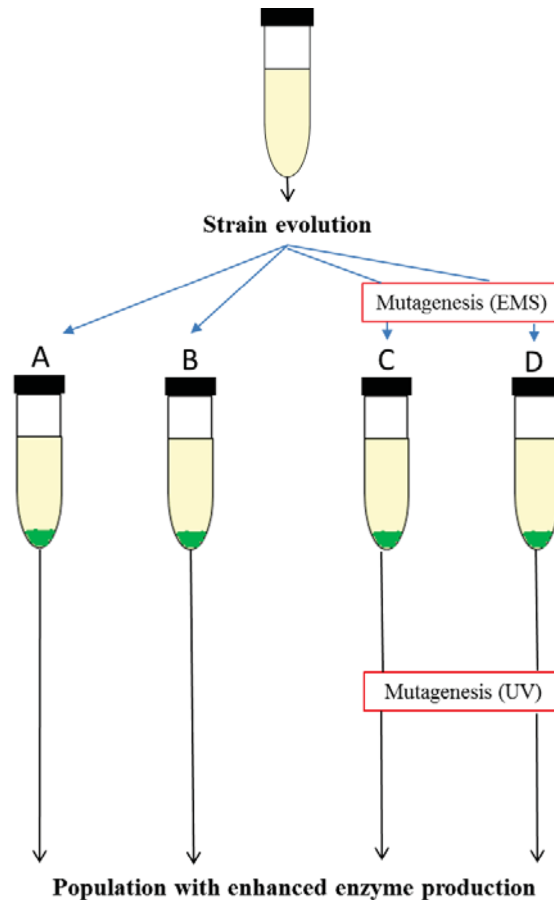

Fig 4.2. Schematic diagram illustrating parallel lineages A-D. Each strain was inoculated into four separate lineages; two parallel lineages were mutagensised (lineages C and D), and two parallel lineages remained non-mutagenised (lineages A and B).

The feasibility of improving enzyme activity and production using non-GM techniques to a level that is cost effective in large-scale production was unclear. However, the approach of screening multiple strains in combination with a variety of mutagenesis treatments may increase the probability of obtaining improved strains.

The aim of this chapter was to generate at least two strains with significantly greater abilities to degrade cellulose, hemicellulose or forage material in culture compared to their parent strains prior to mutagenesis and/or positive selection. The FEs from these strains would then be analysed using proteomic methods (Chapter 5), and such strains could form the basis for the development of a ruminant feed treatment.

4.2 Results and discussion

4.2.1 Mutagenesis optimisation

4.2.1.1 EMS mutagenesis optimisation

The results from the EMS mutagenesis optimisation experiment (Section 2.2.7.2) indicated that candidate strains treated with 0.5% EMS for 15 min at 37˚C resulted in the desired kill rate of approximately 50% (Table 4.1), except for *B. fibrisolvens* MD2001, which had a kill rate of 88%. Treatment with EMS at higher concentrations between 1% and 5% resulted in a kill rate that appeared to kill all of the cells for each of the strains. See Section 2.2.12.7 for calculations.

Table 4.1. The kill rates of candidate strains treated with 0.5% EMS for 15 min at 37˚C.

Species	Strain	EMS (%)	Average CFUs [a]	Standard error [a]	Kill rate
Bacteroides sp.	Ga6A1	0	3.9E+08	4%	
Bacteroides sp.	Ga6A1	0.5	1.93E+08	12%	50%
Butyrivibrio fibrisolvens	MD2001	0	1.07E+09	5%	
Butyrivibrio fibrisolvens	MD2001	0.5	1.27E+08	3%	88%
Ruminococcus albus	AD2013	0	9.57E+08	4%	
Ruminococcus albus	AD2013	0.5	6.3E+08	13%	34%

[a] Values were calculated using three replicates

At the time, a 50% kill rate was chosen so that there would still be a reasonable proportion of viable cells left to propagate and continue to grow in the selection medium. It was possible that this kill rate may have been too low and resulted in a low proportion of cells that were not mutagenised, or that the surviving cells contained mutations that would not enhance fibre degradation. As such, a higher kill rate of, say ~95%, would have generated more desired mutants, but there was a risk that all cells may have been killed. To compensate for using a lower kill rate, in future experiments, cells could be subjected to numerous rounds of mutagenesis with EMS in an attempt to increase the chances of generating desirable mutants.

The EMS treatment method was complex, and required several anaerobic manipulations, washes and incubations where technical variation could have been introduced. The EMS solution was quite viscous and tended to sit at the bottom of the tubes. It was therefore imperative that the EMS solution was mixed gently immediately before it was added to the cells, and that tubes were incubated horizontally with gentle shaking in order to prevent the EMS from settling at the bottom of the tubes. In saying that, tubes spent a large amount of time upright with the EMS sitting on the bottom of the tube as they were moved in and out of the anaerobic chamber's airlock for the duration of the cycle (approximately 5 min). Also, due to the time taken to manoeuvre within the anaerobic chamber, which was also dependant on the number of tubes processed during each optimisation experiment, cells processed first would have been exposed to EMS longer than the cells processed last. This time of EMS exposure would have also differed between optimisation experiments. Although this is difficult to rectify without processing the cells one at a time, this was taken into consideration when analysing the optimisation results from mutagenesis with EMS.

4.2.1.2 UV radiation mutagenesis optimisation

The results from the UV radiation mutagenesis optimisation experiment (Section 2.2.7.4 and Section 2.2.12.8) indicated that treating candidate strains with UV radiation from a transilluminator for 45 min at RT resulted in the desired kill rate of approximately 95% (Table 4.2).

Table 4.2. The kill rates of bacterial strains exposed to UV radiation for 0, 45 and 60 min.

Species	Strain	UV exposure (min)	Kill rate	Standard error [a]
Butyrivibrio fibrisolvens	MD2001	0	0.72%	3.32%
Butyrivibrio fibrisolvens	MD2001	45	96.5%	5.40%
Butyrivibrio fibrisolvens	MD2001	60	98.0%	100%
Ruminococcus albus	AD2013	0	13.9%	9.76%
Ruminococcus albus	AD2013	45	98.5%	12.9%
Ruminococcus albus	AD2013	60	82.1%	20.0%
Bacteroides sp.	Ga6A1	0	n/a	5.82%
Bacteroides sp.	Ga6A1	45	-	-
Bacteroides sp.	Ga6A1	60	n/a	100%

[a] Standard errors were calculated using three replicates

n/a denotes that no CFUs were detected

The final CFU counts for this experiment showed greater variability than preferred, thus it was decided to use a UV radiation exposure time of 40 min at RT for each strain. An exposure time of 40 min was chosen to err on the side of caution as an exposure time of 45 min resulted in kill rates close to 100%, and these results were highly variable.

No CFUs for *Bacteroides* sp. Ga6A1 were observed, and this was likely due to technical error. Standard culture dilutions of 10^{-4}, 10^{-5} and 10^{-6} were used for all strains, but it was likely to have been too high for Ga6A1, and it is recommended that a broader range of dilutions (e.g. from 10^{-2}) should be used in any future experiments to accurately measure treatments where high kill rates result.

4.2.2 Improving fibrolytic activity

4.2.2.1 Mutagenesis and selection for improved fibrolytic activity

The three candidate strains were used to generate four lineages (labelled A - D) from a common starting culture. Lineages A and B were duplicates that were not subjected to mutagenesis, and served as negative controls to highlight any effect that mutagenesis had on the fibrolytic activities of the candidate strains. At the same time, through spontaneous mutation, there was a likelihood of obtaining strains with improved fibrolytic activity through

the experimental regime. Lineages C and D were duplicates that were subjected to mutagenesis with EMS and UV radiation in combination with serial sub-culturing on RG.

Based on the results from the EMS optimisation experiment (Section 4.2.1.1), lineages C and D from each of the candidate strains were subjected to treatment with 0.5% EMS for 15 min at 37˚C, and then subcultured daily for 14 consecutive subcultures on RG. At day 15, lineages C and D were exposed to UV radiation for 40 min based on results from the UV radiation optimisation experiment (Section 4.2.1.2), with tubes being rotated every 15 min, and further subcultured daily on RG for another 15 times. SN samples were taken for each strain and lineage periodically throughout the experiment, with biochemical protein and activity assays performed on SN samples from the first subculture (subculture 1) and the last subculture (subculture 31). Only two time points were analysed to minimize the number of samples that needed to be processed, but these two time points in particular were selected because they would give a clear indication of whether there was any increase in fibrolytic activity.

4.2.2.2 Mutagenesis kill rates

Unfortunately, the CFU data for the EMS experiment was lost, thus the kill rate for the EMS mutagenesis could not be estimated, apart from that it must have been less than 100%, given that lineage C and D growth ensued after this process for all strains. This lost data, again, was due to values being recorded on a separate piece of paper that was subsequently misplaced or discarded. Moving forward, all data was recorded in a laboratory book that could be decontaminated, and all data, both individual values and averages, were transferred to an Excel spreadsheet.

The UV mutagenesis kills rates are shown in Table 4.3. Although the kill rate for each strain was close to the desired rate of 95%, the methodology produced variable results as indicated by some of the large standard error percentages obtained. This could be due to a variety of factors, including inconsistent UV exposure because of interference from the RG substrate in the media, or varied exposure timings between processing the first sample and the last sample. Furthermore, by CFU counts, *B. fibrisolvens* MD2001 exhibited a kill rate of 100% (Table 4.3, lineage C), but the lineage was still viable after mutagenesis, demonstrated by its successful subculturing. Thus, the kill rates calculated at are at best an estimate of the actual kill rates in the corresponding lineages.

Table 4.3 The kill rates of lineages C and D of each bacterial candidate after a 40 min exposure to UV radiation at RT.

Species	Strain	Lineage	UV exposure (min)	Average CFU (CFU/mL)	Standard error [a]	Kill rate
Bacteroides sp.	Ga6A1	C	0	1.52E+08	2%	
Bacteroides sp.	Ga6A1	C	40	8.33E+06	24%	94.5%
Bacteroides sp.	Ga6A1	D	0	1.16E+08	3%	
Bacteroides sp.	Ga6A1	D	40	2.07E+07	11%	82.2%
Butyrivibrio fibrisolvens	MD2001	C	0	7.87E+09	4%	
Butyrivibrio fibrisolvens	MD2001	C	40	3.33E+06	26%	100%
Butyrivibrio fibrisolvens	MD2001	D	0	8.43E+09	4%	
Butyrivibrio fibrisolvens	MD2001	D	40	1.23E+07	28%	99.9%
Ruminococcus albus	AD2013	C	0	6.47E+08	13%	
Ruminococcus albus	AD2013	C	40	4.10E+07	6%	93.7%
Ruminococcus albus	AD2013	D	0	5.30E+08	8%	
Ruminococcus albus	AD2013	D	40	1.20E+07	17%	97.7%

[a] Standard errors were calculated using three replicates

4.2.2.3 Protein concentrations

The protein concentrations of the SNs from subculture 1 and subculture 31 were measured using the Bradford assay (Sections 2.2.8.3 and 2.2.12.1) and are shown in Table 4.4.

Table 4.4. Protein concentrations in candidate strain SNs using the Bradford assay.

Species	Strain	Subculture	Lineage	Protein concentration (mg/mL) [a]	Standard error [b] (%)
Bacteroides sp.	Ga6A1	1	A	0.07	0.00%
Bacteroides sp.	Ga6A1	1	B	0.13	0.31%
Bacteroides sp.	Ga6A1	1	C	0.10	4.54%
Bacteroides sp.	Ga6A1	1	D	0.07	0.53%
Bacteroides sp.	Ga6A1	31	A	0.05	0.58%
Bacteroides sp.	Ga6A1	31	B	0.04	3.38%

Bacteroides sp.	Ga6A1	31	C	0.05	6.38%
Bacteroides sp.	Ga6A1	31	D	0.05	27.1%
Butyrivibrio fibrisolvens	MD2001	1	A	0.07	1.05%
Butyrivibrio fibrisolvens	MD2001	1	B	0.04	0.00%
Butyrivibrio fibrisolvens	MD2001	1	C	0.07	2.75%
Butyrivibrio fibrisolvens	MD2001	1	D	0.05	0.53%
Butyrivibrio fibrisolvens	MD2001	31	A	0.06	3.62%
Butyrivibrio fibrisolvens	MD2001	31	B	0.07	2.21%
Butyrivibrio fibrisolvens	MD2001	31	C	0.08	1.23%
Butyrivibrio fibrisolvens	MD2001	31	D	0.06	7.56%
Ruminococcus albus	AD2013	1	A	0.06	5.30%
Ruminococcus albus	AD2013	1	B	0.06	1.63%
Ruminococcus albus	AD2013	1	C	0.13	0.90%
Ruminococcus albus	AD2013	1	D	0.06	0.89%
Ruminococcus albus	AD2013	31	A	0.07	0.86%
Ruminococcus albus	AD2013	31	B	0.10	2.15%
Ruminococcus albus	AD2013	31	C	0.09	2.34%
Ruminococcus albus	AD2013	31	D	0.02	9.70%

[a] Protein concentration of SN samples after concentrating

[b] Standard error was calculated using two replicates

It was noted that the standard errors for all samples were extremely low, except for *Bacteroides* sp. Ga6A1, lineage D at subculture 31. The reason for this is unclear; however, the protein concentration calculated for this sample was as expected.

4.2.3 Measuring the cellulolytic and xylanolytic activity of candidate strains at subculture 1 and subculture 31.

4.2.3.1 Sigmacell cellulose-degrading specific activities of candidate strain SNs

The cellulolytic activity of each of the candidate strains was assayed as in Section 2.2.9, except that Sigmacell cellulose was used instead of FP to the same final concentration (Section 2.2.9.4). Sigmacell cellulose produced a much more even substrate suspension than FP, and it was thought that the presence of more readily available cellulose binding sites could result in higher cellulolytic activity. This time, only specific activity (μmol $min^{-1}mg^{-1}$) was used as a measure of activity in order to take into account the varying amounts of protein

that were used for each sample (see Section 2.2.12.3 and Section 2.2.12.4 for calculations). Table 4.5 shows the results of cellulolytic activity assessment for the evolved lineages.

Table 4.5. Relative total sugar released from the SN of candidate strains on Sigmacell cellulose after 1 subculture and 31 subcultures.

			Subculture 1		Subculture 31			
Species	Strain	Lineage	Specific activity (µmol $min^{-1}mg^{-1}$)	Standard error [a]	Specific activity (µmol $min^{-1}mg^{-1}$)	Standard error [a]	Percentage Increase (%)	P value [b]
Bacteroides sp.	Ga6A1	A	17.0	8.46%	11.9 [d]	16.1%	-29.8%	0.348
Bacteroides sp.	Ga6A1	B	9.14	7.79%	25.0	6.66%	173%	6.17E-05
Bacteroides sp.	Ga6A1	C	11.1	2.36%	22.6	2.46%	104%	5.66E-07
Bacteroides sp.	Ga6A1	D	16.0	11.3%	21.6	8.90%	36%	3.70E-02
Butyrivibrio fibrisolvens	MD2011	A	12.8	6.98%	14.1 [d]	7.22%	10%	0.188
Butyrivibrio fibrisolvens	MD2011	B	18.6 [d]	1.13%	13.7	2.62%	-26.2%	1.16E-05
Butyrivibrio fibrisolvens	MD2011	C	12.6	5.68%	13.0	6.62%	3%	0.371
Butyrivibrio fibrisolvens	MD2011	D	15.3 [d]	5.39%	20.0	5.36%	31%	0.006
Ruminococcus albus	AD2013	A	9.81 [c,d]	8.16%	10.6 [d]	16.4%	8%	0.348
Ruminococcus albus	AD2013	B	10.9 [d]	4.95%	13.0	5.64%	18%	0.034
Ruminococcus albus	AD2013	C	6.13 [d]	9.19%	12.5	8.73%	104%	0.001
Ruminococcus albus	AD2013	D	12.3 [d]	7.46%	32.1 [d]	18.8%	161%	0.009

[a] Standard error was calculated using four replicates

[b] P value was determined using a 1 tail, type 2, T-test

[c] value below LOD score (see Section 2.2.12.5 for calculations)

[d] value below LOQ score (see Section 2.2.12.6 for calculations)

Ten lineages appeared to have an increase in cellulolytic activity, with five being statistically significant ($P<0.05$). *Bacteroides* sp. Ga6A1 lineage B had the greatest increase in cellulolytic activity by 173%, followed by *R. albus* AD2013 lineage D with an improvement of 161% (Table 4.5). It was unclear whether mutagenesis had any considerable impact on generating mutants with enhanced fibrolytic activity as two of the lineages with significant increases had been subjected to positive selection only, without mutagenesis.

The use of an even suspension of cellulose does not appear to have made much difference to the amount of cellulolytic activity measured compared to the use of FP (Table 3.5). The specific activities calculated in this assay were still low compared to the OSX values (Table 4.6). Despite this, the majority of samples displayed activities that were above the limits of detection and quantitation for the assay (see Sections 2.2.12.5 and 2.2.12.6), including the top two ranking strains which are of the most interest.

4.2.3.2 Xylanolytic specific activities of candidate strain SNs

The xylanolytic activity of each of the candidate strains was assayed using OSX as described in Section 2.2.9.4 (Table 4.6). Again, activity was expressed only as specific activity (µmol $min^{-1}mg^{-1}$).

Table 4.6. Relative total sugar released from the SN of candidate strains on OSX at subculture 1 and subculture 31.

			Subculture 1		Subculture 31			
Species	Strain	Lineage	Specific activity (µmol $min^{-1}mg^{-1}$)	Standard error [a]	Specific activity (µmol $min^{-1}mg^{-1}$)	Standard error [a]	Percentage Increase (%)	P value [b]
Bacteroides sp.	Ga6A1	A	50.7	16%	14.9 [c,d]	52%	-71%	0.009
Bacteroides sp.	Ga6A1	B	34.3	8.7%	65.4	9.7%	90%	0.002
Bacteroides sp.	Ga6A1	C	23.3	2.0%	55.0	11%	136%	0.002
Bacteroides sp.	Ga6A1	D	81.4	6.9%	57.5	5.8%	-29%	0.005
Butyrivibrio fibrisolvens	MD2011	A	19.6 [c,d]	1.7%	19.1 [c,d]	2.6%	-3%	0.210
Butyrivibrio fibrisolvens	MD2011	B	28.4 [c,d]	5.9%	17.0 [c,d]	4.6%	-40%	4.28E-04
Butyrivibrio fibrisolvens	MD2011	C	19.2 [c,d]	1.7%	18.3 [c,d]	2.2%	-5%	0.065
Butyrivibrio fibrisolvens	MD2011	D	23.9 [c,d]	6.6%	27.6 [d]	3.5%	15%	0.048
Ruminococcus albus	AD2013	A	123.5	4.3%	134	4.3%	9%	0.108
Ruminococcus albus	AD2013	B	101 [d]	9.7%	84.8	4.5%	-16%	0.089
Ruminococcus albus	AD2013	C	78.7	6.7%	104	2.7%	32%	0.003
Ruminococcus albus	AD2013	D	153 [d]	3.1%	87.6 [c]	5.7%	-43%	4.09E-05

[a] Standard error was calculated using four replicates

[b] P value was determined using a 1 tail, type 2, T-test

[c] value below LOD score (see Section 2.2.12.5 for calculations)

[d] value below LOQ score (see Section 2.2.12.6 for calculations)

Based on the results from this experiment, five strains appeared to have an improvement in xylanolytic activity, with four of these being statistically significant. *Bacteroides* sp. Ga6A1 lineage C had the most statistically significant improvement in cellulolytic activity by 136%, followed by *Bacteroides* sp. Ga6A1 lineage B with an improvement of 90%. Again, it was unclear whether mutagenesis had any considerable impact on obtaining mutants with enhanced fibrolytic activity as two of the lineages with significant increases had been subjected to positive selection only, without mutagenesis.

Interestingly, in this assay the *R. albus* AD2013 strains had specific activities almost 10 fold higher than the other two candidate strains. This was interesting because such a difference in xylanolytic activity was not seen during the initial biochemical analysis, and *R. albus* AD2013 was not predicted to have high xylanolytic activity based purely on its genome sequence.

Saluzzi, et al. (35) were able to improve the fibre-degrading activity of *R. flavefaciens* strain 17 by 60% in 24 hr incubations after 23 consecutive subcultures on RG. Substrate degradation was measured as an increase in dry matter solubilisation. After 31 subcultures on RG, this study was able to show improvements in cellulolytic and xylanolytic activity, with increases as high as 173% and 136% respectively. Although both studies have measured the increase in fibrolytic activity using different measures, both studies demonstrated the potential of improving fibrolytic activity using non-GM methodologies.

In an effort to enhance fibrolytic activity even further, strains in this study could be subcultured on RG for a longer period of time. The increased duration of selection may improve the likelihood of generating strains with improved fibrolytic activity. Furthermore, rumen bacterial strains could be subjected to multiple rounds of EMS and UV radiation mutagenesis in combination with longer subculturing, which may further boost the chances of obtaining improved strains. Any improvements in fibrolytic activity should then be re-confirmed, and this may require more technical replicates in order to detect finer increases. Whole genome sequencing and comparative genomics is then recommended to identify any genetic changes that are responsible for any increases in fibrolytic activity.

4.2.3.3 *p*NP-conjugated assays

The SNs of candidate strains from subculture 1 and subculture 31 were similarly assayed using *p*NP-conjugated substrates as described in Section 3.2.3.4, except that two substrates were excluded, *p*NP acetate and *p*NP α-D maltoside, as these substrates were no longer available.

Table 4.7. Candidate strain SNs incubated with *p*NP-conjugated substrates that represent major biochemical linkages in the plant cell wall.

Species	Strain	Lineage	Subculture	Sub1: *p*NP α-L arabinopyranoside	Sub2: *p*NP β-D fucopyranoside	Sub3: *p*NP α-D galactopyranoside	Sub4: *p*NP β-D glucopyranoside	Sub5: *p*NP α-D glucopyranoside	Sub6: *p*NP α-D mannopyranoside	Sub7: *p*NP β-D mannopyranoside	Sub8: *p*NP α-L rhamnopyranoside	Sub9: *p*NP β-D glucuronide	Sub11: *p*NP α-L arabinofuranoside	Sub13: *p*NP β-D xylopyranoside	Sub14: *p*NP β-D cellobioside	Sub15: *p*NP α-L-fucopyranoside
Bacteroides sp	Ga6A1	A	1	-	-	-	0.41	-	-	-	-	-	0.47	-	-	-
Bacteroides sp	Ga6A1	A	31	-	-	0.08	0.54	0.10	-	-	++	-	0.62	-	-	+
Bacteroides sp	Ga6A1	B	1	-	-	-	0.63	0.20	-	-	-	-	0.49	-	-	-
Bacteroides sp	Ga6A1	B	31	-	-	-	0.56	0.21	-	-	-	-	0.67	-	-	-
Bacteroides sp	Ga6A1	C	1	-	-	-	0.45	0.11	-	-	-	-	0.39	-	-	-
Bacteroides sp	Ga6A1	C	31	-	-	-	0.44	-	-	-	-	-	0.36	-	-	-
Bacteroides sp	Ga6A1	D	1	-	-	-	0.35	0.09	-	-	-	-	0.62	-	-	-
Bacteroides sp	Ga6A1	D	31	-	-	-	0.27	0.08	-	-	-	-	0.42	-	-	-
Butyrivibrio fibrisolvens	MD2001	A	1	+	0.09	0.42	0.14	+	+	+	++	+	0.18	0.23	+	0.13
Butyrivibrio fibrisolvens	MD2001	A	31	+	0.02	0.11	0.03	+	+	+	+	+	0.07	0.04	+	0.16

Butyrivibrio fibrisolvens	MD2001	B	1	+	+	0.36	0.12	+	+	+	++	+	0.17	0.19	+	+
Butyrivibrio fibrisolvens	MD2001	B	31	+	+	0.14	0.05	+	+	+	++	+	0.09	0.06	+	0.25
Butyrivibrio fibrisolvens	MD2001	C	1	-	0.12	0.38	0.19	-	-	-	0.10	-	0.26	0.34	-	-
Butyrivibrio fibrisolvens	MD2001	C	31	-	0.01	0.17	0.06	-	-	-	0.03	-	0.14	0.06	-	-
Butyrivibrio fibrisolvens	MD2001	D	1	-	0.10	0.53	0.17	-	-	-	0.09	-	0.28	0.29	-	-
Butyrivibrio fibrisolvens	MD2001	D	31	-	0.01	0.19	0.04	-	-	-	0.03	-	0.12	0.07	+	+
Ruminococcus albus	AD2013	A	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Ruminococcus albus	AD2013	A	31	++	++	++	++	++	++	++	++	++	++	++	1.14	++
Ruminococcus albus	AD2013	B	1	+	+	+	-	-	-	-	-	-	+	+	0.85	-
Ruminococcus albus	AD2013	B	31	++	++	++	++	++	++	++	++	-	++	+	1.20	-
Ruminococcus albus	AD2013	C	1	-	-	-	-	-	-	-	+	-	-	-	-	-
Ruminococcus albus	AD2013	C	31	-	+	+	+	+	+	+	+	+	+	+	-	+
Ruminococcus albus	AD2013	D	1	-	+	+	-	-	-	-	-	-	+	+	-	-
Ruminococcus albus	AD2013	D	31	++	++	++	++	++	++	++	++	++	++	++	++	++

Activity was recorded as an average absorbance value at 405 nm. Minor activity was recorded as a + or ++.

In relation to the initial screen (Table 3.7), when comparing activities on *p*NP-conjugated substrates, the candidate strains appeared to be active on the same substrates, although activities varied. A striking exception to this was *B. fibrisolvens* MD2001, which appeared to have been active on α-D glucopyranoside in the initial screen, but not in this assay. Conversely, the original strain was not active on β-D fucopyranoside or α-L fucopyranoside in the initial screen, but the parallel lineages showed activity on these substrates in this assay. As these results were also relevant for strains at subculture 1, this would suggest that the ability to degrade these substrates was present in the ancestor strain used to start the lineages. It is difficult to explain why this occurred, especially without the use of replicates in the initial pilot screen, and it would have been desirable to repeat these assays to confirm these results.

In addition, although candidate strains appeared to be active on the same substrates before and after 31 subcultures on RG, the maximum OD reached for strains after 31 subcultures was much lower than the maximum OD reached after 1 subculture. As the amount of protein used in the assay was normalised for each treatment, it was difficult to explain why this occurred. As each sample taken after 31 subcultures appeared to be slightly darker than samples taken after 1 subculture, it was possible that artefacts from the culturing on RG were present in the treated sample. This may have interfered with the accurate measurement of protein concentrations, which would have therefore resulted in lower specific activities. A simpler possibility could be that the increase in cellulolytic and xylanolytic activity after 31 subcultures on RG was a trade off that resulted in a decrease in the maximum ODs reached. Should this experiment be repeated in the future, a more detailed analysis quantifying the effect of RG degradation in the SN on protein and enzyme activity could be carried out in an attempt to explain such an observation.

It was also worth noting that in this experiment, *R. albus* AD2013 strains showed barely any detectable activity on all substrates, despite being active on four substrates in the initial screen. This was likely due to the lower amount of protein that was used in this assay with samples being diluted to the same concentration of 0.02-0.09 (Table 4.4), compared to a protein concentration of 0.222 mg/mL used in the initial screen (Table 3.3). As the activities in the initial screen were already quite low, it was possible that using a lower concentration of protein in this assay resulted in lower activity that was barely detectable and would not have met the LOQ, although LOQs were not calculated for this assay.

Overall, there appeared to be no apparent improvement in the activities of the evolved lineages on the *p*NP-conjugated substrates, so data from this assay was not taken into account when choosing strains to focus on for proteomic work (Chapter 5).

4.2.4 Candidate lineages selected for proteomic analysis

Based on the biochemical assays which measured the *in vitro* cellulolytic and xylanolytic activity of each lineage, two lineages that appeared to show a good improvement in fibrolytic activity were chosen for proteomic analysis of secreted proteins. *Bacteroides* sp. Ga6A1 lineage C was chosen for proteomic analysis as it showed a good improvement in cellulolytic activity (104%, Table 4.5) and xylanolytic activity (136%, Table 4.6). *Bacteroides* sp. Ga6A1 lineage B had the highest improvement in cellulolytic activity (173%, Table 4.5) and the second highest improvement in xylanolytic activity (90%, Table 4.6). These improvements made it a good candidate to pursue further, but lineage C was chosen instead solely because this lineage was mutagenized, and this could have resulted in some interesting genetic changes that may have impacted the proteome.

R. albus AD2013 lineage C was the other lineage selected for proteomic analysis. It showed an improvement of 104% in cellulolytic activity (Table 4.5) and a 32% increase in xylanolytic activity (Table 4.6), however, it was selected as the second strain for proteomic analysis as it was from a different species, and had one of the largest improvements on both Sigmacell cellulose and OSX compared to the other candidate strains.

4.3 Chapter summary

Mutagenesis in combination with an extended positive selection on fibrous substrates was used in an attempt to obtain rumen bacterial strains with improved fibrolytic activity so that they could potentially form the basis for the development of a feed treatment to be used for NZ dairy cows.

Candidate strains were subcultured daily on RG for a total of 31 subcultures in 4 parallel lineages (A-D) for each of the three candidate strains, and EMS and UV radiation were used

to mutagenise lineages C and D using conditions optimised for each strain (Section 4.2.1.1 and Section 4.2.1.2).

The SN from each lineage after the 1st and the 31st subculture was harvested and analysed for improvements in fibrolytic activity using the same biochemical assays used during the initial screening (Section 3.2.3), except Sigmacell cellulose was used instead of FP in order to increase the sensitivity of the assay. However, the protein samples were not as concentrated as those used in the initial experiments as proteins were harvested from 10 mL of culture instead of 20 mL.

There was no apparent improvement in activity on *p*NP-conjugated substrates after 31 subcultures, with or without mutagenesis, and instead, there appeared to be less activity. It was possible that this could have been a trade off for the improved cellulolytic and xylanolytic activity, however, this data was not used to select strains for proteomic analysis. Based on the cellulolytic and xylanolytic activity assays, *Bacteroides* sp. Ga6A1 lineage C and *R. albus* AD2013 lineage C were selected to pursue further due to their statistically significant increases in fibrolytic activity, their different taxonomies and because both lineages were mutagenised.

It was hypothesized that proteomic analysis would identify protein-level differences that may account for improved fibrolytic activity, whether this was as a result of mutations in fibre-degrading genes, differential gene expression, or at the very least, it would aid in identifying rumen bacterial proteins involved in fibre-degradation, as the identities of such enzymes are unconfirmed for these strains.

Chapter Five: Proteomic Analysis of Candidate Strains with Improved Fibrolytic Activity

5.1 Introduction

Proteins in the SN from *Bacteroides* sp. Ga6A1 lineage C and *R. albus* AD2013 lineage C, both grown on RG, were separated by 1D SDS PAGE. Bands shown in Figure 5.3 were excised, destained, reduced and alkylated. Bands were then subjected to in-gel digestion with trypsin before the peptides were extracted with two washes of 50% methanol made with 0.1% in formic acid and the washes combined. These peptides were then concentrated by Speedvac, separated by Nano-reverse phase liquid chromatography and identified by MS/MS. The results from this data identified the expression of any new proteins, or the complete loss of others, resulting from passaging 31 subcultures of these two strains of bacteria on RG. Potential FEs were also identified based on available genome sequences.

5.2 Results and discussion

5.2.1 SDS PAGE gels

SDS PAGE gels, and in particular 1D SDS PAGE gels, are useful for separating proteins making up a complex sample based on their size. As SDS binds to each protein in a constant weight ratio, smaller proteins travel faster through the gel matrix than larger proteins when voltage is applied. By including an appropriate molecular weight (MW) standard, it is possible to estimate the size of any unknown proteins of interest. In order to analyse the secretomes of *Bacteroides* sp. Ga6A1 lineage C and the *R. albus* AD2013 lineage C, 10 mL of each culture was concentrated to approximately 30 µL using Vivaspin™ ultrafiltration units. They were then washed with 3 mL of 50 mM MES buffer, pH 6.5 (Section 2.1.4.12) to remove the excess salt, and the concentrate was analysed by SDS PAGE (7.5% acrylamide, Section 2.2.6) (Fig 5.1).

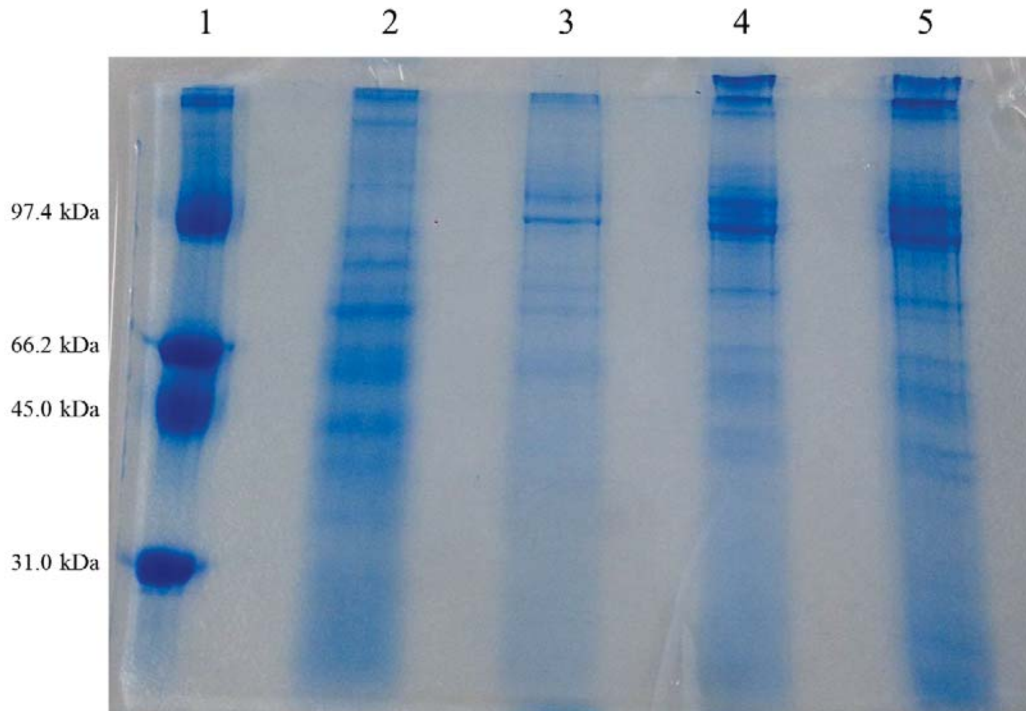

Fig 5.1. 1D SDS PAGE gel of candidate strains before and after treatment. Analysis of SN samples from rumen bacterial strains with improved activity after 1 and 31 subcultures run on a 7.5% SDS-PAGE gel. The gel was stained with colloidal Coomassie (Section 2.2.6.2). Lane 1: Low MW protein ladder (Bio-Rad). Lane 2: SN of *Bacteroides* sp. Ga6A1 lineage C after 1 subculture. Lane 3: SN of *Bacteroides* sp. Ga6A1 lineage C after 31 subcultures. Lane 4: SN of *R. albus* AD2013 lineage C after 1 subculture. Lane 5: SN of *R. albus* AD2013 lineage C after 31 subcultures.

The majority of proteins for each secretome had MWs greater than 45 kD. Although distinct bands were observed, there was still a large smear of protein throughout each lane that could be a result of lipid contamination, high salt concentration, protein aggregation or proteolysis.

Comparing the bands within subculture 1 to subculture 31 for *Bacteroides* sp. Ga6A1 lineage C (Fig 5.1, lane 2 and 3), showed there were obvious differences, specifically the absence of three or four bands between 70 kD and 100 kD, and the presence of two bands around 97 kD. However, as the sample in lane 5 appeared to be less concentrated than its control, it was difficult to be certain about this. There appeared to be no obvious differences between the subculture 1 and subculture 31 of the *R. albus* AD2013 lineage C samples, although in this case, the concentration of the control (Fig 5.1, lane 4) appeared to be slightly lower than that of the passaged sample (Fig 5.1, lane 5). Samples were therefore re-concentrated and the gels

re-run to improve both resolution and loading to allow peptide analysis by mass spectrometry (Fig 5.2).

For the next gel, the last 1 mL aliquot for each treatment, which had been stored at -80°C separately from aliquots used for biochemical analysis, were thawed and concentrated to approximately 50 µL using ultrafiltration (Vivaspin™ 20, 10,000 MW cut-off). Each sample was loaded across two lanes in an attempt to increase the amount of protein available for mass spectrometry (Fig 5.2). The second gel showed bands that were much more distinct and although not perfect, in view of time restraints, it was used for secretome analysis.

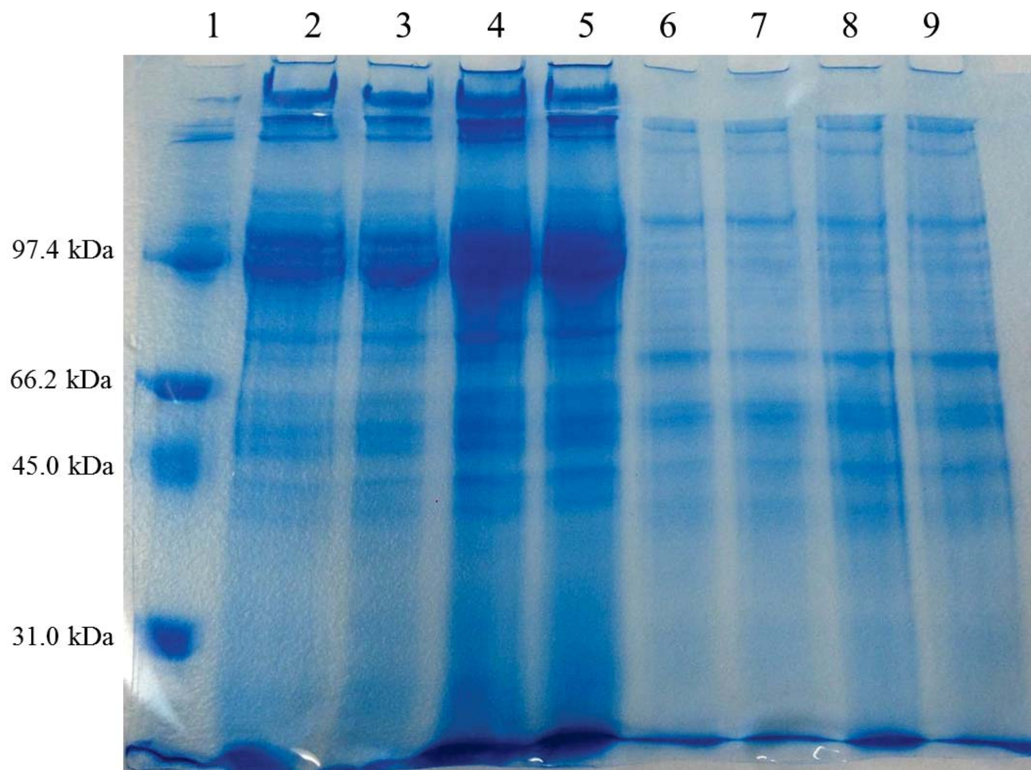

Fig 5.2. Repeat 1D SDS PAGE gel of candidate strains before and after treatment. Analysis of SN samples from rumen bacterial strains with improved activity after 1 and 31 subcultures run on a 7.5% SDS gel and stained with colloidal Coomassie (Section 2.2.6.2). Lane 1: Low MW protein ladder (Bio-Rad). Lanes 2 and 3: Concentrated SN of *R. albus* AD2013 lineage C after 1 subculture. Lanes 4 and 5: Concentrated SN of *R. albus* AD2013 lineage C after 31 subcultures. Lanes 6 and 7: SN of *Bacteroides sp*. Ga6A1 lineage C after 1 subculture. Lanes 8 and 9: SN of *Bacteroides sp*. Ga6A1 lineage C after 31 subcultures.

The protein bands of *Bacteroides* sp. Ga6A1 lineage C and R. *albus* AD2013 lineage C appeared to be similar to what was seen in the first gel (Fig 5.1). However, it was clear that there was a remarkable difference between the secretomes of *Bacteroides sp.* Ga6A1 lineage C taken after 31 subcultures on the first gel (Fig 5.1, lane 3) and that shown in Fig 5.2, (lanes 8 and 9). Whereas the original gel indicated that there may have been a difference in protein expression after 31 subcultures on RG, the second gel showed no obvious differences in protein expression after the same number of subcultures. This was quite puzzling, as the samples used for each gel were taken from the same tube at the same time, and should have displayed similar protein profiles. It was possible that proteolysis could have resulted in the bands of interest being degraded over time, thus changing the band pattern and intensity in the initial gel. An alternative explanation was that the differences between gels were due to sample contamination of the original sample, although this is unlikely. As the 1 mL secretome aliquot used in Fig. 5.2, was from a frozen sample that had not been thawed and used for other tests, it is probable that this sample gave a better representation of the original culture SN. Although it looked as though there were no obvious differences in protein expression for both *R. albus* AD2013 and *Bacteroides* sp. Ga6A1 after 31 sub-cultures on RG, a proteomic analysis of the bands was still carried out, as enzyme assays showed a clear difference.

Should this experiment be repeated in the future, it is paramount that a protease inhibitor is added immediately before processing samples, extra care is taken to avoid contamination of samples, and protein concentrations are normalised at each step in order to highlight differences in protein expression.

5.2.2 In-gel trypsin digest

In an attempt to generate peptide fragments for mass spectrometry, bands were excised from the SDS PAGE gel, as indicated in Fig 5.3, destained with 1x ABC/ 80% acetonitrile, and reduced and alkylated with iodoacetamide before being subjected to in-gel digestion with trypsin (Section 2.2.11.1). Distinct bands present in the SN after 1 subculture (untreated) and 31 subcultures (treated) were processed separately for *Bacteroides* sp. Ga6A1 lineage C and *R. albus* AD2013 lineage C, and protein smears within each lane were pooled for each strain across both treatments to increase the chances of identifying proteins within the smear. The

duplicate lanes were combined and processed as one sample, instead of two, in order to ensure there was enough protein to be detected. In hindsight, this was foolish as it prevented any statistical analysis for reliability of results, or quantitation.

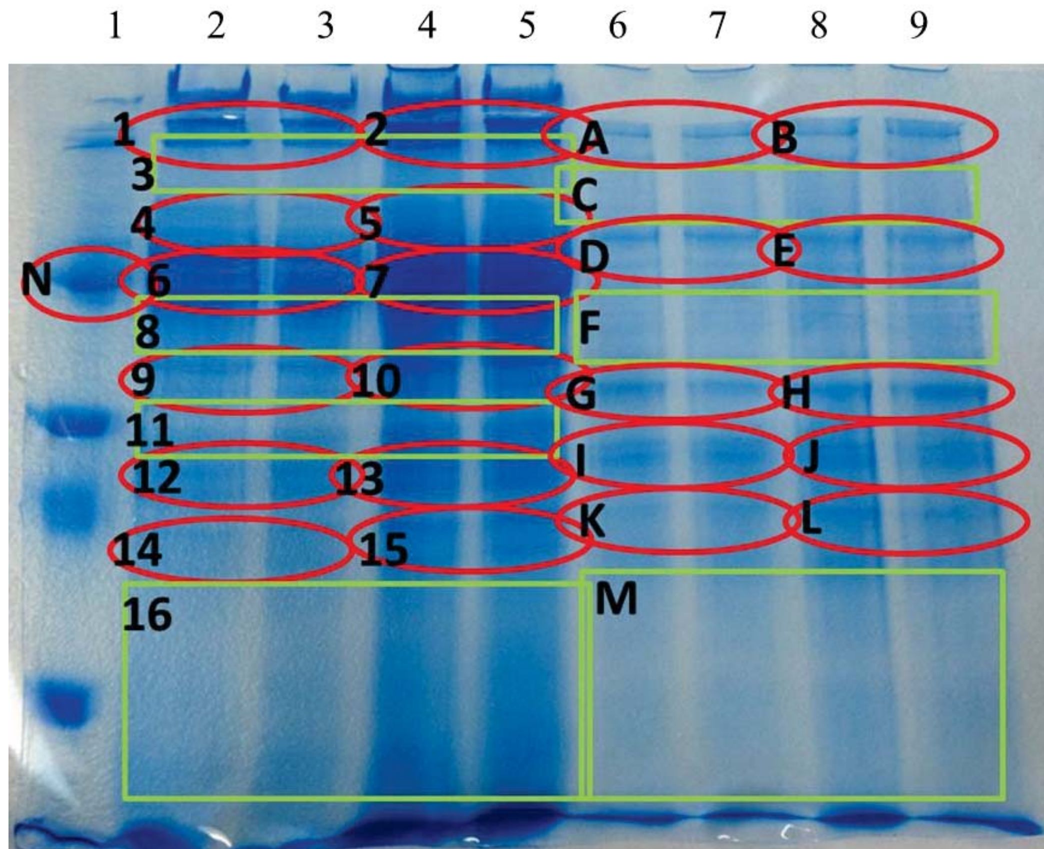

Fig 5.3 Schematic diagram showing the location of samples for analysis and their corresponding codes for identification. The protein markers are the same as in Fig 5.2. Lane 1: Low MW protein ladder (Bio-Rad). Lanes 2 and 3: SN of *R. albus* AD2013 lineage C after 1 subculture. Lanes 4 and 5: SN of *R. albus* AD2013 lineage C after 31 subcultures. Lanes 6 and 7: SN of *Bacteroides* sp. Ga6A1 lineage C after 1 subculture. Lanes 8 and 9: SN of *Bacteroides* sp. Ga6A1 lineage C after 31 subcultures. Codes 1-16 and A-N correspond to the codes used to identify the samples for further analysis.

5.2.3 Identification of FEs using mass spectrometry

Samples were run on the Q-Exactive plus mass spectrometer, (Thermo Fisher scientific, Bremen, Germany) at Massey University by a research technician (Section 2.2.11.2 and

Section 2.2.11.3). The analysis was done by categorising the bands shown in Figure 5.3 into one of 3 categories: a) no visible bands, which describes the smear of protein, b) untreated, which included samples with distinct protein bands taken after 1 subculture on RG, or c) treated, which included samples with distinct protein bands taken after 31 subcultures on RG (Table 5.1 and Table 5.2). Although this was straightforward for *R. albus* AD2013 samples, it was slightly difficult for *Bacteroides* sp. Ga6A1 samples, as "Sample D" was destroyed during the in-gel digestion with trypsin.

Table 5.1. List of 1D SDS PAGE IDs (Fig 5.3) for *Bacteroides* sp. Ga6A1, and the corresponding categories and IDs assigned for analysis using mass spectrometry.

1D SDS PAGE gel ID	Category	Mass range (kD)	Mass spectrometry ID
A	Untreated	>97	Sample 1
B	Treated	>97	Sample 2
C	No visible bands	>97	Sample 3
D	Not processed	>97	Not processed
E	Treated	>97	Sample 4
F	No visible bands	66-97	Sample 5
G	Untreated	66-97	Sample 6
H	Treated	66-97	Sample 7
I	Untreated	45-66	Sample 8
J	Treated	45-66	Sample 9
K	Untreated	31-45	Sample 10
L	Treated	31-45	Sample 11
M	No visible bands	31-45	Sample 12
N	Not analysed	~97	Standard

Table 5.2. List of 1D SDS PAGE IDs (Fig 5.3) for *R. albus* AD2013, and the corresponding categories and IDs assigned for analysis using mass spectrometry.

1D SDS PAGE gel ID	Category	Mass range (kD)	Mass spectrometry ID
1	Untreated	>97	Sample 1
2	Treated	>97	Sample 2

3	No visible bands	>97	Sample 3
4	Untreated	>97	Sample 4
5	Treated	>97	Sample 5
6	Untreated	>97	Sample 6
7	Treated	>97	Sample 7
8	No visible bands	66-97	Sample 8
9	Untreated	66-97	Sample 9
10	Treated	66-97	Sample 10
11	No visible bands	45-66	Sample 11
12	Untreated	45-66	Sample 12
13	Treated	45-66	Sample 13
14	Untreated	31-45	Sample 14
15	Treated	31-45	Sample 15
16	No visible bands	<45	Sample 16

A large proportion of proteins identified in the data set of each strain were proteins that can be found either within the cell, such as proteins involved in DNA replication or transcription, specifically DNA ligases, DNA gyrases and ribosomal proteins, proteins involved in starch utilisation such as SusC and SusD, or proteins attached to the cell membrane, such as sulfatases. (Appendix 1, CD "Mass spectrometry data"). This suggests that there was a significant amount of cell lysis, probably because the anaerobic cultures were processed aerobically, or because a protease inhibitor was not added to samples immediately before processing. However, some degree of cell lysis will always occur naturally and was to be expected.

For this analysis there was no attempt to analyse whether proteins present in both untreated and treated samples were up or down regulated using spectral counting methods. There were two main reasons for this: firstly, when the analysis was started, the appropriate software was not available, and secondly, there were no replicates. Initially 1,949 proteins were identified in the *R. albus* AD2013 lineage C sample which was reduced to 687 when proteins were selected that had a minimum of two unique peptides and a FDR of less than 1 %. Analysis of the *Bacteroides* sp. Ga6A1 lineage C sample secretome on the other hand initially identified 15,705 proteins, but only 229 with high confidence. This was perhaps not surprising as the intensity of the gel bands for the *Bacteroide*s samples was a lot lower than for the

Ruminococcus samples. Thus, all proteins in both secretomes were identified on the basis of containing at least 2 unique peptides, and with a false discovery rate of less than 1%. In order to reduce these to a more manageable number, they were then sorted manually. Firstly, proteins with carbohydrate hydrolase, protease and peptidase activities that occurred in both the treated and untreated samples were grouped together, as these were expected to be secreted by rumen bacteria and were of interest to this project. Of the remaining proteins, only those with a predicted MW consistent with their MW band on the SDS gel (Fig 5.2) were included in the list, and of these, only proteins that were present in either the untreated or treated samples were kept. Hypothetical proteins were identified, where possible, using BLAST and any PFAM domains identified noted. The resulting protein list was then examined for enzymes that may play a role in fibre degradation or proteolytic activity.

5.2.3.1 Identification of FEs from *Bacteroides* sp. Ga6A1

Of the 15,705 proteins identified in the *Bacteroides* sp. Ga6A1 samples, 229 survived filtering, and pie charts were constructed to illustrate the predicted molecular functions (Fig 5.4), cellular components (Fig 5.5) and biological processes (Fig 5.6) of these proteins.

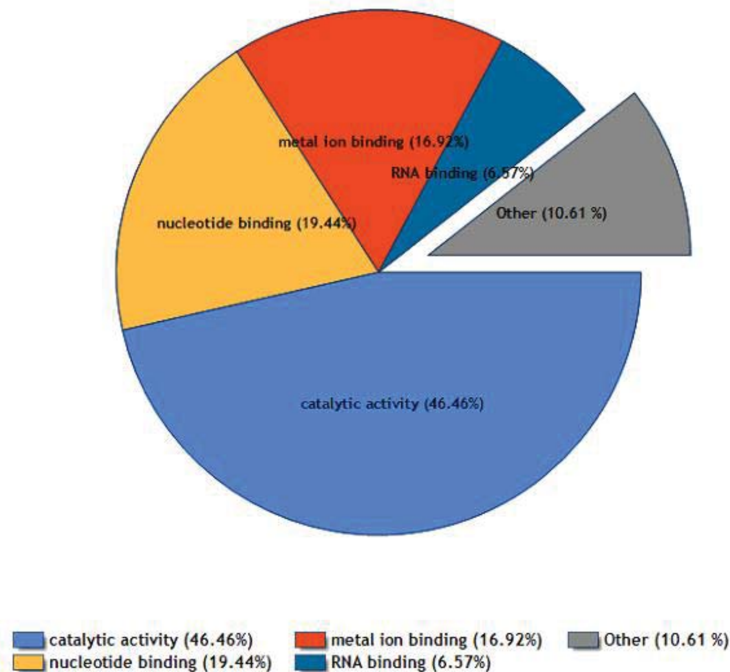

Fig 5.4: The proportion of proteins assigned to various predicted molecular functions for *Bacteroides* sp. Ga6A1 lineage C.

A large proportion of proteins identified in the *Bacteroides* sp. Ga6A1 lineage C samples were predicted to have catalytic activity (46.6%), followed by nucleotide binding (19.4%), metal ion binding (16.9%) and RNA binding (6.6%). The remaining 10.6% had a predicted molecular function described as "other", and it was possible that any FEs expressed by *Bacteroides* sp. Ga6A1 could have been found within this grouping.

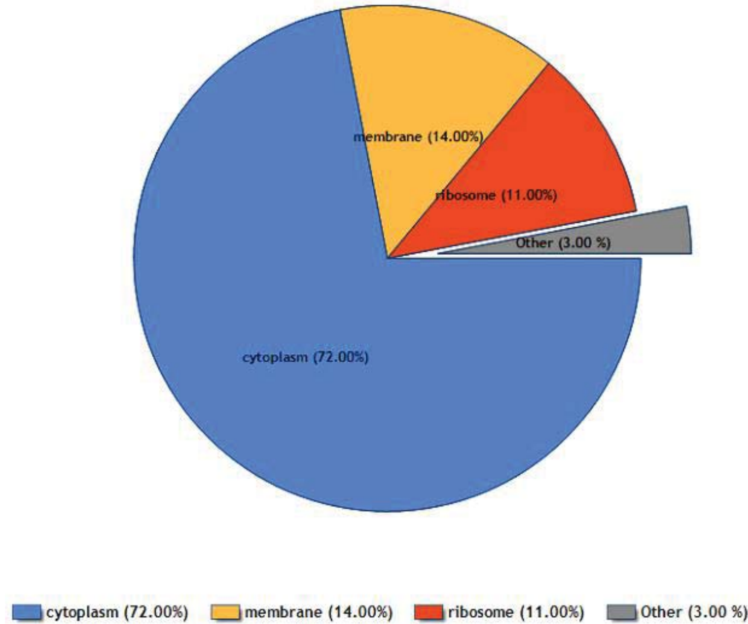

Fig 5.5: The proportion of proteins assigned to various predicted cellular components for *Bacteroides* sp. Ga6A1 lineage C.

The majority of proteins identified in the *Bacteroides* sp. Ga6A1 lineage C samples were predicted to be located in the cytoplasm (72.0%), with the remainder in the cell membrane (14.0%) and ribosome (11.0%). This suggested that secreted enzymes were likely to be a part of the "other" category (3.0%), and FEs could have been present within this category. Although it would seem that less than 3% of the proteins identified in the "other" category may have been FEs that were secreted, this percentage was similar to the amount of genome which is devoted to fibre degradation in *B. proteoclasticus* B316, for example (6%) (19). The large number of proteins identified as being located within the cytoplasm, or associated with the membrane, was consistent with the large degree of proteolysis that was apparent in the SDS PAGE gels (Fig 5.1 and Fig 5.2).

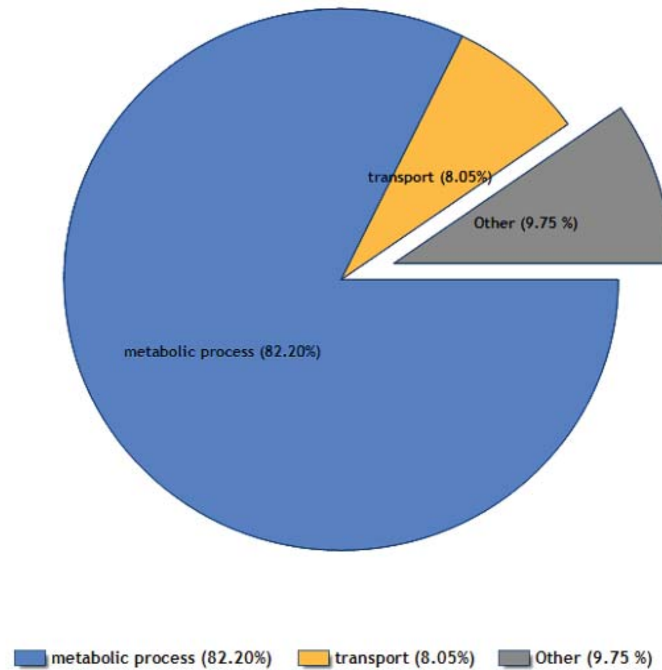

Fig 5.6: The proportion of proteins assigned to various predicted biological processes for *Bacteroides* sp. Ga6A1 lineage C.

Most of the proteins identified in the *Bacteroides* sp. Ga6A1 lineage C samples were predicted to be involved in metabolic processes (82.2%). The rest were divided between those having transport functions (8.1%) and those having "other" functions (9.8%). These "other" proteins could include FEs.

Once the data was filtered to show proteins that were present either before or after treatment, 29 remained. These proteins included Ton B outer membrane proteins, Sus C proteins, tRNA ligases and 30S ribosomal proteins. No proteins involved in fibre degradation were identified. FEs and GHs were present in the original data set, but they contained less than two unique peptides and were subsequently discarded. This result was consistent with the SDS PAGE gels (Fig 5.1 and Fig 5.2) which showed faint protein bands and a protein smear, and this was indicative of proteolysis.

It was surprising that no FEs were detected at all, and it was possible that this was due to limitations within the proteomics experiment. There were a few crucial steps where enzymes, and in particular FEs, could have been lost. As already discussed, as a protease inhibitor was

not added to samples immediately before processing, FEs could have been degraded by proteases during processing as proteases were detected in the secretome.

Another important limitation from this experiment that needs to be addressed was the lack of biological and technical replicates. Biological replicates would require the identification of proteins in the SN from parallel lineages of candidate strains grown on RG for 31 subcultures. Inclusion of biological replicates would increase the confidence in the identification of enzymes found in the secretome, but due to technical difficulties in maintaining these cultures through the number of subcultures required, the number of independent subcultures required for a reliable statistical analysis was not possible within the time constraints of the project. An attempt was made to include technical replicates, albeit only two for each treatment, but these replicates were combined before the in-gel digestion with trypsin to increase the protein concentration of proteins to be identified due to the weak bands on the SDS PAGE gels. These results can therefore only be regarded as a preliminary investigation to see if it was possible to use such methods to successfully identify the enzymes responsible for the increased activity observed.

In addition, it was worth noting that proteomic analysis is only as good as the annotation of the nucleotide databases as seen by the relatively large number of hypothetical or uncharacterised proteins that were identified (4 of the 29 proteins that survived all filtering were uncharacterised). Furthermore, some of the filters were applied manually based on individual judgement, which may have eliminated proteins that may have been present. For example, the MW cut-offs used to filter the proteins detected within each band from the mass spectrometry data were only approximate. This could result in different protein profiles, which would therefore have an impact any conclusions made. Including appropriate biological and technical replicates would have helped mitigate the influence these limitations may have had on the results from this experiment.

5.2.3.2 Identification of FEs from *R. albus* AD2013

Of the 1,949 proteins identified in the *R. albus* AD2013 samples, 687 survived filtering, which was substantially higher than what was seen in *Bacteroides* sp. Ga6A1 lineage C samples. Pie charts were constructed to illustrate the predicted molecular functions (Fig 5.7), cellular components (Fig 5.8) and biological processes (Fig 5.9) of these proteins.

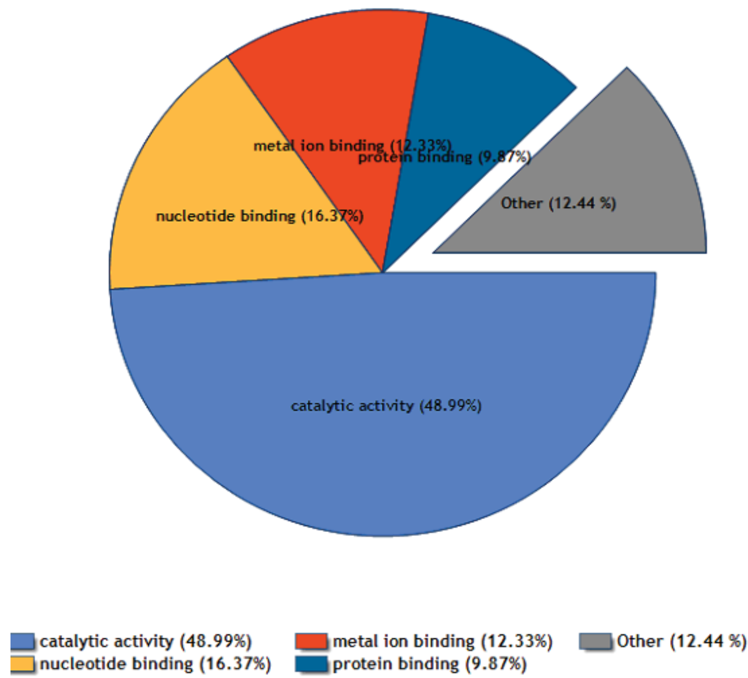

Fig 5.7: The proportion of proteins assigned to various predicted molecular functions for *R. albus* AD2013 lineage C.

In similar proportions to *Bacteroides* sp. Ga6A1 lineage C, proteins were predicted to have catalytic activity (49.0%), nucleotide binding (16.4%), metal ion binding (12.3%) and protein binding (9.9%). The remaining 12.4% appears to have a predicted molecular function described as “other”, and it is likely that any FEs expressed by *R. albus* AD2013 could be found within this grouping.

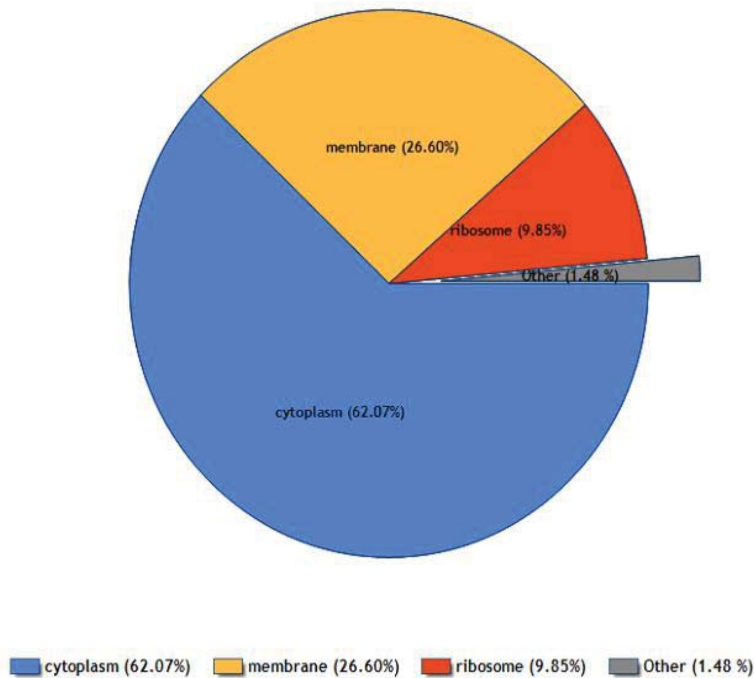

Fig 5.8: The proportion of proteins assigned to various predicted cellular components for *R. albus* AD2013 lineage C.

The majority of proteins identified in the *R. albus* AD2013 lineage C samples were predicted as being cytoplasmic (62.1%), with the remainder identified as membrane (26.6%) and ribosomal proteins (9.9%). This suggests that secreted FEs might have been found in the "other" category (1.5%). These proportions were similar to what was found for the *Bacteroide*s sp. Ga6A1 lineage C and were consistent with the large degree of proteolysis that was apparent in the SDS PAGE gels (Fig 5.1 and Fig 5.2).

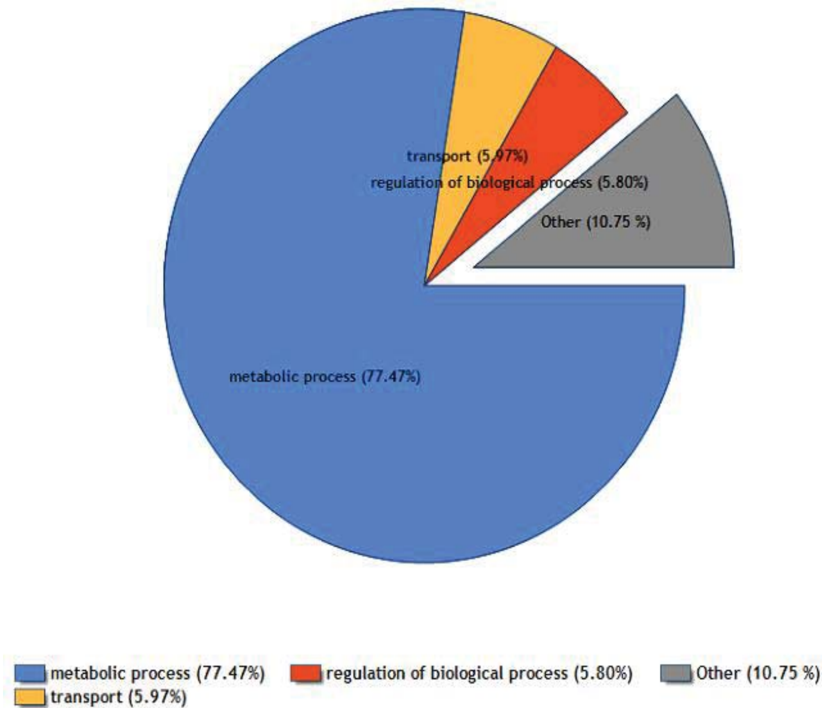

Fig 5.9: The proportion of proteins assigned to various predicted biological processes for *R. albus* AD2013 lineage C.

Most of the proteins identified in the *R. albus* AD2013 lineage C samples were predicted to be involved in metabolic processes (77.5%). A few were identified as being involved in transport (6.0% similar to *Bacteroides* sp. Ga6A1), but also included proteins that were predicted to be involved in the regulation of biological processes (5.8%). This means that approximately 10.8% of proteins were identified as "other", and could include FEs.

Once the data from the *R. albus* AD2013 samples were filtered accordingly, there were only 87 proteins that could be identified either before or after treatment. These included a variety of proteins that are normally located within the cell, including DNA gyrase subunits, sulfatases, phosphotransferases and transcriptional regulators. However, 26 of the proteins identified appeared to be involved in carbohydrate degradation, which included such proteins as GHs, β-xylanases, cellobiosidases, glucuronoxylanases and endoglucanases (Table 5.3) but it was likely that most of these proteins were located within the cell as they were all predicted to be involved in the metabolic process.

Table 5.3. List of potential FEs identified in *R. albus* AD2013 lineage C samples using mass spectrometry.

Protein identified	Accession number	Molecular weight (kD)	Biological process
Cellulose 1,4 β-cellobiosidase	A0A011WK75	97.2	Metabolic process
Cellulose 1,4 β-cellobiosidase	E6UFU2	96.2	Metabolic process
Glycoside hydrolase	A0A011VX14	92.8	Metabolic process
β-xylanase	A0A011WKN5	91.6	Metabolic process
Endo-1,4-β-xylanase	E6UB84	86.2	Metabolic process
α-galactosidase	A0A011V0U3	84.7	Metabolic process
β-glucosidase	E6UA77	84.1	Metabolic process
Glycoside hydrolase family 43	A0A011W0Y0	83.6	Metabolic process
Glycoside hydrolase	A0A011UY44	82.2	Metabolic process
Glucuronoxylanase	A0A011UWH9	79.3	Metabolic process
Glycoside hydrolase family 5	E6UCH0	75.5	Metabolic process
Endoglucanase	A0A011UCD5	75.2	Metabolic process
1,4-β-xylanase	A0A011WPY7	74.6	Metabolic process
β-1,4-endoglucanase	A0A011WPL0	71.6	Metabolic process
Cellulose 1,4 β-cellobiosidase	A0A011V4L2	67.4	Metabolic process
Glycoside hydrolase family 5	A0A011VXC4	67.1	Metabolic process
Glycoside hydrolase	A0A011VSK7	67.0	Metabolic process
Glycoside hydrolase	A0A011VSL4	66.7	Metabolic process
Endoglucanase	A0A011UA54	65.8	Metabolic process

Arabinogalactan endo-β-1,4-galactanase	A0A011WSJ1	59.6	Metabolic process
Glycoside hydrolase family 43	A0A011VYP1	59.6	Metabolic process
α-N-arabinofuranosidase	A0A011UJF8	51.8	Metabolic process
Endoglucanase	A0A011UFY5	45.7	Metabolic process
Endoglucanase	A0A011WP22	45.5	Metabolic process
Endo-1,4-β-xylanase	A0A011VXT8	39.6	Metabolic process
β-glucanase	A0A011WK98	30.1	Metabolic process

Despite this, the range of FEs identified from the *R. albus* AD2013 samples was intriguing, and could partially explain the high amount of activity observed from ruminococci strains in the biochemical assays (Sections 3.2.3 and 4.2.3). β-glucosidases, endoglucanases, GHs, β-glucanases are all important enzymes that act in synergy to break down cellulose, and their detection is consistent with the predicted cellulolytic activity of *R. albus* AD2013 (Table 3.1). Interestingly, β-xylanases and endo-1,4-β-xylanases were also identified, and their expression could explain why *R. albus* AD2013 performed well in the biochemical assay that measured xylanolytic activity, although this does not rule out the presence of multifunctional cellulases that could break down xylan as well.

Bacterial fibrolytic systems usually encode for different versions of the same FEs, so it was possible that a variety of β-xylanases and endoglucanases were identified as some proteins appeared to show up numerous times and in different bands. It was also possible that these proteins could have been degraded into numerous peptide fragments that were dispersed throughout the SDS gel, resulting in fragments of the same protein being detected in different bands throughout the gel. Therefore, it was difficult to make accurate conclusions about the number of FEs detected. However, compared to the lack of FEs identified from *Bacteroides* sp. Ga6A1 samples (Section 5.2.3.1), the repertoire of FEs identified in *R. albus* AD2013 samples, which confers the ability to degrade an extensive variety of plant cell wall linkages, was impressive and may explain why *R. albus* is considered a major player of fibre degradation in the rumen.

As an example, a GH (UniProt accession number A0A011VSL4) was identified that had 3 unique peptides and a coverage of 4.6%. It's predicted MW of 66.7 kD was consistent with the MW of the band it was located in (sample 8: 66-97 kD, Table 5.2), and the protein was present both before and after treatment. As there was no way to measure abundance, it was impossible to detect if it was upregulated in response to 31 subcultures on RG. In retrospect, it was a bad decision to combine the before and after treatment bands for the protein smears, but at the time it seemed like a good idea in order to ensure the adequate concentration of peptides were obtained.

At first glance, it would appear that for *R. albus* AD2013 at least, the proteomic experiment was a success. However, on further analysis, it was extremely difficult to make any accurate conclusions with only the available information. It was also possible that these results could

have been an artefact of the experiment, with the detection or absence of proteins influenced by such factors as proteolysis, low protein concentrations, or cross contamination. Had the appropriate number of technical and biological replicates been used, it would have been possible to disregard such factors. Although a great deal of information was gathered from the first attempt at employing these proteomic methods, it was clear that the experiment should be repeated.

5.3 Chapter summary

Proteomic methods were employed in an attempt to identify the FEs expressed by *Bacteroides* sp. Ga6A1 lineage C and *R. albus* AD2013 lineage C, as these strains were shown to have a significant increase in fibrolytic activity after 31 subcultures on RG (Section 4.2.3). 1D SDS PAGE was used to separate the proteins within the SN samples taken after one subculture on RG (untreated) and after 31 subcultures (treated) for both strains. Protein bands were then removed from the gel in slices and subjected to in-gel digestion with trypsin in order to generate peptides that were identified using mass spectrometry and MS/MS.

The results from the mass spectrometry run detected a variety of FEs that targeted a range of biochemical linkages within the plant cell wall for *R. albus* AD2013 samples, while no FEs were detected in *Bacteroides* sp. Ga6A1 samples. This was not surprising as *R. albus* AD2013 was shown to have higher fibrolytic activity in the biochemical assays (Sections 3.2.3 and 4.2.3). However, it was impossible to explain which FEs may have resulted in the significant increase in activity seen in either strain.

Although it was thought that a lot of protein may have been lost due to the lack of protease inhibitors added before processing the samples, or because low-bind affinity tubes were not used during the in-gel digestion with trypsin, it was clear that the results would have greatly benefited from the inclusion of appropriate biological and technical replicates. With this in mind, it is very important that any future work that specifically employs proteomic methods should have an appropriate number of biological and technical replicates.

Chapter Six: Conclusions and Future Directions

6.1 Conclusions

With the lack of commercially available ruminant feed treatments in NZ, and the potential to develop a feed additive that could be used in a NZ farming context, the use of rumen-derived FEs from rumen bacteria was explored.

This study set out to:

1. Screen a diverse range of plant-adherent rumen bacterial strains that were predicted to be fibrolytic for fibrolytic activities *in vitro*, and identify the top ranked strains.
2. Improve the fibrolytic activity of three rumen bacterial strains using a combination of mutagenesis and artificial selection techniques.
3. Identify the suite of FEs that were expressed from strains with improved fibrolytic activity using proteomic methods, including SDS PAGE and mass spectrometry.

A total of 15 pre-selected rumen bacterial strains were grown on 5 different fibrous substrates in an attempt to induce the expression of FEs (Chapter 3). The fibrolytic activity of the resulting 46 strain/substrate combinations was assessed using two separate biochemical assays; one that measured cellulolytic and xylanolytic activity, and another that identified which biochemical linkages in the plant cell wall could be degraded through the use of artificial substrates.

Bacteroides sp. Ga6A1 possessed the greatest cellulolytic activity and *R. albus* AD2013 possessed the greatest xylanolytic activity as measured by the amount of glucose and xylose equivalents released after incubation on FP and OSX (Section 3.2.3). *B. fibrisolvens* MD2001 stood out amongst the five top ranked strains with activity on six *p*NP-conjugated substrates as it had activity on substrates that were of more relevance to major linkages in the plant cell wall (Section 3.2.3). These three strains were therefore chosen as ideal candidate strains to take forward for improving FE production using a combination of positive selection and mutagenesis.

The three candidate strains were subcultured daily for a total of 31 subcultures in 4 parallel lineages (Chapter 4). Lineages A and B were merely subcultured, whereas lineages C and D were subcultured and mutagenised. Mutagenesis consisted of treatment with 0.5% EMS for 15 min at 37˚C and exposure to UV light at RT for 40 min.

The SN from each lineage after the subculture 1 and the subculture 31 was analysed for improvements in fibrolytic activity using the same biochemical assays used during the initial screening (Section 3.2.3), except Sigmacell cellulose was used instead of FP. *Bacteroides* sp. Ga6A1 lineage C was shown to have an increase in cellulolytic and xylanolytic activity of 104% and 136% respectively, while *R. albus* AD2013 lineage C was shown to have an increase in cellulolytic and xylanolytic activity of 104% and 32% respectively. These two strains were selected to pursue further due to their statistically significant increases in fibrolytic activity, their different taxonomies and because both lineages were mutagenised. There were no obvious improvements in activity on *p*NP-conjugated substrates after 31 subcultures for each lineage, so this data was not used to select two strains for proteomic analysis.

Proteomic methods, specifically 1D SDS PAGE, in-gel digestion with trypsin and mass spectrometry, were employed in an attempt to identify the FEs expressed by *Bacteroides* sp. Ga6A1 lineage C and *R. albus* AD2013 lineage C (Chapter 5). 1D SDS PAGE was used to separate the proteins within the SN samples and in-gel digestion with trypsin was carried out to generate peptides that were identified using mass spectrometry and MS/MS.

The results from the mass spectrometry run detected a variety of FEs that targeted a range of biochemical linkages within the plant cell wall for *R. albus* AD2013 samples, while no FEs were detected in *Bacteroides* sp. Ga6A1 samples. Unfortunately, it was not possible to explain which FEs may have resulted in the significant increase in activity seen in either strain.

Overall, this research contributes to a field still in its infancy in NZ. The ease with which the fibrolytic activity of a variety of rumen bacteria can be stimulated and measured *in vitro* was clearly demonstrated in this study. Initial results from the proteomics experiment also give an indication of the potential variety of FEs that could be identified by rumen bacterial strains. Generating non-GM isolates with improved fibrolytic activity could be used successfully

within a NZ dairy farming context to improve animal productivity, and this will provide financial benefit for both dairy farmers, and NZ's economy.

6.2 Future directions

Bacteroides sp. Ga6A1 lineage C and *R. albus* AD2013 lineage C were shown to have the greatest statistically significant improvement in fibrolytic activity after 31 subcultures on RG in combination with mutagenesis. However, these rumen bacterial strains could be subcultured on RG for a longer period of time in order to further improve fibrolytic activity, and potentially generate observable differences in protein expression not seen in this study. This may, or may not, include repeated rounds of mutagenesis with EMS and UV exposure. It would be important to keep in mind the difficulty of maintaining pure anaerobic lineages in culture medium for an extended period of time as the successful subculturing of strains for 31 days reported in this thesis was only achieved after trouble-shooting persistent contamination issues.

Additionally, the SNs and/ or CPs generated from these strains with improved activity could be tested in a rumen *in vitro* experiment, which would determine the suitability of each strain to be used as a feed additive. This experiment would involve pre-treating forage with the processed SNs and/ or CPs fraction from each strain, and then measuring the gas production and fatty acid release after the addition of fresh rumen fluid over 24 or 48 hrs. However, methods to generate higher concentrations of protein would need to be investigated first.

As already mentioned, this project serves as a foundation for the use of FEs derived from rumen bacterial strains to be used potentially as feed additives, so another route of exploration could involve determining the fibrolytic potential of new rumen bacteria not assayed in this study in the hopes of finding even better fibre degraders. The fibrolytic capacity of rumen fungi should also be considered, as literature has shown that there are potent fibre degraders within this group and FEs produced from rumen fungi should also be suited to the anaerobic conditions of the rumen.

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