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Cultivation and community
composition analysis of plant-adherent
rumen bacteria

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ABSTRACT

Ruminants have a symbiotic relationship with the complex community of microbes that reside within their rumen. These microbes are able to break down recalcitrant plant material that would otherwise be indigestible by the host. Ruminal bacteria that attach to the ingested plant material are important for the degradation of plant fibre. The number of bacteria cultured from the rumen is estimated to represent only some 10% of the total diversity. This has led to the belief that a large proportion of bacteria in the rumen are unculturable. In this study, liquid media that mimic the physico-chemical composition of the rumen, were used in combination with dilution to a single cell, to obtain >1000 cultures of anaerobic bacteria from the plant-adherent fraction of bovine rumen contents from 20 rumen samples. The phylogenetic affiliation of 828 of these cultures was assessed by comparative analysis of partial 16S rRNA gene sequences. There were 626 unique sequence types (V1-V3 of the 16S rRNA gene), and 200 of these isolates were novel (<96% similarity to a previously-cultured bacterium). The near full-length 16S rRNA gene sequences from 186 selected isolates (representing 801 of the total sequenced isolates) were classified into 14 families including two potentially new families, 77 genera including 59 potentially new genera, and 122 species including 103 potentially new species.

The total bacterial communities in the same rumen samples were characterised using FLX-titanium 16S rRNA gene amplicon pyrosequencing of the V1-V3 region of the 16S rRNA gene. These data were then compared with the isolates that had been cultured. The majority of the isolates and amplicon sequences were associated with the phyla Firmicutes and Bacteroidetes. Sequences were grouped into operational taxonomic units (OTUs) at 96% sequence similarity. At this level, 32% of the plant adherent community (i.e., total pyrosequences) and 7.7% of the observed diversity (i.e., unique OTUs) were in OTUs that contained a newly-cultivated isolate. More OTUs (169) contained a sequence from an isolate cultured for the first time in this study compared to the number of OTUs (103) that contained sequences from previously-isolated bacteria. The isolates gained in this study can begin to bridge the gap between the cultured and the uncultured.

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NON-STANDARD ABBREVIATIONS

BLASTN	basic local alignment sequence tool (nucleotide)
bp	base pairs
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
h	hour
Ltd	Limited
M	molar
min	minutes
m/s	meters per second
ML	maximum likelihood
MPN	most probable number
NJ	Neighbor Joining
OTU	operational taxonomic unit
PCR	polymerase chain reaction
QIIME	quantitative insight into microbial ecology
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
U	unit
UV	ultraviolet
VFA	volatile fatty acid
s	seconds
SDS	sodium dodecyl sulfate
L	litre
mL	millilitre
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-indolyl- β -D-galactopyranoside
nt	nucleotide(s)

Chapter 1 Literature review

1.1 Introduction – importance of agriculture to New Zealand

Over 41% of land in New Zealand is used for farming (FAOSTAT, 2012), and agricultural exports are key to the New Zealand economy. The main products come from farmed ruminants: sheep, cattle, deer and goats. In the year ending in March 2013, exports of ruminant products, which include meat, dairy and wool, were valued at NZ\$ 19 billion. This represented more than 40% of New Zealand's total exports (Statistics New Zealand, 2013).

New Zealand farming systems are predominantly pasture based whereas in many parts of the world animals are fed concentrate diets and live for at least part of the year in restricted accommodation. As of June 2012 there were 3.7 million beef cattle, 6.4 million dairy cattle and 31.3 million sheep (Statistics New Zealand, 2012). The combined national herd of dairy cows alone consumes more than half a million tonnes of fresh pasture each day. Typical New Zealand farm pasture is dominated by a mix of ryegrass and clover species, which contain a large proportion of fibre (Holden *et al.*, 1994; Kusmartono *et al.*, 1997; Waghorn *et al.*, 2004). Ruminant animals, like all higher eukaryotes, do not produce the enzymes necessary for degrading plant fibre. They are only successful at using forage plants as a food source because of the complex microbiota residing within the rumen, the forestomach modification that is the site of microbial fermentation of the forage before it enters the proper mammalian digestive system. Thus, the economic prosperity of New Zealand relies heavily on the microbial processes occurring in the rumen. Study of the rumen and its microbes will contribute to better understanding of this important biological system. A better understanding could lead to opportunities to improve the efficiency of the rumen, and thereby improve animal productivity or reduce environmental impacts, which could lead to gains for New Zealand.

1.2 Ruminant animals

Ruminants are considered the dominant herbivores on earth (Hungate, 1975) with a wide geographic distribution across many climates. Ruminants are from the order Artiodactyla and represent close to 200 species. They range in size from 3-6 kg (dik-dik) to more than 1000 kg (buffalo) (Weimer *et al.*, 2009). The majority of the species (and population numbers) are from the families Bovidae and Cervidae (Hackmann *et al.*, 2010). All domesticated ruminants (cattle, goats, sheep, water buffaloes, oxen and yaks) belong to the family Bovidae with the exception of deer, which are from the family Cervidae. The process of microbial fermentation has helped shape the evolution of these animals (Clauss *et al.*, 2010). Ruminants have a unique digestive arrangement that allows microbial fermentation to occur in the foregut.

1.3 Ruminant digestive system

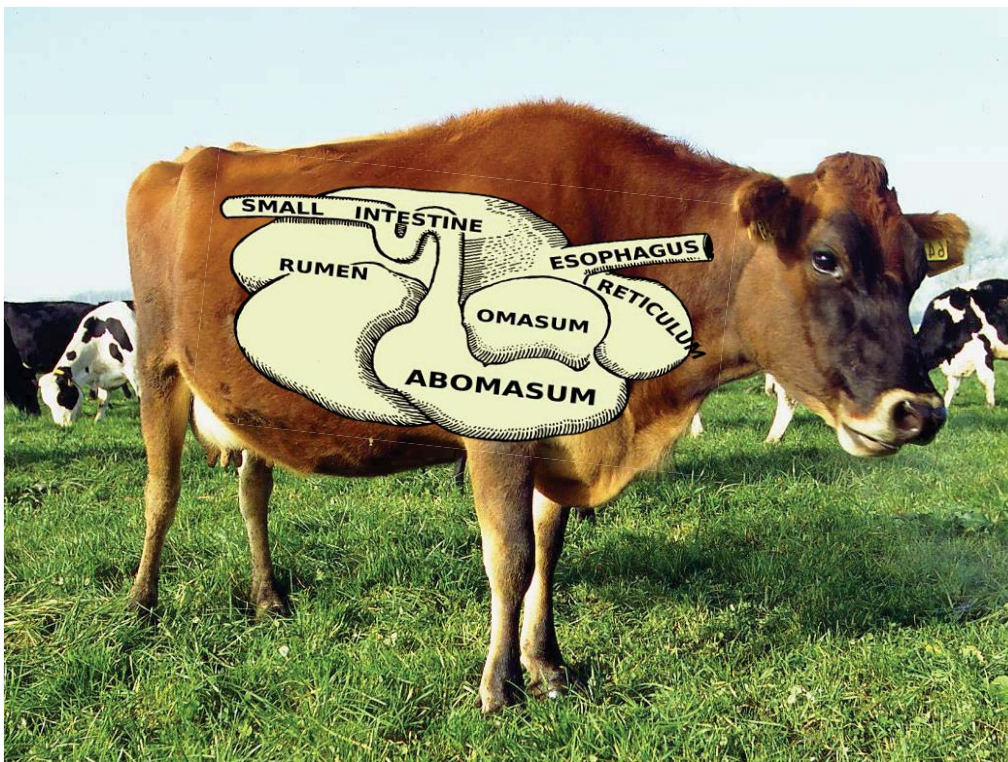


Figure 1-1. The first four compartments of the ruminant digestive system.

The reticulo-rumen (commonly referred to as the rumen) consists of the first two compartments of the so-called four stomachs in ruminants (Figure 1-1) and provides the habitat for a vast number of microbes, estimated at 10^{11} cells per ml. It is a large organ that can account for 12-25% of the live weight of the ruminant (Waghorn, 2002). An adult cow's rumen typically contains 60-120 kg of digesta and that of a sheep 4-8 kg of digesta (Waghorn *et al.*, 2007). The rumen environment is essentially anaerobic, with ~0.5% O₂, ~65% CO₂ and ~35% CH₄ (v/v) in the gas phase (Waghorn *et al.*, 2007). Oxygen introduced with ingested material is quickly metabolised by a small but active population of facultative anaerobes. The rumen has a stable temperature of ~39°C, and digesta flow of 120-200 L per day for cows and 10-30 L per day for sheep (Waghorn *et al.*, 2007). Ruminants normally spend 5-8 h each day eating and 6-10 h each day ruminating, where the ingested feed matter is further reduced in size. Grazing ruminants chew ingested plant material, breaking up fibre and exposing plant cell walls, which allows their fermentation by microbes. The rumen does not secrete any digestive juices, as the epithelial wall contains no glandular cells. Degradation of plant matter is accomplished by the microbes residing in the rumen. The ingested plant material is buffered by copious amounts of saliva, around 70-150 L per day for cows and 10-15 L per day for sheep (Waghorn *et al.*, 2007). Apart from providing fluid, the large amount of saliva produced helps maintain the optimal pH (5.8-6.8) for microbial fermentation (Williams *et al.*, 2001).

Due to its size, the passage of feed is slowed in the rumen, allowing time for the microbes to break down the ingested matter. The time that forage material remains in the rumen depends on the quality of the forage, with leafy new growth taking less time to be broken down by the microbes than stems. Materials in the rumen partition into zones based on their specific density, with gas rising to the top, and freshly eaten and fermenting digesta sitting on top of the fluid phase. Contractions of the rumen constantly mix the digesta, and, as fermentation progresses, plant fragments are reduced to smaller size until they can pass with the liquid phase out of the rumen (Bowen, 2009; Russell *et al.*, 2001; Waghorn *et al.*, 2007). Retention of solid digesta is aided by the omasum which traps large feed particles in the laminae. These large particles are subsequently recirculated back into the rumen. The small particles of digesta and rumen

microbes that pass out of the rumen are digested by the animal in the abomasum, which is the true stomach where peptic digestion occurs and is similar in structure to the stomach in monogastric animals.

1.4 Forage fibre

1.4.1 Forages

Managed pastures used for commercial farming in New Zealand are predominantly mixtures of ryegrass and white clover. The diet of the ruminant becomes the substrate for the microbiota, so the chemical nature of pasture has an important impact on its microbial digestion. The nutritive and chemical value of pasture depends on factors affecting plant growth, such as season, plant variety and stage of plant maturity. Plant matter is made up primarily of fibre (55-70%) and protein (15-22%), with minerals (e.g. potassium and calcium), lipids (e.g., waxes and cell membranes), soluble sugars, nucleic acids, and secondary compounds (e.g., condensed tannins) making up the remainder (Waghorn *et al.*, 2007). Fibre comes from plant cell walls and provides structural rigidity and strength to the plant. The quantity, structure and chemical composition of the fibre differs between plants, within a plant over time, and in different parts of a plant.

1.4.2 Fibre

Plant cell walls are strengthened with fibre, which includes cellulose, hemicelluloses, lignin and pectin. Fibre is a tough matrix of overlapping bundles of cellulose with hemicellulose and pectin (Figure 1-2) bonded together with lignin. The lignin is not degraded to any great extent in the rumen (Balch *et al.*, 1954), but the cellulose, hemicellulose and pectin are degraded by attached microbes. Cellulose and lignin are the most abundant natural polymers on Earth (Pérez *et al.*, 2002). Cellulose is a linear polymer comprised of anhydroglucose units. The structure of native crystalline cellulose is comprised of chains of β -[1,4] linked D-glucose subunits arranged in parallel to form microfibrils (Baker *et al.*, 2000; Cosgrove, 2005).

Hemicelluloses are a group of complex semi-soluble polysaccharides including xyloglucans, xylans and mannans. They are a heterogeneous mix of branched polymers with a backbone that resembles cellulose. They are composed of 5-carbon sugars, such

as xylose, or 6-carbon sugars, including glucose (Wang *et al.*, 1981). Their exact structure varies with plant species. Hemicelluloses are tightly bound to cellulose but do not form microfibrils (Cosgrove, 2005).

Pectins are another heterogeneous group of complex polysaccharides. They include rhamnogalacturonans I and II, homogalacturonan, arabinans, galactans and other polysaccharides. Pectins can covalently link together and can form cross linkages to both cellulose and hemicellulose (Cosgrove, 2005). Both pectins and hemicelluloses can be modified with acetyl and methoxy groups, thus adding to the complexity of fibre.

Lignin is a heterogeneous biopolymer comprised of phenylpropane units in a random 3-dimensional form. It fills the spaces between cellulose, hemicellulose and pectin in the secondary cell walls of plants and forms cross-links strengthening the structure (Iiyama *et al.*, 1994). Lignification occurs in most but not all secondary plant cell walls. The lignins in grasses are bonded by ferulate and diferulate cross-links, *p*-coumarate cyclodimers, and possibly benzyl ester and ether cross-links (Grabber *et al.*, 2004). Lignin is not degraded to any extent by the rumen microbiota and, because of its hydrophobicity and cross-linking to the other cell wall components, it minimizes the accessibility of microbial enzymes to cellulose and hemicellulose. Hence, in general lignin is associated with reduced digestibility of forages.

Nearly the entire range of plant polysaccharides, which includes fibre (cellulose, hemicellulose and pectin), starch and fructans, is able to be hydrolysed by the rumen microbial population. The process of fibre degradation by rumen microbes is complicated and not completely understood, in part due to the complex nature of fibre. The speed at which fibre can be digested is a limiting factor of animal performance (Waghorn, 2002).

As more is learnt about the rumen microbial ecosystem, the possibility of improving the rumen fermentation increases (Krause *et al.*, 2003). Fibre is not completely digested in the rumen. Some fermentable fibre passes through the rumen and is lost in the faeces. Ruminants exhibit natural variation in fermentation efficiency and methane production and some of this variation can be explained by differences in the rumen microbiota (Zhou *et al.*, 2009). Understanding the factors that lead to these differences could pave the way to strategies for improving rumen function.

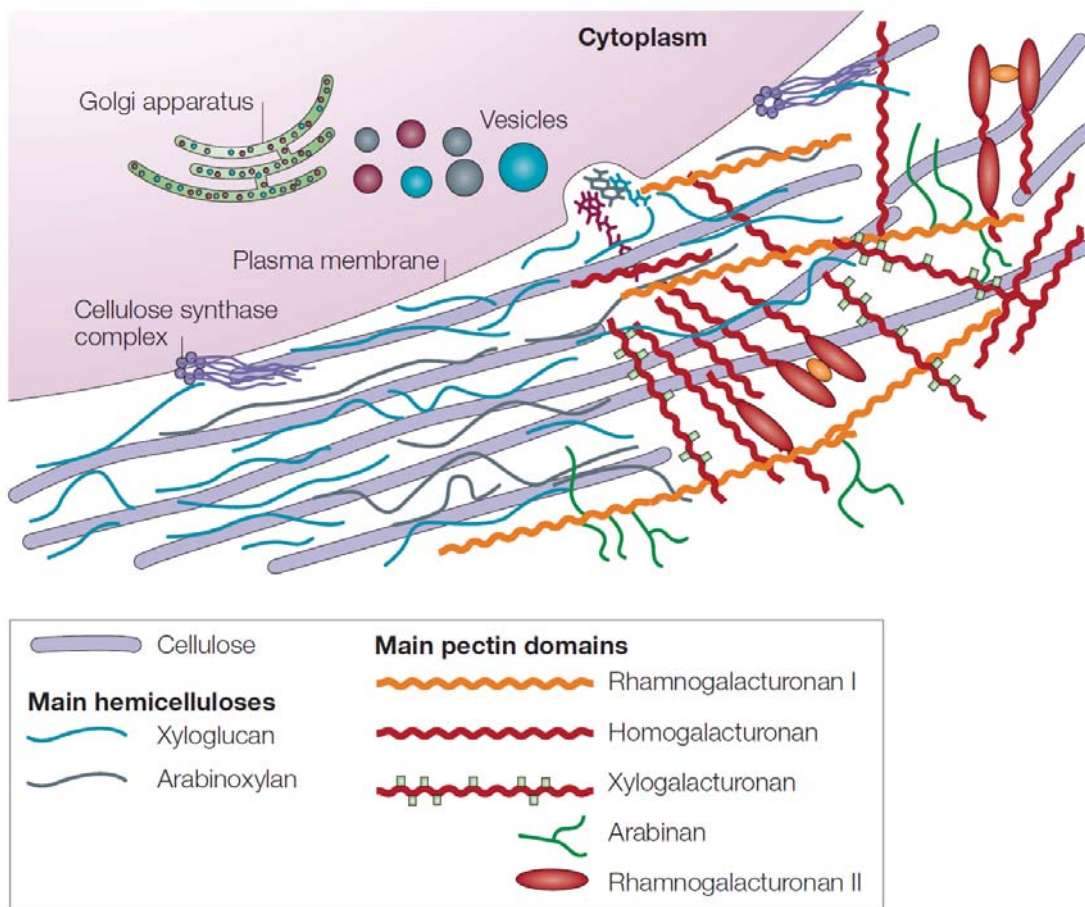


Figure 1-2. Generalized structure of the primary plant cell wall (not lignified). Cellulose microfibrils are shown as purple rods. The hemicellulose–cellulose network is shown on the left part of the cell wall without pectins, which are emphasized on the right part of the figure. The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I (not shown) and rhamnogalacturonan II. Pectin domains are believed to be covalently linked together and to bind to xyloglucan by covalent and non-covalent bonds. The figure is from Cosgrove (2005) with permission.

1.5 Rumen microbiota

The microbiota of the rumen is comprised of bacteria (members of the domain Bacteria; $8-10 \times 10^{10}/\text{ml}$), archaea (members of the domain Archaea; $10^7-10^9/\text{ml}$), and smaller numbers of members of the domain Eukarya; namely protozoa ($10^4-10^6/\text{ml}$), and fungi ($10^4-10^5/\text{ml}$) (Morvan *et al.*, 1996; Waghorn *et al.*, 2007). Most of the microbiota are strictly anaerobic but the small fraction (1000 times fewer) of facultative anaerobes plays an important role, quickly removing any oxygen introduced into the rumen and thereby maintaining the anoxic environment (Hungate, 1975). The anaerobic microbiota starts colonising the digestive tract soon after birth of the ruminant animal (Fonty *et al.*, 1987). Bacteria dominate the microbiota in terms of numbers, but protozoa can account for more than 50% of the microbial biomass (Waghorn *et al.*, 2007). The presence of protozoa is not essential for the functioning of the rumen, and the microbiota of defaunated animals is still able to degrade plant material. Rumen protozoa are predators of bacteria and can cause a loss of nitrogen to the animal by removing bacterial protein. Defaunated animals show increases in bacterial protein leaving the rumen and also decreases in methane production (Jouany, 1996).

Methanogens are the only representatives of archaea present in the rumen and are responsible for the production of the important greenhouse gas methane (Janssen *et al.*, 2008). The dominant mechanism of methane formation is by oxidising H_2 and reducing CO_2 (Whiticar, 1999). Most of the hydrogen produced from fermentation by bacteria, protozoa and fungi is used by these methanogens. This is the major way hydrogen is eliminated and the maintenance of low partial pressure of hydrogen is thought to be essential for the proper functioning of the rumen. Some plant cell wall degrading bacteria are inhibited by high partial pressures of hydrogen (Wolin *et al.*, 1997). Methanogens are often associated with the rumen protozoa, growing attached to their surfaces, thereby explaining the decrease in methane in defaunated animals (Finlay *et al.*, 1994; Krumholz *et al.*, 1983).

Fungi from the rumen were the first strictly anaerobic fungi to be discovered (Orpin *et al.*, 1997). Fungi are more prevalent in pasture-fed animals than in animals on grain-based diets. They may be important for fibre digestion as they can degrade cellulose and pectin, and are even thought to be able solubilise lignin, although they do

not metabolise it. However, it has not been clearly demonstrated that fungi significantly impact ruminal fibre digestion (Akin *et al.*, 1990; Waghorn *et al.*, 2007).

1.6 Rumen bacteria

1.6.1 Rumen bacterial functions

The primary consequence of the activity of the rumen bacteria is the conversion of ingested feed material into bacterial biomass and fermentation end products that can be used by the ruminant. Microbes within the rumen can digest around 70-80% of the fibre associated with consumed forage (Kusmartono *et al.*, 1997). The major fermentation products are short-chain fatty acids (VFAs), such as acetic, propionic, and butyric acids (Figure 1-3), which are taken up across the rumen wall by specific transporters, and microbial cells, H₂ and, CO₂. The amounts of VFAs produced can vary with diet but the main fermentation products are acetic, propionic and butyric acids at approximately a ratio of 60:30:10. There are other products formed by rumen fermentation, but many of these are in turn metabolised and converted to one of the major end products mentioned above by members of the rumen consortia. Most of the lactic acid formed is converted into acetic and propionic acids, and much of the succinic acid formed is converted into propionic acid, allowing neither to accumulate to any extent in the normally-functioning rumen (Hungate, 1975). The rumen microbes grow and exit the rumen into the lower digestive tract, and can serve as a source of protein for the animal. Together, the VFAs and the microbes provide 70-85% of the nutrients absorbed by the ruminants (Bergman, 1990; van Houtert, 1993; Waghorn *et al.*, 2007). The type and quantity of fermentation products produced greatly impact on the growth and development of the ruminant and hence its productivity.

Once established, the rumen microbiota forms a relatively stable community. The prevalence of particular bacterial species can change over time within an animal or may be different between animals or diets, but overall microbial numbers remain relatively constant (Crater *et al.*, 2007). Even though diet, host animal and geographic location have an effect on the species compositions of rumen communities, microbes from different ruminant species from disparate parts of the world and on various diets all produce VFAs, CO₂ and CH₄ as the major fermentation products (Hungate, 1975). This indicates there are similarities in microbial function despite a great deal of variation in specific community members.

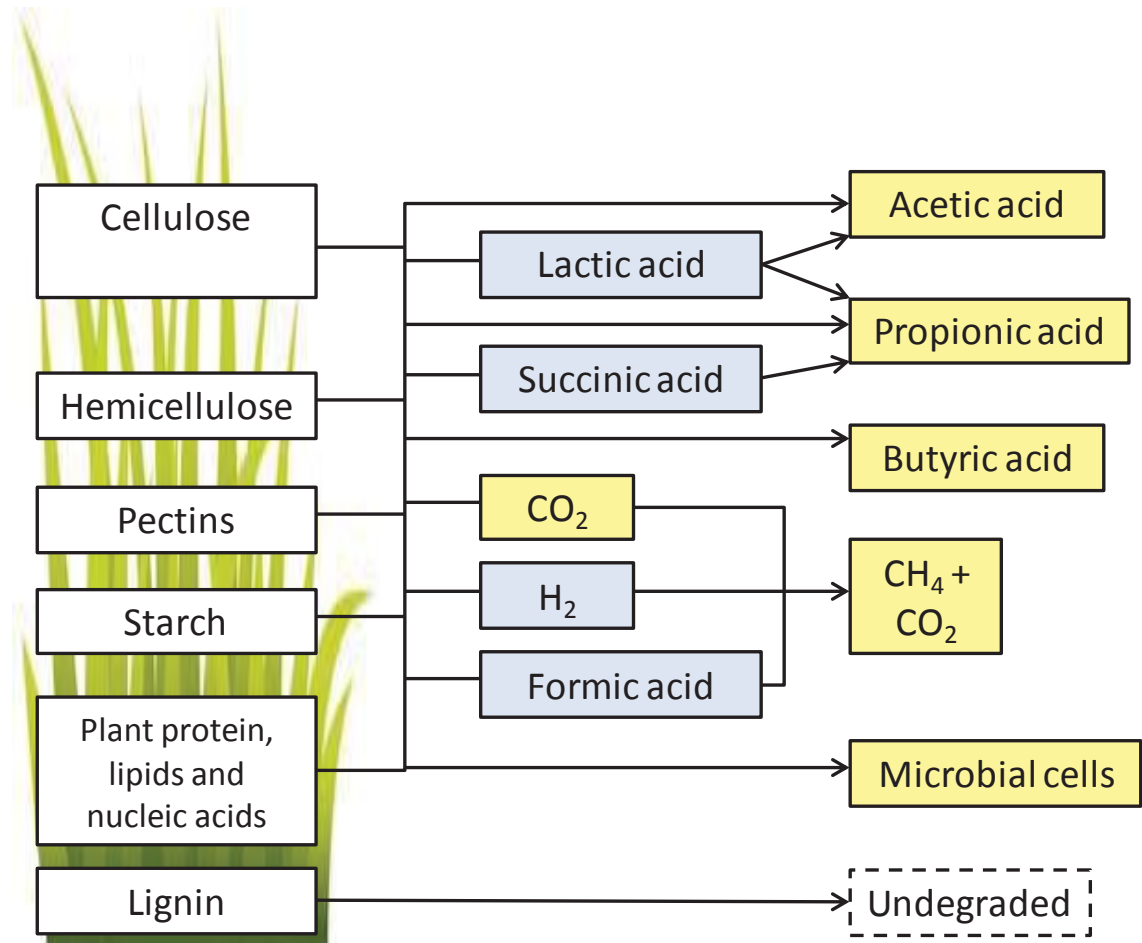


Figure 1-3. General scheme of plant polysaccharide fermentation in the rumen. Products in yellow shaded boxes are final products of ruminal fermentation, while compounds in blue shaded boxes are usually intermediates. The figure is modified from Weimer *et al.* (2009).

Rumen bacteria can be described as belonging to one of several functional groups based on their substrate preference, such as fibrolytic, amylolytic, and proteolytic types, which preferentially digest structural carbohydrates, non-structural carbohydrates, and protein, respectively. Viable counts of cellulolytic, amylolytic and proteolytic bacteria from swamp buffalo rumen contents were respectively 5.1×10^{10} , 1.1×10^9 and 1.0×10^8 colony forming unit per ml (Wanapat *et al.*, 2009). The fermentation end products of the main well-characterised ruminal bacteria are shown in Table 1-1. *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Prevotella* spp. are considered to be the main fibrolytic bacteria in the rumen.

The best-studied cellulose degraders are *F. succinogenes*, *R. flavefaciens* and *R. albus*, while *B. fibrisolvans* and *Prevotella* spp. are active against hemicellulose (Russell *et al.*, 2001; van Houtert, 1993). Pectin is degraded by *F. succinogenes*, *Prevotella ruminicola*, *B. fibrisolvans*, *Eubacterium cellulosolvans*, *Lachnospira multiparus* and *Succinivibrio dextrinosolvans* (Marounek *et al.*, 1999; van Houtert, 1993). Numbers of bacteria affiliated with the family Veillonellaceae increase upon a dietary switch from high roughage to high grain, implicating a role in the degradation of high starch feeds (Tajima *et al.*, 2000).

Table 1-1. The characteristics of the main well characterised ruminal bacteria.

Species	Major substrates ^a	Major products ^a
<i>Anaerovibrio lipolytica</i>	GL, SU	A, S, P
<i>Butyrivibrio fibrisolvans</i>	ST, CE, HC, PC, SU	B, F, A, H ₂
<i>Clostridium aminophilum</i>	AA	A, B
<i>Clostridium sticklandii</i>	AA	A, <i>i</i> , B, P
<i>Eubacterium ruminantium</i>	HC, DX, SU	A, F, B, L
<i>Fibrobacter succinogenes</i>	CU	S, F, A
<i>Lachnospira multiparus</i>	PC, SU	L, A, F, H ₂
<i>Megasphaera elsdenii</i>	L, SU	P, A, B, <i>i</i> , H ₂
<i>Peptostreptococcus anaerobius</i>	AA	A, <i>i</i>
<i>Prevotella</i> spp.	ST, PC, XY, SU	S, A, F, P
<i>Ruminobacter amylophilus</i>	ST	S, F, A, E
<i>Ruminococcus albus</i>	CE, HC	A, F, E, H ₂
<i>Ruminococcus flavefaciens</i>	CE, HC	S, F, A, H ₂
<i>Selenomonas ruminantium</i>	ST, DX, SU, L, S	L, A, P, B, F, H ₂
<i>Streptococcus bovis</i>	ST, SU	L, A, F, E
<i>Succinivibrio dextrinosolvans</i>	PC, DX, SU	S, A, F, L

^a Abbreviations: A, acetate; AA, amino acids; B, butyrate; *i*, branched chain VFAs; CE, cellulose; DX, dextrans; E, ethanol; F, formate; GL, glycerol; H₂, hydrogen; HC, hemicellulose; L, lactate; P, propionate; PC, pectin; ST, starch; SU, sugars; S, succinate; XY, xylan. Data from Russell & Rychlik (2001).

1.6.2 Bacterial diversity

The study of the rumen microbial community often involves assessment of the abundance and diversity of the constituent components, their activities, and the relationships of each of the community members with each other and the ruminant host. Major advances in the field of rumen microbiology have come with the development of techniques to isolate and culture strict anaerobes (Hungate, 1947, 1950; Hungate *et al.*, 1966) and the more recent proliferation of molecular ecological techniques to enable community analysis without need for cultivation in the laboratory. The study of pure cultures has elucidated many of the important metabolic pathways in the rumen, whereas phylogenetic analysis of the 16S rRNA gene has allowed the characterisation of the whole rumen community (Edwards *et al.*, 2004; Kim *et al.*, 2011c; Sirohi *et al.*, 2012).

The number of bacterial species found in the rumen is not known, but probably is >500. An estimate based on a molecular study of a large-scale 16S rRNA gene clone library was 698 species (Sundset *et al.*, 2007). In a combined analysis of published 16S rRNA gene sequences, Edwards *et al.* (2004) estimated 341 OTUs of bacteria within the rumen ecosystem. This outcome was obtained with a conservative 95% gene sequence similarity cut-off, so number of species this represents is not clear. Because species are often delineated at 97% sequence identity (Schloss *et al.*, 2004), this is probably an underestimate. A meta-analysis of the pooled publically-available (in the RDP database) 16S rRNA gene sequences of rumen origin revealed 5271 species-level OTUs (Kim *et al.*, 2011c). Published estimates based on 16S rRNA gene pyrotag analysis are generally higher: around 1000 species (Hess *et al.*, 2011), 1,903 to 2,432 species-level OTUs (Fouts *et al.*, 2012), 4896 species-level OTUs (Jami *et al.*, 2012a), 1027 and 662 species on alfalfa and triticale diets respectively (Kong *et al.*, 2010), and 502-787 on a wheat diet, and 946 - 1641 on a hay diet (Pitta *et al.*, 2009). Apparent differences in bacterial community structure are caused by diet (Kocherginskaya *et al.*, 2001; Pitta *et al.*, 2009; Tajima *et al.*, 2001; Wanapat *et al.*, 2009), geographic area (Ramsak *et al.*, 2000), animal species (An *et al.*, 2005), growth stage (Welkie *et al.*, 2009; Wu *et al.*, 2012), photoperiod (McEwan *et al.*, 2005) heat stress (Tajima *et al.*, 2007; Uyeno *et al.*, 2009)

but not rumen sampling technique (Li *et al.*, 2009; Lodge-Ivey *et al.*, 2009). These factors will add to the large global diversity of the rumen microbiome.

Even though differences in rumen community structure are seen between different conditions and studies, the dominant phyla remain remarkably similar across all ruminants (Table 1-2) and even in the hind gut of some monogastric animals (Yamano *et al.*, 2008). Most studies show Firmicutes as the dominant phylum, followed by Bacteroidetes and only minor contributions from other phyla. Indeed Firmicutes and Bacteroidetes together account for more than 90% of the 16S rRNA gene sequences from gut environments including ruminants, hindgut fermenters and monogastric animals (Flint *et al.*, 2008). Only four studies found that Firmicutes were not the most prevalent phylum, three of those were from animals fed a concentrate diet (An *et al.*, 2005; Jami *et al.*, 2012a; Li *et al.*, 2012a; Pope *et al.*, 2012). It is tempting to speculate the higher level of Bacteroidetes could be a result of the diet. Some studies that have found that the proportion of Bacteroidetes is increased on concentrate based diets (Kocherginskaya *et al.*, 2001; Sadet-Bourgeteau *et al.*, 2010) but Tajima *et al.* (2000) found the proportion of Bacteroidetes is decreased after transition to a corn based diet. A metagenomic study by Brulc *et al.* (2009) highlighted the differences the method used to examine rumen communities can have on apparent community structure. Community composition was examined using next generation sequencing of the metagenome using the 454 pyrosequencing method, 16S rRNA gene clone libraries and environmental gene tags (EGTs) on the same sample. The three methods resulted in apparent contributions of the dominant phylum, Firmicutes, of 55%, 81% and 36%, respectively, to the total rumen bacterial community in one animal.

The remainder of the rumen bacteria are mainly in the phyla Proteobacteria, Fibrobacteres and Spirochaetes. Recent studies using next generation sequencing technologies have found members of Tenericutes, Verrucomicrobia, Synergistetes, Actinobacteria, Chloroflexi, and candidate divisions TM7 and SR1 in low numbers (Kong *et al.*, 2010; Wu *et al.*, 2012). Analysis of the combined deposited 16S rRNA gene sequences has revealed the presence of 19 phyla in the rumen, but it is unclear whether all of these are normal residents of the rumen (Kim *et al.*, 2011c). It has been suggested that the core rumen microbiome contains just eight phyla (Wu *et al.*, 2012).

In the rumen, different bacterial species specialise in degrading different substrates, and therefore the animal's diet has a large influence on the rumen microbiota. Diet has a greater impact on community composition than animals (Sundset *et al.*, 2007) or communities detected in different rumen fractions (Kim *et al.*, 2011b). A study on the ruminal communities of Norwegian reindeer showed a significant difference in the communities between artificial and natural feeding conditions in the same animals but no difference between geographically separate subspecies on different natural diets. In fact, the reindeer populations were even similar to gazelle microbiota from a different continent (Sundset *et al.*, 2007). This demonstrates that the populations within the rumen are remarkably similar in all ruminants throughout the world if they are feeding on natural feed but are markedly disturbed if fed a concentrate diet. The rumen environment is specially adapted and stable for these populations and even though there are species differences there is remarkable similarity at the level of phylum, class and even order. The rumen bacterial community is also resilient, returning to its original composition after being perturbed by external factors (Li *et al.*, 2012a).

The major differences seen in rumen communities are at the genus and species level. Some rumen microbial taxa (below the level of phylum) may be host specific. For example, a study on yak rumen bacterial community structure revealed clusters of 16S rRNA gene sequences that were distinct from those found in other ruminants (An *et al.*, 2005). Analysis of the rumen community of Svalbard reindeer also revealed host specific taxa (Pope *et al.*, 2012). There is evidence for a core rumen microbiome with variation in taxa abundance in animals on the same diet. Bacterial populations examined by automated ribosomal intergenic spacer analysis (ARISA) from 16 cattle shared an average similarity of 75% of OTUs between cow pairs and 19% of the OTUs were present in all the cows (Jami *et al.*, 2012b). A small core set of six OTUs (grouped at 97% similarity) was found in different ruminant species (reindeer and cow) indicating the similarities at the OTU level are very small (Pope *et al.*, 2012).

Members of *Butyrivibrio*, *Prevotella*, poorly classified members of Lachnospiraceae, *Ruminococcus*, *Treponema*, *Fibrobacter*, *Saccharofermentans*, *Succinivibrionium*, *Sporobacter*, *Moryella*, *Anaerovorax* and *Pseudobutyrvibrio* appear in many clone libraries, suggesting they form part of the core rumen microbiome (Kong

et al., 2010; Wu *et al.*, 2012). *Prevotella* was the most abundant rumen genus-level taxon in many studies (Callaway *et al.*, 2010; Edwards *et al.*, 2004; Jami *et al.*, 2012a; Kim *et al.*, 2012; Li *et al.*, 2012a; Pitta *et al.*, 2009; Wu *et al.*, 2012). *Prevotella*'s dominance has been confirmed by quantitative PCR, which showed that *Prevotella* 16S rRNA genes were an order of magnitude more abundant than each of the other main rumen bacteria including *Ruminococcus albus*, *R. flavefaciens*, *Ruminobacter amylophilus*, *F. succinogenes* or *Butyrivibrio*. Many of these are likely to be uncultured *Prevotella* species, as only 0.4-10% of the *Prevotella* gene copies could be attributed to known *Prevotella* species (Bekele *et al.*, 2010; Kim *et al.*, 2012; Stevenson *et al.*, 2007).

Microbes start to colonise the ruminant gastrointestinal tract soon after birth (Fonty *et al.*, 1989; Skillman *et al.*, 2004). Cellulolytic bacteria establish in the rumen a week after birth (Fonty *et al.*, 1987). The microbial community of the developing rumen in calves is different from the fully developed rumen, but the developing calf rumen contains many of the bacterial species needed for the fully developed rumen function (Wu *et al.*, 2012). It appears that the bacteria needed for efficient fermentation of plant material are present in the pre-ruminant animal ready to fill the niche once it becomes available.

The rumen is one of the best studied gastrointestinal microbial ecosystems, and much has been learnt from cultivation of some of its microbes and determination of their functions. Newer molecular biological approaches are providing data that greatly expand upon and yet challenge our current understanding of the rumen and its microbes. It is now evident that microbial diversity is much greater than originally estimated, and that perhaps only 7% of the microbes that actually reside in the rumen have been cultivated (Kim *et al.*, 2011c). Published estimates of the proportion of cultured bacterial diversity are presented in Table 1-3. New diversity is being uncovered with each study (Fouts *et al.*, 2012; Kim *et al.*, 2011b; Pope *et al.*, 2012). Increasing amounts of 16S rRNA gene survey data are being deposited in publically available databanks as sequencing becomes faster and cheaper, yet recent studies are still revealing previously unseen genera, indicating we are still at the early stages of cataloguing the rumen microbiome. Kong *et al.* (2010) classified 32 genera from cattle fed high fibre diets and only eight were in common with the combined 42 genera previously described from rumen clones. All but one clone from three wild African ruminant species plus one

domesticated species from the same location were not similar (<91%) to a known bacterium (Nelson *et al.*, 2003). More massively parallel sequencing efforts with sequences from different species of ruminants on different diets and different geographical locations are needed to understand the full diversity of the rumen. A currently active project, the Global Rumen Census, aims to undertake such a sequencing effort covering a wide range of ruminant species, breeds, feeds, and locations (www.globalrumencensus.org.nz).

Table 1-2. Phylum-level composition of rumen bacterial communities in various ruminants revealed by analysis of 16S rRNA gene libraries.

Study	Country	Source	Phyla represented	
Whitford <i>et al.</i> (1998)	Canada	Holstein cattle (5 animals) rumen fluid	Firmicutes	55.0%
			Bacteroidetes	26.8%
			Proteobacteria	4.8%
		Holstein cattle (5 animals) clarified rumen fluid	Firmicutes	11.1%
			Bacteroidetes	80.0%
			Proteobacteria	7.4%
		Fibrobacteres	1.8%	
Tajima <i>et al.</i> (1999)	Japan	Holstein cow rumen fluid	Firmicutes	52.4%
			Bacteroidetes	38.1%
			Proteobacteria	4.7%
			Spirochaetes	2.4%
			unaffiliated	2.4%
		Holstein cow rumen solids	Firmicutes	71.4%
		Bacteroidetes	26.2%	
		Spirochaetes	2.4%	
Tajima <i>et al.</i> (2000)	Japan	Dry Holstein cows (8) on a high roughage diet	Firmicutes	90.2%
			Bacteroidetes	3.9%
			Proteobacteria	3.9%
			Actinobacteria	2.0%
		Dry Holstein cows (8) in transition between diets	Firmicutes	72.4%
			Bacteroidetes	22.4%
Dry Holstein cows (8) on a grain diet	Actinobacteria	3.4%		
	Firmicutes	95%		
		Bacteroidetes	2.4%	
Koike <i>et al.</i> (2003b)	Japan	Sheep, fibre-associated fraction	Firmicutes	44%
			Bacteroidetes	43%
Edwards <i>et al.</i> (2004)	Many	Ruminants	Firmicutes	54%
			Bacteroidetes	40%
An <i>et al.</i> (2005)	Qinghai-Tibetan plateau china	Yak grazing at high altitudes	Firmicutes	54.12%
			Bacteroidetes	30.93%
			Spirochaetes	4.64%
			Fibrobacteres	3.09%
			Archaea	7.22%
An <i>et al.</i> (2005)	China	Jinnan cattle (3 animals) fed a concentrate diet	Bacteroidetes	45.18%
			Proteobacteria	26.9%
			Firmicutes	22.3%
			Fibrobacteres	3.55%
			Spirochaetes	0.5%
Sundset <i>et al.</i> (2007)	Norway	Svalbard reindeer	Firmicutes	76.7%
			Bacteroidetes	22.5%
Liu <i>et al.</i> (2009)	China	Guangxi buffalo	Firmicutes	56.66%
			Bacteroidetes	36.66%
			Fibrobacteres	3.33%
			Spirochaetes	1.72%

Study	Country	Source	Phyla represented	
Kong <i>et al.</i> (2010)	Canada	Holstein cattle fed alfalfa hay	Firmicutes	48.2%
			Bacteroidetes	45.6%
			Proteobacteria	2.2%
			Fibrobacteres	0.4%
			Tenericutes	0.2%
			Actinobacteria	0.2%
			Chloroflexi	1.2%
	SR1	0.2%		
	Canada	Holstein cattle fed triticale straw	Unclassified	1.2%
			Firmicutes	53.3%
			Bacteroidetes	39.9%
			Proteobacteria	2.3%
			Fibrobacteres	0.2%
			Tenericutes	0.2%
Chloroflexi			0.8%	
TM7	1.0%			
Unclassified	2.3%			
Kim <i>et al.</i> (2011c)	Many	All sequences from rumen origin in RDP (as of Nov 2010)	Firmicutes	57.8%
			Bacteroidetes	26.7%
			Proteobacteria	6.9%
			16 minor phyla	<3%
Kim <i>et al.</i> (2011b)	Not specified	Holstein and Jersey cattle (4 animals)	Firmicutes	69.7%
			Bacteroidetes	10.4%
			Spirochaetes	2.7%
			Proteobacteria	13%
			Verrucomicrobia	0.7%
Unclassified	2.7%			
Jami & Mizrahi (2012a)	Israel	Holstein/Friesian cattle (16 animals) fed 30% roughage and 70% concentrate	Bacteroidetes	51%
			Firmicutes	41.6%
			Proteobacteria	5.5%
			Actinobacteria	0.9%
			Tenericutes	0.7%
Other	0.4%			
Li <i>et al.</i> (2012a)	USA	Holstein cattle fed total mixed rations	Bacteroidetes	70.9%
			Firmicutes	22.2%
			Proteobacteria	3.3%
			Fibrobacteres	1.5%
			Spirochaetes	1.1%
Pope <i>et al.</i> (2012)	Svalbard Archipelago	Svalbard Reindeer	Bacteroidetes	61.0%
			Firmicutes	27.0%
			Chloroflexi	5.0%
			Proteobacteria	0.2%
			TM7	0.6%
			Plantomycetes	0.2%
			Spirochaetes	0.06%
			Actinobacteria	0.02%
			Other	4.0%

Table 1-3. Similarity of rumen bacterial 16S rRNA genes to those of already cultured organisms.

Study	Number of sequences	Percentage of bacterial 16 rRNA gene clones (percentage identity to a known sequence)	Close to a cultured species	Close to a genus with a cultured species	Not close to a cultured representative
Tajima <i>et al.</i> (1999)	84	6% ^a (>98%) ^b	38% (90-98%)	56% (<90%)	
Tajima <i>et al.</i> (2000)	51	2.0% (>97%)	N.D. ^c	N.D.	
	58	31.0% (>97%)	N.D.	N.D.	
	41	2.4% (>97%)	N.D.	N.D.	
Koike <i>et al.</i> (2003b)	91	23.1% (>97%)	35.2% (90-97%)	41.7% (<90%)	
Nelson <i>et al.</i> (2003)	30	N.D.	N.D.	100% (<91%)	
	38	2.6% (>98%)	N.D.	97.4% (<91%)	
	54	N.D.	N.D.	100% (<91%)	
	78	N.D.	N.D.	100% (<91%)	
	52	N.D.	N.D.	100% (<91%)	
Edwards <i>et al.</i> (2004)	NA	N.D.	11% (> 95%)	89% (<95%)	
An <i>et al.</i> (2005)	194	N.D.	N.D.	54.6% (<90%)	
	197	N.D.	N.D.	35.5% (<90%)	
Sundset <i>et al.</i> (2007)	121	7.5% (>97%)	N.D.	N.D.	
Kong <i>et al.</i> (2010)	1014	N.D.	N.D.	58.4% (<97%)	
Kim <i>et al.</i> (2011c)	13,478	6.5% (≥97%)	N.D.	N.D.	

^a Proportion of clones

^b Identity to the sequence of a cultured organism

^c N.D., data not available

1.6.3 Bacterial attachment to feed

Rumen bacteria can be arbitrarily separated into four groups according to their habitat: planktonic (or free living) in the rumen fluid, associated with feed particles, associated with the rumen epithelium, and attached to the surface of protozoa (Koike *et al.*, 2003b; McAllister *et al.*, 1994). Feed particle associated bacteria can be further divided into loosely associated bacteria and tightly adherent bacteria (Larue *et al.*, 2005).

The bacteria that attach to plant material are thought to be primarily responsible for plant fibre digestion. When the fibrolytic bacteria were prevented from adhering to plant fibres, degradation was inhibited, demonstrating adherence is a precursor to significant fibre digestion (Weimer *et al.*, 1993). Adherence to plant matter also helps bacteria evade predation by protozoa and increasing the time spent in the rumen, as the feed particles are retained in the rumen until they are small enough (<2 mm) to exit (Weimer, 1996). Little is known about the mechanism by which rumen bacteria attach to the feed or the order they colonise freshly ingested feed.

Most rumen bacteria have enzymatic activity against a variety of polysaccharides, but only a specialised group can ferment the insoluble plant cells wall components. Switching from a natural diet, with high fibre content, to a comparatively low fibre diet, caused a decrease in numbers of fibrolytic bacteria like *F. succinogenes* and *R. flavefaciens*, xylanolytic bacteria like *E. ruminantium* and an increase in *Streptococcus bovis* and members of the *Selenomonas ruminantium-Mitsuokella multiacida* group (Tajima *et al.*, 2001). *R. flavefaciens* is one of the most studied rumen bacteria and is thought to be one of the main fibre degraders. It is a Gram positive coccus belonging to the order Clostridiales of phylum Firmicutes. *R. flavefaciens* strains are able to degrade various forms of cellulose, including crystalline and amorphous (Krause *et al.*, 1999). Insoluble crystalline cellulose cannot be degraded by a single enzyme and requires the simultaneous binding of multiple enzymes to hydrolyse it (Schwarz, 2001). *R. flavefaciens* produces an extracellular enzyme complex called a cellulosome which consists of cellulolytic enzymes bundled together by scaffoldin proteins. The enzymes are anchored in the complex by their dockerin domains to complementary cohesin modules on the scaffoldin (Flint *et al.*, 2008). The whole complex is anchored to the cell

and also binds to the cellulose substrate. Due to its close proximity to the cellulose, the cell can benefit from the breakdown of cellulose. *F. succinogenes* is another highly cellulolytic bacterium but there is no evidence for the production of cellulosome structures by this organism and it appears to employ a new, as yet undefined, fibre degrading mechanism (Ransom-Jones *et al.*, 2012).

1.6.4 Diversity and activity of fibre-attached bacteria

High quality feeds like spring growth pasture offer a greater nutritional content, as well as being fermented fast, which means that these spend a relatively short period of time in the rumen and allow the animal to eat more as the rumen empties. Late summer pasture growth and tropical grasses have a higher proportion of insoluble fibre, which takes longer for the microbes to ferment and slows the passage of feed in the rumen. The periods of optimal pasture growth are seasonal and brief. In their natural settings, ruminant animals must survive periods on less digestible forages. In nutrient limiting conditions, proportionally less of the feed material is converted into microbial biomass and more into VFAs (Hungate, 1975). The rate at which fibre is degraded is the limiting step in rumen fermentation. It is here where fibre degrading bacteria play a crucial role and specifically the bacteria that adhere to the fibre, as these are thought to be responsible for fibre degradation.

It has been established that there are differences in the species composition of the bacteria that attach to the feed particles compared to those that live free floating in the fluid of the rumen (de Menezes *et al.*, 2011; Kim *et al.*, 2011b; Larue *et al.*, 2005; Stiverson *et al.*, 2011). This difference was noticed before the common use of the 16S rRNA gene to examine community composition. Firmly-attached bacteria were chemically different from liquid associated bacteria but the loosely-attached or fibre associated bacteria were similar to the liquid-associated bacteria (Legay-Carmier *et al.*, 1989). The differences between bacterial populations in the different rumen fractions were reported to be greater than between different animals (Kong *et al.*, 2010). Overall a much greater diversity in the adherent fraction was reported compared with the liquid fraction (Kong *et al.*, 2010; Larue *et al.*, 2005). A greater diversity of bacteria was found on more complex high fibre diets such as C4 perennial grasses (Kong *et al.*, 2010;

Pitta *et al.*, 2009). This seems reasonable, as each bacterial species is able to degrade a somewhat different range of substrates therefore more complex substrates support the growth of more species.

Bacterial communities attached to feed particles have been reported to have higher proportion of Firmicutes to Bacteroidetes than the rumen fluid (Tajima *et al.*, 1999). Fouts *et al.* (2012) found that *Butyrivibrio* and *Blautia*, both Firmicutes, were more commonly found in the fibre adherent fraction. The numbers of attached bacteria were found to reach a maximum one hour after feeding and ~70% of the total rumen bacteria attach to feed particles (Legay-Carmier *et al.*, 1989). Tajima *et al.*, (1999) found sequences from *R. flavefaciens* and *Eubacterium siraeum* associated with rumen solids but not the rumen fluid. *Ruminococcus* was also found in the adherent fraction by Kim *et al.* (2011b), although other members of Ruminococcaceae were also found in the liquid fraction (de Menezes *et al.*, 2011). *R. flavefaciens* adhered to damaged hay fragments specifically along the edges of pits caused by bacterial degradation (Shinkai *et al.*, 2007a).

Member of Fibrobacteres were found to be enriched in the fibre adherent fraction on cattle fed total mixed rations in two pyrosequencing studies (de Menezes *et al.*, 2011; Pitta *et al.*, 2009). Analysis of *Fibrobacter* 16S rRNA gene sequences revealed that this genus falls into four distinct phylogenetic groups (Amann *et al.*, 1992). The attachment of *F. succinogenes* to hay stems was monitored by real time quantitative PCR and visualised by fluorescence *in situ* hybridization (Shinkai *et al.*, 2007a). *F. succinogenes* firmly adhered to the cut edges and the undamaged surfaces of the hay. Members of phylogenetic group 1 were the most prevalent of the *F. succinogenes* groups and a major member of the total plant adherent community. This group appeared to be a primary coloniser as it was detected on hay fragments where few other bacteria were visible. Members of phylogenetic group 2 were seen amongst mixed communities of bacteria adhered to the hay fragments, group 3 was not detected and group 4 was not examined (Shinkai *et al.*, 2007a).

F. succinogenes, *R. flavefaciens* and *R. albus* were quantified by real time quantitative PCR in swamp buffalos (8.0×10^6 , 5.5×10^5 and 2.6×10^4 copies/ml of rumen fluid, respectively) (Wanapat *et al.*, 2009). Stiverson *et al.* (2011) found that *R. flavefaciens* was the most abundant of the three followed by *R. albus* then *F.*

succinogenes ($4.3 - 5.9 \times 10^7$, $3.1 \times 10^7 - 6.5 \times 10^8$ and $2.1 \times 10^4 - 2.7 \times 10^5$, copies/ μg of metagenomic DNA respectively). The higher values were from the adherent fraction from sheep fed hay and the lower were from the liquid fractions of sheep feed corn or hay.

R. flavefaciens, *F. succinogenes* and *R. albus* are thought to be the major fibre degrading bacteria and are also some of the most studied of the rumen bacteria. However, they only account for a small percentage of the rumen community (Michalet-Doreau *et al.*, 2002). Evidence now suggests that groups of uncultured organisms may also play equally important roles in the rumen, but most of what we know about fibre degradation comes from the study of the *R. flavefaciens*, *F. succinogenes* and *R. albus* (Stiverson *et al.*, 2011). Uncultured bacteria were also predicted to have important roles in fibre degradation because several large groups of uncultured bacteria were more abundant in the plant-adherent fraction than in the liquid fraction (Kim *et al.*, 2012).

The fibre fermenting bacteria do not absorb all of the sugars released from carbohydrate hydrolysis, which seems to be disadvantageous for their own growth, but does support the growth of a more complex community via crossfeeding (Krause *et al.*, 2003). This may be advantageous to the total community, allowing the production of more growth factors by the rumen consortia. For example, cellulolytic bacteria need to be able to degrade hemicellulose and pectin linkages to gain access to the cellulose microfibrils, but they do not always use the products, which then become available for other bacteria. A study by Koike *et al.* (2003) looked at 16S rRNA gene libraries generated from bacteria that attached to orchard grass or alfalfa incubated in the rumen. Although around 77% of the clones had no close cultured relative, of those 16S rRNA gene sequences that had greater than 97% match to a cultured bacterium, most belonged to the fibrolytic species *B. fibrisolvans* (57%). The remaining matches were with *P. ruminicola*, *F. succinogenes*, and the non-cellulolytic species *Selenomonas ruminantium*, *Schwartzia succinovorans*, *Succinivibrio dextrinosolvans*, and *Pseudobutyrvibrio ruminis*. This is further evidence that non-cellulolytic species also play a role in fibre degradation, possibly by cross feeding from fibre breakdown products.

Butyrivibrio appears to be an important fibre-attached group. Supporting this idea, *Butyrivibrio* were more abundant in sheep fed hay, a diet with high fibre content,

compared to sheep supplemented with corn (Stiverson *et al.*, 2011). Tajima *et al.* (1999) reported 86% of 16S rRNA gene sequences detected in rumen solids had no close cultured relative (<97% similarity), but 67% of those with a close cultured relative were related to *Butyrivibrio fibrisolvens*. 16S rRNA gene sequences from clone libraries related to *B. fibrisolvens* (>97% similarity) made up 14.2% and 13.2% of the total clones in each library of Tajima *et al.* (1999) and Koike *et al.* (2003) respectively.

Pyrosequencing the adherent and liquid-associated rumen bacterial communities have shed light on the differences of these two niches. Some genera that are abundant in one study were found to be minor components in another. These differences could reflect variations in experimental design or differences in the communities due to diet or animal variation. More studies are needed to understand global differences between adherent and non-adherent rumen communities and identify core members.

1.7 Techniques to identify rumen bacteria - what makes a species

The question of how many bacterial species are there in the rumen can only be answered when there are defined methods for deciding what makes a new species or what belongs to an existing species. The rules for naming bacteria are covered by the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992), but the definition of a species is not standardised among researchers. Indeed, the concept of a bacterial species is still debated (Cohan, 2002; Fraser *et al.*, 2009). The species definition for higher eukaryotes cannot be applied to asexually reproducing prokaryotes. The definition of a species quoted by the *ad hoc* committee for the re-evaluation of the species definition in bacteriology is ‘a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions (Stackebrandt *et al.*, 2002). The *ad hoc* committee for the re-evaluation of the species definition in bacteriology recommended the use of sequencing housekeeping genes, DNA profiling techniques and DNA arrays as promising methods of species determination, but only if they can be verified with DNA/DNA relatedness studies which are still the gold standard for defining new species. Whole genome DNA–DNA hybridisation studies have shown genomes that share 70% or more homology are

nearly always members of the same species as determined by phenotypic analysis (Wayne *et al.*, 1987).

Traditionally, phenotypic approaches were used to identify bacteria based on their physical properties such as morphology, growth characteristics, cell wall composition, carbohydrate utilisation as well as the end products of fermentation. The process of identifying a new isolate was time consuming and required a high level of expertise and was not always accurate. Recent use of molecular markers like the 16S rRNA gene to identify bacteria offers information on the genetic basis of evolution and relatedness. Over time, new molecular techniques have superseded phenotypic methods of classification and, in some cases, demonstrated that phenotype is not always a good indicator of phylogenetic relatedness (Hugenholtz *et al.*, 1998; Krause *et al.*, 1996; Rappé *et al.*, 2003). However, phenotype plus genotype are still necessary to make decisions about the cut off points in sequence data for species delineation (Stackebrandt *et al.*, 2002).

As classification of bacteria moves away from phenotypic characterisation and towards nucleic acid sequence comparisons, many previously-named strains have been renamed. It has become obvious that some genera like *Clostridium*, *Prevotella* and *Butyrivibrio* need to be dissected and reclassified. New bacterial diversity is still being uncovered with each study and the organisation of the taxa very much a work in progress. As the taxonomic framework of known bacteria improves, the ability to accurately identify new isolates by comparisons to the known bacteria is increased. Sequence assignments from datasets missing a representative from one genus can increase error rates of family level assignments by 50-100% (Liu *et al.*, 2008b). Currently, the taxonomic assignments of new isolates will only be as good as the database of reference sequences they are being compared to. This is confounded by conflicting nomenclature present in the literature. Comparisons of 16S rRNA (gene) sequences from multiple curators have found mismatching taxonomic nomenclature even at the phylum level (DeSantis *et al.*, 2006b). The development of curated databases of 16S rRNA gene sequences like Greengenes (DeSantis *et al.*, 2006b; McDonald *et al.*, 2012), the Ribosomal Database Project (RDP) (Cole *et al.*, 2009; Maidak *et al.*, 2001) and SILVA (Pruesse *et al.*, 2007) has improved conflicting taxonomies produced from poor quality data, but introduced new ones. Greengenes provides annotated, aligned and

chimera checked full length 16S rRNA (gene) sequences that are compatible with a number of different analysis formats. The Greengenes classifier outperformed BLAST and the RDP classifier with better coverage and accuracy when tested with three environmental datasets (Liu *et al.*, 2008b). SILVA is a publically-available dataset with quality controlled, aligned rRNA sequences from all three domains of life. Regular updates allow the wealth of new sequences to be included in the ever-expanding dataset (Pruesse *et al.*, 2007). At the time of writing, RDP is on release 10, update 30, and has 2,578,902 aligned and annotated bacterial and archaeal 16S rRNA (gene) sequences.

Removal of chimeric sequences in the databases is important for correct alignment with new sequences and probe design. Approximately 3% of culture independent sequences submitted to NCBI were chimeras and even 0.2% of culture-derived sequences were chimeric (DeSantis *et al.*, 2006b). Another issue is that two thirds of 16S rRNA gene sequences deposited into GenBank are only classified to the domain level, most being from environmental surveys. These sequences can drown BLAST searches, leaving the user none the wiser about the phylogenetic affiliation of the query sequence. The Greengenes database serves to partially correct this, by classifying a higher percentage of the sequences (75% by at least one rank) but it is still not complete. Reconciling the various 16S rRNA (gene) databases will improve taxonomic assignments, as it has been shown the choice of database used has a larger effect on the results than the assignment method (Liu *et al.*, 2008b).

While sequencing technologies have improved at a high rate, bringing down the costs per base and dramatically increasing the throughput, technology to analyse the massive amounts of data created is lagging behind (Sboner *et al.*, 2011). When defining what is in the rumen, a suitable level of relatedness needs to be chosen to answer the questions being asked. At one extreme, the rumen bacteria could be grouped into around eight major phyla (Wu *et al.*, 2012), which does not give enough information about the complexity of the community. At the other extreme, there are approximately $\sim 10^{16}$ bacterial cells in a bovine rumen, which is far too much complexity to be able to analyse (Figure 1-4). Comparing the genomes of every bacterium in the rumen would be the ultimate analysis, but the expense and the technology currently available means this is not feasible and would result in more information than is needed for identification. Data have to be reduced to a certain taxonomic level in order to obtain meaningful and

manageable high level summaries. Cataloguing of full and partial 16S rRNA genes from rumen communities results in thousands of different phlotypes that need to be grouped in operational taxonomic units (OTUs) in order to be analysed. It remains to be decided by the individual researcher at what phylogenetic level it is practical to examine the rumen community.

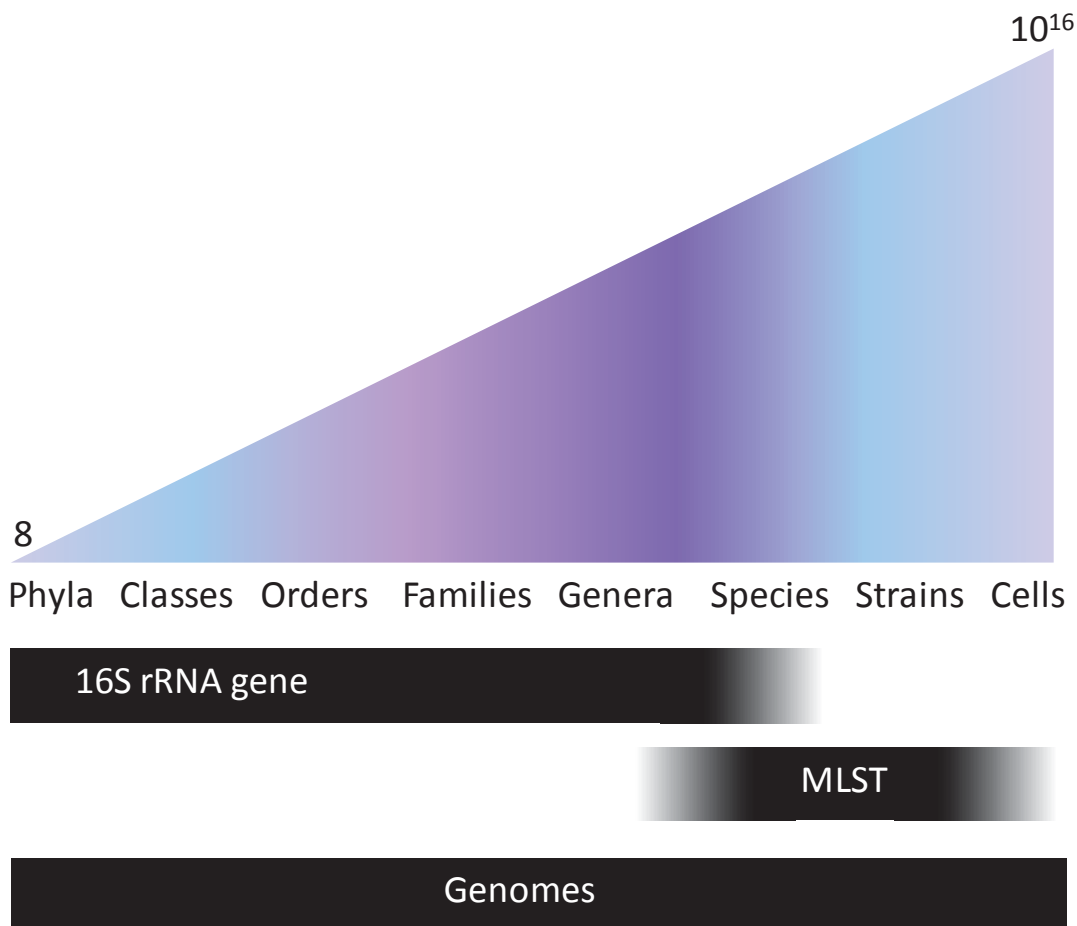


Figure 1-4. Schematic representation of the number of phlotypes of rumen bacteria grouped at various taxonomic levels (indicated by the vertical height of the wedge). The diversity of bacteria present in the rumen could be examined at any level complexity, from the phylum level to each individual bacterial cell. The black bars represent the power each technique has to discriminate differences between bacteria phlotypes at the various levels. The 16S rRNA gene is able to reliably discriminate differences to about the genus or species level. Concatenated sequence from conserved housekeeping genes (multi-locus sequence typing; MLST) can discern differences between species and strains. Analysis of complete genomes or extracted subsets of information (core genomes) cover the complete range of community complexity.

Ecosystem richness predicted from 16S rRNA gene sequences is likely to be an underestimate and depends on the cut-off value selected. Definitions of what is a new species and what is a new genus depend on what arbitrary value of 16S rRNA gene sequence similarity is taken as a cut-off. There are no defined limits for cut-off values, but generally greater than 97% 16S rRNA gene sequence similarity to a previously named bacterium indicates the target sequence is probably from the same species, and greater than 95% similarity indicates the target sequence is from an organism in the same genus (Schloss *et al.*, 2004). The figure of 97% similarity, widely used for demarcating a species, was determined by Stackebrandt & Goebel (1994), who found that strains with greater than 3% 16S rRNA gene sequence divergence were nearly always members of a different species as determined by DNA-DNA hybridisation. In contrast, strains that have less than 3% divergence may or may not be of the same species. Therefore using 97% sequence similarity to estimate species richness from environmental samples will calculate the minimum species number, as species with less than 3% sequence divergence will not be detected with this method. The sequence divergence between different phyla is typically 20-25% (Hugenholtz *et al.*, 1998).

These similarity cut-off values apply when the whole 16S rRNA gene sequence is being compared, but many techniques, especially those that employ next generation sequencing, compare only partial 16S rRNA gene sequence reads. The variability of the 16S rRNA gene is not evenly distributed along its length and the portion of the gene targeted for analysis can affect its taxonomic assignment. Different regions vary greatly in their ability to recapture the taxonomic assignments inferred from the full length 16S rRNA genes (Liu *et al.*, 2008b). By comparing assignments of truncated known sequences with the assignments of their full length counterparts, the best criteria for the use of short 16S rRNA gene reads can be calculated. Liu *et al.* (2008b) found the V2-V3 region most accurately represented the full length 16S rRNA gene sequence when compared by BLAST, both Greengenes and RDP classifiers, and two tree based methods. Longer (446-652 bp) fragments of the 16S rRNA gene, that are more representative of the read length that can be obtained with current next generation sequences, were examined by Kim *et al.* (2011a). Here primers that cover the V1-V4 provided the most accurate estimates, and it was noted that relaxing the cut-off values to 96% similarity also increased the accuracy of the assignment of the short sequences

compared to the full length. Schloss (2010) went one step further and considered many of the parameters used to analyse 16S rRNA gene sequences. Alignment quality, methods used to calculate pairwise differences in sequences, sequence filtering as well as the region of the 16S rRNA gene sampled all affected estimates of α - and β -diversity, leading to the recommendation that pyrotags be considered as markers rather than being directly comparable to species groupings. The α - and β -diversity are commonly sought pieces of information in ecology studies relating to the community structure. The α -diversity refers to the diversity seen in a single community or sample and the β -diversity the differences seen between multiple communities or samples.

Whole genome sequencing of bacteria enables the identification of genes that are conserved across taxa, providing alternatives or additions to the 16S rRNA gene for the identification of bacteria (Jolley *et al.*, 2010). To improve upon the resolution of typing with just one conserved marker gene (e.g. 16S rRNA gene), techniques such as multilocus sequence typing (MLST) have been proposed as an alternative for bacterial identification (Maiden *et al.*, 1998). MLST compares sequence variation at several housekeeping gene loci. However, due to the diversity of bacteria across the domain, each set of MLST targets has to be developed for each particular group of related bacteria, limiting each scheme developed to discerning differences within a genus or even sub-genus group (Maiden, 2006). MLST has been mainly used for clinically important pathogenic bacteria, where accurate typing to the species or strain level is important.

Ribosomal multilocus sequence typing (rMLST) is similar to the MLST approach, comparing sequence variation from the 53 genes that encode bacterial ribosome protein subunits (*rps* genes). These genes are targeted because they are found in all bacteria; are distributed around the bacterial chromosome, and encode proteins which are functionally conserved. This scheme is proposed to resolve bacteria at all taxonomic levels with a higher resolution than the 16S rRNA gene (Jolley *et al.*, 2012). Phylogenies constructed from *Neisseria* species showed the same taxa groupings constructed from the 53 *rps* genes compared to a much more extensive comparison with 246 gene sequences of comparable coding sequences shared amongst the genus *Neisseria* (Bennett *et al.*, 2012). This method required the comparison of 21,398 nucleotide bases and was performed on isolates whose complete genome had already

been sequenced. Although it appears it can be used to accurately type bacteria, the prerequisite of acquiring the large amount of sequence necessary for the comparison rules it out as a quick, efficient method for initial identification.

Comparison of the 16S rRNA gene provides a good starting point for bacterial identification, as taxonomic affiliation, sometimes to the species level, can be obtained provided there are closely related reference sequences. Once a new strain is placed within the framework of known taxa, other methods like DNA-DNA relatedness combined with phenotype can further fine-tune the placement against its closest neighbours. Uncultured bacteria can also be classified with more certainty with full length 16S rRNA gene sequences obtained from primers designed from the partial 16S rRNA gene sequences (Stiverson *et al.*, 2011). In the future, a newly defined species is likely to need a complete genome sequence as part of its formal description (Jones, 2012).

1.8 Molecular ecology techniques to study rumen communities

The rumen bacterial community is very complex and largely comprised of unknown members. The use of molecular ecology techniques answers questions about the nature of these communities *in vivo*. Most of the molecular techniques rely on comparisons made with the 16S rRNA gene from DNA which is extracted directly from the environment. These approaches allow rapid and sensitive analysis of the rumen bacterial communities (Deng *et al.*, 2008; Sirohi *et al.*, 2012). Molecular ecology techniques can be used alone but are often used in combination with each other to offer more insight on community structure and function. Some of the techniques used to study rumen bacterial communities are briefly described in the following sections

1.8.1 Automated ribosomal intergenic spacer analysis

Automated ribosomal intergenic spacer analysis (ARISA) is a high throughput technique utilising the natural variability of the ribosomal intergenic spacer (ITS) region to compare microbial communities. The ITS is amplified by PCR with a fluorescence-labelled forward primer. The fragment sizes are then measured using an automated electrophoresis system resulting in a complex fingerprint profile (Fisher *et al.*, 1999).

ARISA profiles represent the bacterial community composition, with each band representing at least one community member. ARISA was used to determine that the plant adherent and planktonic bacterial communities were different (Larue *et al.*, 2005) and to look at changes in the plant adherent and liquid rumen bacterial populations at different time points after feeding (Welkie *et al.*, 2009). It was also used to examine the effects of animal variation by assessing the similarity of rumen communities in 16 cattle on the same diet (Jami *et al.*, 2012b). It gives an estimate of the degree of similarity between communities, without revealing the identities of the species within it.

1.8.2 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a rapid PCR-based method for studying the structure of microbial communities. It is based upon the differing migration of PCR products in an electrophoresis gel. The V3 region of the 16S rRNA gene is typically used because of its length and species-specific heterogeneity. PCR is performed, with one primer of the pair having the addition of a GC-rich clamp to prevent complete dissociation of the denatured fragments. The PCR products are run on a gel containing a linear gradient of denaturant (urea and formamide) and separated based on their melting behaviour due to their sequence composition (Muyzer *et al.*, 1998). The pattern of bands creates a fingerprint where each band theoretically represents a species (Muyzer *et al.*, 1993). An adaptation of this method is temperature gradient gel electrophoresis (TGGE), which uses a linear temperature gradient instead of a denaturing gradient (Muyzer *et al.*, 1998). McEwan *et al.* (2005) used DGGE to assess the bacterial and ciliate communities in sheep. Similarly Wanapat *et al.* (2009) examined the rumen communities of buffaloes on various diets. Li *et al.* (2009) used DGGE to determine changes in community composition due to sampling procedures and determined that the bacterial community is highly similar among different locations within the rumen and over time. Additional information can be obtained from DGGE analysis by excising and sequencing the individual bands from the gel, as demonstrated by Kocherginskaya *et al.* (2001) when looking at the rumen community of steers fed hay or corn diets. That allowed the identity of bacteria from which individual bands to be estimated. DGGE is particularly useful to quickly compare ruminal bacterial

communities before applying other molecular ecology techniques like clone libraries or pyrosequencing, which are more expensive and time consuming. Kim *et al.* (2011b) used DGGE to visualize the impact that diet and rumen fractions had on rumen communities and then further examined the differences using clone libraries of the 16S rRNA gene. A limitation of this technique is that it intrinsically lacks reproducibility between experiments (band patterns of samples separated on different gels and days), making it hard to make comparison between gels. This is due to influence of minute technique and condition changes on the obtained band pattern and can be due to sample handling, the formation of primer dimers, variable staining or instability due to long run times (Sigler *et al.*, 2004; Sirohi *et al.*, 2012). Moreover, this technique is not able to resolve all of the community members. In general, DGGE will only display the PCR fragments obtained from the predominant species present in the community and therefore will show only major shifts in species composition (Muyzer *et al.*, 1998). Co-migration of PCR products from bacteria with multiple non-identical copies of the 16S rRNA gene can further complicate interpretation of gels.

1.8.3 Real time quantitative PCR

Real-time PCR (also known as quantitative PCR, qPCR, qRT-PCR and RT-qPCR) is commonly used to quantify the abundance of a microorganism or group of related microorganisms in environmental samples. It involves the real time detection of PCR products as amplification occurs, based on a correlation between the time taken to reach a threshold concentration of PCR product and the starting concentration of the target template. The resulting amplification curve can then be compared to those generated by known concentrations of standards to estimate the abundance of the target product in the total microbial community. qPCR has been used to confirm the abundance and population dynamics of species within the rumen (Kim *et al.*, 2012). Wanapat *et al.* (2009) measured the abundance of *R. flavefaciens*, *R. albus* and *F. succinogenes* in swamp buffalos on various diets. Stiverson *et al.* (2011) used this technique to quantify eight cultured bacterial species and five uncultured bacteria. Tajima *et al.* (2001) used real time PCR to study the dynamics of major bacterial populations during dietary transition from hay to grain. Some limitations of this

technique are biases due to PCR efficiencies and the limitation of coverage and specificity due to inherent difficulties in primer design.

1.8.4 Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphisms (TRFLP) allow the fingerprinting of a community by analyzing the polymorphism of a particular gene (usually the 16S rRNA gene). This technique is used for profiling microbial communities based on the variable position of a restriction site closest to a labelled end of the 16S rRNA gene. It involves amplification by PCR of the target gene from DNA extracted from rumen samples, followed by digestion with restriction enzymes. The size of the terminal fragments are detected using a DNA sequencer which generates a profile based on the fragment lengths. TRFLP is high throughput, rapid and gives semi-quantitative information about the diversity of a community. TRFLP has been used to study the effect of subacute ruminal acidosis on the rumen microbiome (Khafipour *et al.*, 2009).

1.8.5 16S rRNA gene clone libraries

Clone libraries of the 16S rRNA gene have been used extensively to catalogue the species present in the rumen. Clone libraries are constructed from PCR amplification of DNA extracted from rumen samples using universal primers targeting all bacteria or specific bacterial groups, followed by cloning into a suitable vector and sequencing. Analysis is performed based on the frequency of unique clone types (OTUs) and their phylogeny. qPCR has shown that the abundance of rRNA gene clones in libraries is not always an accurate measure, overestimating species abundance of a number of groups in hay and corn fed sheep (Stiverson *et al.*, 2011).

Chimeric sequences can be a problem and will show a novel lineage where one does not exist. Chimeric sequences were found in 3% of reads deposited in GenBank from environmental uncultured samples (DeSantis *et al.*, 2006b). Clone libraries have revealed much about the presence and abundance of bacterial genera, but have in part been superseded by next generation sequencing technologies as the number of clones

required to fully describe the bacterial community would make this a time consuming approach. However, clone libraries have the advantage of enabling the near full length 16S rRNA gene to be used for phylogenetic analysis, which has greater discriminating power than the partial sequences obtained from current next generation sequencing platforms. Kim *et al.* (2011b) showed that small clone libraries can still reveal novel species. Currently, knowledge of large groups of as yet uncultured bacteria that can be placed phylogenetically and studied using other methods, like fluorescence *in situ* hybridisation, comes mainly from 16S rRNA gene sequences detected in clone libraries.

1.8.6 Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) can be used to directly visualise and enumerate target bacteria within a mixed community. It involves the hybridisation of a DNA oligonucleotide probe (designed from the 16S rRNA gene) to the ribosome through permeabilized microbial cells (Hugenholtz *et al.*, 2002). The fluorescently-labelled probe can then be visualised or quantified using epifluorescence microscopy, confocal laser scanning microscopy, or flow cytometry. Probes can be designed to be specific for groups of bacteria or even species. FISH was used to visualise populations of *F. succinogenes* and *R. flavefaciens* adherent to hay (Shinkai *et al.*, 2007a). Some limitations of FISH are that it is not as sensitive as PCR based techniques for detecting targets and knowledge of the target organism is required in order to design probes. There are also limitations of coverage and specificity due to inherent difficulties in probe design. Care has to be taken with the hybridisation conditions to ensure that they are not too relaxed, allowing the probes to bind to non-target species, or too stringent, which can result in weak signals.

1.8.7 Pyrosequencing

Pyrosequencing, a form of next generation sequencing, is a high throughput DNA sequencing technique. It is based on monitoring DNA synthesis. When a new base is added, the incorporation creates a bioluminescent signal which is detected. With the addition of oligonucleotide nucleotide barcodes, multiple samples can be analysed in a

single run. Sequencing platforms such as the Roche 454 FLX Titanium are capable of producing 400-600 megabases per 10 hour run allowing sequencing at a massive depth. Pyrosequencing has been used to analyse the variation in rumen communities in animals fed the same diet (Jami *et al.*, 2012a), the effect of adding distillers grain to the rumen community (Callaway *et al.*, 2010), and the effects of changing the diet from bermudagrass to wheat on the fibre adherent and fluid borne communities (Pitta *et al.*, 2009).

Pyrosequencing is a powerful tool for molecular ecologists, allowing the acceleration of “omics” technologies such as metagenomics. The large number of reads allows even minor constituents of the rumen community to be detected. However, as with any technique, there are limitations, and the sequencing error rate becomes a problem in studies of microbial diversity. When single genomes are being sequenced, the redundancy in the consensus assembly can compensate for these sequencing errors. With the sequencing of PCR amplicons of 16S rRNA genes, there is no assembly and each read is treated as a separate entity. Errors in sequence reads (pyrotags) create false diversity. Stringent data cleanup of pyrosequencing is needed to ensure the accuracy of the results. Removing reads with ambiguous bases and abnormal read lengths is not enough to ensure accurate diversity measures (Kunin *et al.*, 2010a). Multiple methods and tools are now available for improving the accuracy and denoising pyrosequencing datasets (Chou *et al.*, 2001; Gobet *et al.*, 2010; Huse *et al.*, 2010; Kunin *et al.*, 2010b; Li *et al.*, 2012b; Quince *et al.*, 2011) Which of these tools to use is up to the individual researcher and can create differences in downstream analysis. Care must be taken when comparing results from different studies.

1.9 Tools for analysing molecularly detected communities

The reduction of price and increase in capacity of sequencing has lead to the increased popularity of culture independent surveys directly from environmental samples using the 16S rRNA gene. A consequence of this is the rapid accumulation of very large amounts of data that need to be analysed. In 2007, approximately 10,000 rRNA sequences were deposited in public databases each month (Pruesse *et al.*, 2007). Many tools for analysing large volumes of 16S rRNA gene sequence data have been developed and most are free to use and open source.

SSuMMo, the Small Subunit Markov Modeler, is based on a database of hidden Markov models, trained with the SILVA reference database (Leach *et al.*, 2012). The QIIME (Quantitative Insights Into Microbial Ecology) pipeline covers all steps of data processing from denoising and demultiplexing raw sequence data to taxonomic and phylogenetic profiles and diversity measures (Caporaso *et al.*, 2010b). Quality control, and many different alignment and sequencing assignment methods are offered allowing users to choose their own settings. The QIIME pipeline uses the updated Greengenes taxonomy to retrain the RDP classifier, a naive Bayesian classifier, for taxonomic assignments. This has been shown to increase classification resolution over the use of RDP for environmental survey sequences (Werner *et al.*, 2012). RDP-II (<http://rdp.cme.msu.edu/>), Greengenes (<http://greengenes.lbl.gov/>) and SILVA (<http://www.arb-silva.de/>) are curated databases of annotated 16S rRNA genes. Each has a suite of online tools for the alignment and classification of 16S rRNA (gene) sequences and probe design features. RDP-II (Wang *et al.*, 2007), Greengenes (DeSantis *et al.*, 2006a) and SILVA (Pruesse *et al.*, 2012) each have their own method for aligning sequences. The ARB software suite was developed for large scale rRNA analysis (Ludwig *et al.*, 2004). Both Greengenes and SILVA are databases that are compatible with the ARB software suite.

1.10 Effect of methodology on community detection

Each step of the process of using 16S rRNA gene fragments for community analysis can potentially have an effect on the final diversity observed. These steps include the way the samples are collected (Rochelle *et al.*, 1994), the DNA extraction method (Wintzingerode *et al.*, 2006; Wu *et al.*, 2010), DNA concentration (Chandler *et al.*, 1997), PCR and cleanup steps (Acinas *et al.*, 2005; Osborne *et al.*, 2005), the choice of primers (Baker *et al.*, 2003; Engelbrektson *et al.*, 2010; Huws *et al.*, 2007), portion or variable regions of 16S rRNA gene sampled (Liu *et al.*, 2008b), sequencing methods (Claesson *et al.*, 2010), library size (Gihring *et al.*, 2012) and the many ways to analyse sequence data (Schloss, 2010). In the end, there is no standard methodology for experimental design and often no theoretical basis for one method being more accurate than another.

Schloss (2010) calculated the phylogenetic diversity from 13 different combinations of variable regions of the 16S rRNA gene using five alignment methods: SILVA, Greengenes, RDP, MUSCLE and pairwise alignment. For alignment quality, SILVA performed the best, followed by Greengenes and then RDP, which produced poor alignment over the variable regions. The datasets from the Greengenes alignment showed the greatest α diversity and the pairwise method the lowest. β -diversity for each alignment was calculated using two OTU-based metrics (Jaccard coefficient and Morisita-Horn coefficient) and two phylogenetic based matrices (weighted and un-weighted UniFrac). The alignment type had a significant impact on the β -diversity but no conclusions could be drawn on the biological relevance of the differences seen with the α - or the β - diversity analyses. The way gaps are treated in sequence alignments accounted for 87%-97% of the variation in the data. Again the alignment type and the gap treatment method had a significant impact on the α - and β -diversity but no conclusions could be drawn on the biological meaningfulness. With a lack of theoretical understanding on how to treat gaps, it is difficult to recommend one method over the other. A Lane mask is often applied to filter the variable regions out from 16S rRNA gene sequences to avoid alignment problems. This reduces the amount of data used for analysis and the observed genetic diversity. Because the sequence information in the variable regions is lost when using these filters, the resulting sequences are more similar to each other and the discriminating power to discern lower level taxonomic grouping is reduced.

There are conflicting results as to which variable regions best represents the whole 16S rRNA gene. Based on UniFrac tests, the phylogenies of some partial length 16S rRNA gene sequences were as good as the full length (Liu *et al.*, 2008b), but regression analysis of pairwise differences showed no partial 16S rRNA gene sequence could represent the full length (Schloss, 2010). Using the SSuMMo method for assigning phylogeny, greater than 70% of sequences were correctly identified from fragments as short as 70 nucleotides (Leach *et al.*, 2012). The RDP classifier was able to correctly classify similar percentages of genera, 76.9% for the V1-3 region, 77.15% for the V3-V5 region and 72.65% for the V6-V9 region (Vilo *et al.*, 2012). In most comparisons, the V6 region was a very poor indicator of full length 16S rRNA gene sequence (Kim *et al.*, 2011a; Liu *et al.*, 2008b; Schloss, 2010). It is desirable to obtain the longest read

lengths possible, as longer regions are better indicators than shorter or single V regions. The V1-4 region accounts for 87% of the variation seen in the full length gene (Schloss, 2010).

1.11 Techniques for culturing bacteria

Isolating rumen bacteria is still of great importance. Newer molecular techniques have established the diversity in the rumen that has escaped cultivation thus far, highlighting the need for renewed efforts in cultivation. Although much can be learnt from community analysis based on examining the 16S rRNA gene or even from metagenomic reconstruction of genomes, much of what we know about rumen function is based upon what has been learnt from isolated cultures. It is especially important to try and isolate representatives of the bacteria that can be detected by molecular means in order to determine their role within the ruminal ecosystem. Validation of predicted activities and interactions are best done using cultured strains. However, the majority of bacteria fail to grow in laboratory conditions. This is typical of many environments, not just the rumen. The development of new cultivation techniques and a better understanding of the rumen environment will potentially help in enabling the isolation of some of these 'unculturable' bacteria.

Originally Hungate designed a method to isolate rumen microbes that employed roll tubes, which are essentially a layer of agar within a sealed atmosphere-controlled vessel (Hungate *et al.*, 1973). The oxidation reduction potential of the rumen is very low (−350 mV), and at this potential the concentration of oxygen is less than 10^{-22} M (Hungate, 1975). To culture anaerobic bacteria from the rumen, oxygen needs to be rigorously eliminated from the growth medium. This is assisted by the use of reducing agents like cysteine and sodium sulfide. Anaerobicity is essential, as oxygen is lethal to many rumen bacteria.

From the roll tube method, other methods have evolved to include the use of anaerobic glove boxes and airtight anaerobic jars where an oxygen-free internal environment can be maintained (Cox *et al.*, 1975). With these, media could be used in agar plate format, which is more convenient for observing and picking bacterial colonies. Isolated bacteria can be grown in anaerobic liquid media in vessels sealed with stoppers that do not permit gas diffusion. Components are transferred into the vessels

with sterile syringes that have been flushed with O₂ free gas (usually N₂ or CO₂) to remove most of the O₂ from the headspace. This enables manipulations to be made under normal laboratory conditions without the need for specialist equipment.

It is difficult to replicate the rumen environment if it is not known which of the parameters are important for bacterial growth. One idea to combat this is to grow bacteria in a diffusion chamber within the natural environment (Kaeberlein *et al.*, 2002). The diffusion chamber method employs a semi permeable barrier that the cells cannot pass through, but nutrient and growth factors can. It has been shown to dramatically increase the recovery rates of microbes from marine and freshwater environments (Bollmann *et al.*, 2007), but has not been attempted for rumen microbes and would be technically difficult.

The dilution to extinction method has been used to rapidly isolate a large number of novel rumen bacteria (Kenters *et al.*, 2011). Here 45% of the cultures obtained were not associated with a known genera (<93% 16S rRNA gene sequence similarity). Button *et al.* (1993) established a theoretical basis for this technique, which involves diluting the sample to be used as the inoculum to the point where it is likely ≤ 1 cultivable cell is being introduced into the culture vessel. This was shown to improve the cultivation success of marine bacteria (Quang *et al.*, 1998). A single cell cultivation strategy was able to isolate a greater diversity of anaerobic oral bacteria than conventional plating or a minitrap technique (Sizova *et al.*, 2012). Goodman *et al.* (2011) used the dilution to extinction technique to isolate thousands of anaerobic bacteria from human fecal samples in 384-well trays.

One advantage of the single cell cultivation strategy is that it eliminates competition from fast growing bacterial 'weeds' that can dominate mixed cultures (Cray *et al.*, 2013). Dilution theory is used to calculate the appropriate dilution point in order to have the highest chance of inoculating with a single viable cell while still obtaining a reasonable number of cultures (Figure 1-5). As the number of culturable cells inoculated increases, so does the number of growth positive tubes in that series. If 100% of the series grows, they are statistically unlikely to be derived from a single cell. Using the calculations from Button *et al.* (1993) to achieve about a 90% incidence of growth-positive cultures that are derived from single cells, the inoculum should contain an expected 0.2 cells. The number of tubes that will be growth positive will be 20% of the

total, and the number of tubes containing cultures derived from a single viable cell will be about 18% of the total.

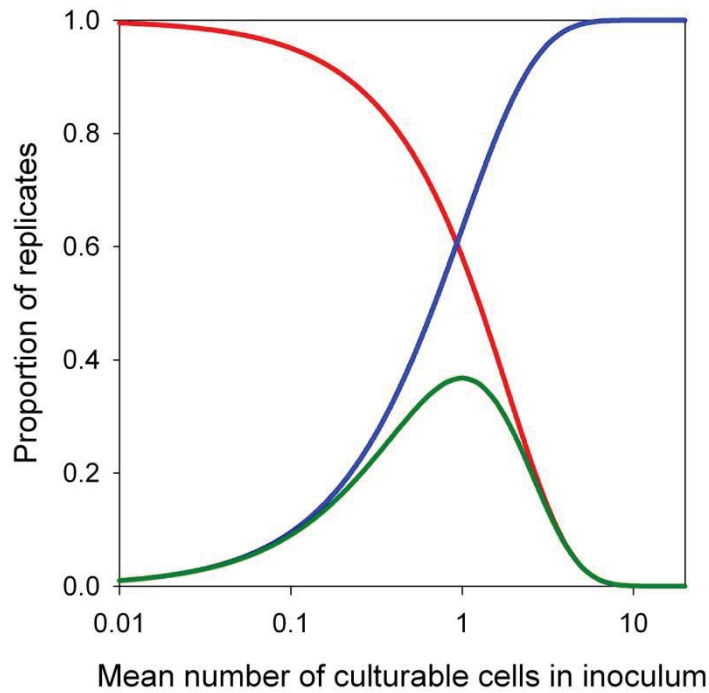


Figure 1-5. The effect of inoculum size on the number of cultures derived from single cells. The blue line represents the proportion of growth positive cultures as a function of the mean number of culturable cells in the inoculum. The red line represents the proportion of positive cultures that will be derived from a single viable cell. The green line represents the calculated proportion of cultures that will be inoculated with a single culturable cell. Modified from Kenters *et al.* (2011).

Recently, high throughput methods to cultivate bacteria have been described. High throughput cultivation is needed to be able to sample the diversity of bacteria present in the rumen. If most rumen bacterial species are present at less than 1% of the population, many cultures will be needed to ‘by chance’ isolate even moderately abundant species. Bruns *et al.* (2003) described a method using a microdrop dispenser system to isolate bacteria in liquid media. A microdrop device creates droplets from the bacterial suspension using a similar process to that of an ink jet printer, depositing the droplets into microtitre plates. This was used on bacteria from freshwater lakes but has never been attempted on anaerobic bacteria. The method described by Zengler *et al.* (2005) is based on the encapsulation of a single cell followed by flow cytometry to sort microcolonies (originated from a single encapsulated cell) into growth media. Diluted cells are mixed with agarose in an emulsion matrix resulting in approximately 10% of formed microcapsules occupied by a single encapsulated cell. Encapsulated cells can be grown together in media or reintroduced into their natural environment. As they cannot escape their capsules, they remain separate yet in close contact with other cells. This allows cross talk between community members, hopefully including signal molecules and growth factors that enable previously unculturable bacteria to grow. Capsules containing microcolonies can then be sorted and grown. This technique has the potential to isolate 10,000 bacteria per sample, but has never been used for culturing rumen bacteria due to the difficulties in maintaining an anoxic environment.

Soil bacteria have been considered to be difficult to culture. However, it has been shown that three factors are significant for isolating many of the so-called unculturable soil bacteria. These are (1) wide separation of cells in the inoculum to avoid negative interactions, which seem to be more important than positive ones, (2) increased incubation time to allow slower growing species to develop to the point when they can be detected, and (3) screening of large numbers of cultures or colonies to allow detection of species that may be abundant in the starting material but may initiate growth under laboratory conditions only rarely (Davis *et al.*, 2005; Davis *et al.*, 2011; Janssen, 2008; Joseph *et al.*, 2003). These approaches could be applied to the rumen to increase the likelihood of isolating part of the missing diversity.

It is desirable to combine molecular biology with microbiology and examine genomes as well as cultured organisms to get a more complete picture of the processes

occurring within the rumen. A modern approach to cultivation is to use knowledge gained from culture independent studies to target the culturing of groups of previously uncultured bacteria. Koike *et al.* (2010) used quantitative real time PCR and FISH to monitor the enrichment of an uncultured group of bacteria designated U2, and specific PCR primers to screen isolates for members of the U2 group. In this way, two isolates were identified (R-25 and B76) of a group that was previously only represented by 16S rRNA gene sequences obtained from culture-independent studies. These organisms are now available for further characterisation and may prove to be an important fibre degrading group in the rumen.

Molecular techniques can also provide clues on how to culture unknown bacteria. For example Bomar *et al.* (2011) used high-throughput sequencing of RNA transcripts (RNA-seq) to characterize the metatranscriptome from the leech gut environment. They determined the dominant uncharacterised organism was utilizing mucin, thus allowing its subsequent cultivation on a medium containing mucin. Pope *et al.* (2011) directed the isolation of a novel succinate-producing bacterium from the wallaby gut using reconstructed bacterial metabolism from metagenomic data. These principles could also be applied to bacteria from the rumen.

New culturing techniques involving microscale cultivation using hollow fibre capillary membranes or microfluidic chips for separating many bacteria in a small volume are being developed (Stewart, 2012) and are promising approaches for the future if they can be adapted to anaerobic environments.

1.12 Medium design

Medium design is a critical aspect of bacterial cultivation. Inability to sufficiently replicate the natural environment in cultivation media is one of the factors leading to the “great plate count anomaly” or the high percentage of uncultured bacterial cells. Sudden changes to the bacteria’s environment can cause them to enter a viable but unculturable state. The first anaerobic rumen media were developed by Hungate (1950). Initial media compositions were dependant on the addition of rumen fluid to add the unknown factors required for the growth of some bacteria. As more was learnt about the nutritional requirements of rumen bacteria, artificial (more defined) media were developed that

were also successful in growing bacteria. However, because the nutritional requirements of previously uncultured bacteria are not known, the requirement for rumen fluid has not been eliminated.

The energy source will have a major impact on which bacteria are cultured. Many rumen bacteria grow with saccharides and polysaccharides. Some degrade amino acids, lactate, or succinate. Some bacteria use a very limited range of substrates as energy sources. For example, *Megasphaera elsdenii* uses lactate and *Ruminobacter amylophilus* only uses starch and its breakdown products (Anderson, 1995).

Ammonia is the major source of nitrogen for bacterial protein synthesis. Values between 3-20 mM resulted in maximal cell growth, although only 3-5 mM were needed in some studies (van Houtert, 1993). Phosphate, though needed for microbial growth, could also be inhibitory to the growth of some bacteria in higher concentrations. A greater proportion of novel bacteria were isolated when the phosphate level was reduced in BM media (Nyonyo *et al.*, 2012). Macro nutrients calcium (Ca), phosphorus (P), sulfur (S), potassium (K), sodium (Na) and magnesium (Mg) are needed for microbial growth. Growth factors that cannot be synthesized by the bacterium are important for the growth of some rumen species. These may include vitamins, amino acids and heme. *Prevotella* spp. require heme for growth unless supplemented with rumen fluid (Avgustin *et al.*, 1997). Many strains of rumen bacteria need VFAs in the growth medium (Hungate, 1975). CO₂ is essential for some bacteria and the pH needs to be similar to that of the rumen, between 6 and 7.

Nutrient sparse media that mimic the natural environment are able to isolate a greater range of bacteria (Zengler *et al.*, 2005). Kenters *et al.* (2011) developed a medium that reflected the concentrations of inorganic components of rumen fluid, which, in combination with dilution theory, was able to isolate previously uncultured species. It was found systematically altering various media components can help isolate a wider range of fastidious anaerobes (Carbonero *et al.*, 2010).

1.13 Cultured bacteria from the rumen

There are close to 100 validly named characterised bacterial species from the rumen (William J. Kelly. personal communication) and perhaps over 200 if

uncharacterised species are included (Kong *et al.*, 2010). The study of these species has formed the basis of much of what is known about the rumen function and activities. Bacteria have been identified that can degrade recalcitrant plant cell wall polysaccharides and their specific mechanism studied in some detail (Flint *et al.*, 2008). Our current understanding of the rumen community is no longer limited by what can be cultured, but there is a need to complement molecular information with functional analyses on cultured species.

Based on recent molecular studies of large-scale 16S rRNA gene libraries, the rumen probably contains >500 bacterial species. However, only a small percentage of these (perhaps as low as 10%) are available as pure cultures to study (Table 1-3). Some of the unknown groups are as large as the groups of well known fibrolytic bacteria like *F. succinogenes*, *R. albus* and *R. flavefaciens* (Kim *et al.*, 2012; Stiverson *et al.*, 2011). Therefore, the microbial community in the rumen is not well represented by the microbes currently in culture and the uncultivated microbes may play important roles in fibre digestion.

Some conspicuous cells observed in rumen fluid have never been cultured. An example of this is the very large cell type termed Quin's oval, which has been named *Quinella ovalis*. These cells (4 μm \times 8 μm) are found in high numbers in sheep fed a high sugar diet, like molasses. Although they have evaded cultivation, their 16S rRNA gene sequence has been determined as well as their pattern of carbohydrate fermentation (Krumholz *et al.*, 1993). This was achieved by separating the large cells by differential centrifugation.

A recent study on human gut microbiota demonstrated that much of the diversity and functionality present in the gut was able to be cultured (Goodman *et al.*, 2011). The bacterial communities in fecal samples were pyrosequenced and anaerobically cultured on agar plates. Any bacteria that had grown after seven days were collectively harvested and pooled to form the 'culturable' community and then pyrosequenced. Clustering analysis revealed 50% of the OTUs detected in the total community were also detected in the cultured community. It is possible that a similar rate of culturability could be possible for the rumen bacterial community.

Since the revelation that most of the rumen bacterial diversity is uncultured, little has been done to systematically isolate the organisms missing from culture collections.

Recent reports describing new rumen species (Cai *et al.*, 2010; Cook *et al.*, 2007; Kraatz *et al.*, 2011; Sundset *et al.*, 2008; Zhang *et al.*, 2009a; Zhang *et al.*, 2009b) have been few in number compared to the estimated number of uncultured rumen species. Recently, a small number of studies have begun to address this problem with the isolation of rumen bacteria using either a targeted (Koike *et al.*, 2010) or general approach (Kenters *et al.*, 2011).

1.13.1 Why it is difficult to culture

The inability of microbiologists to culture some bacteria is not limited to the rumen environment, and is one of the major problems faced by microbiologists (Rappé *et al.*, 2003; Stewart, 2012; Vartoukian *et al.*, 2010). Estimates made from the available datasets of 16S rRNA genes demonstrate that we are only beginning to sample global bacterial species richness (Schloss *et al.*, 2004). The direct microscopic count of rumen bacteria significantly exceeds the cultivable count. In cattle fed forage rations, the culturable count is 10-30% of the microscopic count (Hungate, 1975). The possible reasons for this anomaly include the inability to sufficiently replicate the natural environment of the bacteria in laboratory conditions, and the failure to replicate interactions with other microbes that are a requirement for growth. Cells that clump together also reduce the cultivable count as it is not possible to resolve whether a colony (or a growth-positive culture) is derived from a single cell or many; they will only be counted as one.

Difficulties in isolating targeted groups are amplified if they form only a small fraction of the original population. A large number of isolation attempts needs to be made in order to isolate these bacteria, which can be time consuming. On top of this, some media may not be ideally suited to the bacteria, causing them to have a lower growth rate and therefore be outcompeted by other organisms that are better suited to growth in culture. It is also possible that some of the gaps in cultured rumen bacterial groups may be due to lack of identifying sequence information of previously isolated organisms. It is possible that many of the uncultured groups were isolated by earlier investigators, but that the difficulties in differentiating the isolates and in maintaining them resulted in the cultures not being maintained and preserved.

The study of rumen bacteria by cultivation-based techniques is labour intensive and requires prior knowledge of individual nutritional and growth requirements. In the pre-molecular biology era, much of effort went into isolating pure bacterial cultures. However, their characterisation by classical microbiology was difficult, given that most rumen bacteria are Gram-stain variable and often have similar morphologies and a similar range of metabolic end-products (Table 1-1) (Bryant *et al.*, 1953). Difficulties in identification were overcome by 16S rRNA gene sequence analysis and genome sequencing, thereby making cultivation of new bacterial species easier to direct than it was in the past.

1.13.2 Why culture?

Discrepancies between 16S rRNA gene catalogues obtained using cultivation-independent methods and 16S rRNA genes from cultivated strains demonstrate that we have only a limited view of true ruminal diversity. There is a strong bias towards easily cultivated groups and they may not be the most important groups or the most prevalent groups *in vivo*. It is now possible to obtain genome sequences without the need for cultivation. However, without a pure culture, information from the sequence can only be inferred by what is known about the underlying biochemistry coded for by similar gene sequences from microbes that are available as pure cultures. Having the microbe in pure culture allows much more functional information to be derived from its genome and from studying of its physiology.

1.14 Aims of thesis or research gaps

The rumen harbours a complex ecosystem of micro-organisms that work in unison with the animal to break down fibrous plant material. Many of these microbes are yet to be obtained in pure culture and their roles within the rumen remain unknown. I hypothesise that (1) ruminal bacterial diversity can be represented by simple cultivation approaches and (2) previously uncultured bacteria can be isolated from the rumen, using appropriate cultivation techniques and large (many hundreds) numbers of isolates to get a better representation of the plant-adherent community.

I plan to develop a method to isolate plant-adherent rumen bacteria and test it in a small scale trial. Archaea will not be excluded but are not expected to be cultured. This method then will be used to isolate multiple (~1000) presumptively pure cultures of bacteria from the plant-adherent fraction of rumen contents. I will then use cultivation-independent molecular techniques (pyrosequencing) to determine which bacteria are attached to the plant material in rumen digesta. To assess my hypotheses, gene sequence data from the isolates will be compared with the corresponding data from the non-cultivation based community studies to see how much of the diversity of ruminal plant-adherent bacteria has been obtained in culture.

Chapter 2 Materials and Methods

2.1 Materials

All components are in aqueous solution unless otherwise specified. Sterilisation by autoclaving was carried out 121 °C for 20 min.

2.1.1 DAPI stain

A stock of 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution containing 1 mg/mL of DAPI dissolved in phosphate buffered saline (PBS) was stored at – 20 °C in a light-proof tube. When needed 10µL of DAPI stock was diluted in 50 mL of PBS in a foil-covered falcon tube and stored at 4 °C. This results in a final concentration of 0.2 µg/mL of DAPI.

2.1.2 DGGE 2× Gel loading dye

The 2× concentrated DGGE gel loading dye was obtained by mixing 0.25 mL 2% (w/v) bromophenol blue, 0.25 mL 2% (w/v) xylene cyanol, 7 mL 100% (v/v) glycerol and 2.5 mL of distilled water.

2.1.3 DNA-free water

Distilled water was collected directly from the distillation apparatus, filtered through a 0.2 µm pore size sterile filter into 15 mL falcon tubes and then autoclaved. The water was then irradiated with UV light (254 nm, 6 W) for 8 h.

2.1.4 10× Green loading dye

The 10× green loading dye contains 25% (v/v) Ficoll 400, 100 mM EDTA pH 8.0, 0.15% (w/v) orange G and 0.023% (w/v) xylene cyanol.

2.1.5 PCR reagents

10× Taq Buffer with Mg⁺⁺, Taq enzyme and 10 mM dNTP PCR grade mix were supplied by Roche (cat. YE04728858001, Roche Diagnostics NZ LTD). 50 mM MgCl₂ was supplied by Fermentas (Thermo Fisher Scientific, Vilnius, Lithuania).

2.1.6 Primers

Primers were ordered from IDT (Custom Science, Onehunga, Auckland NZ) or Invitrogen (Life Technologies, Carlsbad, USA), made up as stock solutions to 100 pmol/μL with the addition of DNA-free water and diluted to working concentrations of 10 pmol/μL when required.

2.1.7 1× PBS

For phosphate buffered saline solution (PBS) (Sambrook *et al.*, 1989) the following components were added per 1 L of distilled water: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄; the pH adjusted to 7.4 and then autoclaved.

2.1.8 4% paraformaldehyde solution

The 4% paraformaldehyde solution (4% PFA) was made as previously described by (Hugenholtz *et al.*, 2002). Briefly, 12 g paraformaldehyde powder (VWR 1.04005.1000) was added to 195 mL of warm distilled water and heated to 60 °C on a hotplate in a fume cupboard. Drops of 2 M NaOH were slowly added to the solution until it became clear. Once the paraformaldehyde was dissolved the solution was removed from the heat and 99 mL of 3× PBS added. When cooled to room temperature the pH was adjusted to 7.2 before being passed through a 0.2 μm pore size filter and stored in 50 mL aliquots in sterile falcon tubes at -20°C. When needed, 4% PFA solution was defrosted and used within 24 h.

2.1.9 Modified Karnovsky's fixative

Modified Karnovsky's fixative comprised of 3% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. To make 100 mL of fixative, 2 g of paraformaldehyde powder (VWR 1.04005.1000) was added to 80 mL of water and heated with stirring to 60 °C on a hotplate in a fume cupboard. Drops of 1 M NaOH were slowly added to the solution until it became clear. Once the paraformaldehyde was dissolved the solution was cooled in an ice bath and 2.51 g Na₂HPO₄·12H₂O, 0.41g KH₂ PO₄ and 12 mL of 25% (v/v) glutaraldehyde was added. The pH was adjusted to 7.2 and the volume was then adjusted to 100 mL with water.

2.1.10 50× TAE buffer

50 × concentrated Tris-acetate-EDTA buffer comprised of 2M Tris, 1M acetic acid and 50mM EDTA. To make 1 L add 242g Tris, 57.1 mL Glacial acetic acid, 100 mL 0.5M EDTA, pH 8.0 and make up to 1 L with distilled water. The solution was autoclaved and stored at room temperature.

2.1.11 1× TAE buffer

Tris-acetate-EDTA buffer was obtained by 50-fold dilution of 50× TAE or by making from components. It contains 40 mM tris(hydroxymethyl)aminomethane, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 8.0 with NaOH.

2.2 Media

All media and components were made using anaerobic techniques as described in (McSweeney *et al.*, 2005). All vessels with final media preparations contained butyl rubber stoppers to maintain the anaerobic conditions. Any additions to fully prepared media were done through butyl rubber stoppered vessels using oxygen-free CO₂- or N₂-flushed syringes and needles. Where solidified media was used, 1.5% w/v of Bacteriological Agar (Oxoid, Hampshire, UK) was added to the described media before autoclaving. Base media in all experiments were RM02, BY, CRF or 98-5.

2.2.1 Media solutions

2.2.1.1 Salt solution A

The salt solution A was obtained by dissolving 6g of NaCl, 1.5g of (NH₄)₂SO₄, 3g of KH₂PO₄, 0.79g of CaCl₂·H₂O and 1.2g of MgSO₄·7H₂O in 1 L of distilled water.

2.2.1.2 Mineral solution I (Salt solution 2B)

Either 6g of K₂HPO₄ or 7.86g of K₂HPO₄·H₂O were added to 1 L of distilled water

2.2.1.3 Mineral solution II

The mineral solution II was made by dissolving 12g of NaCl, 12g of (NH₄)₂SO₄, 6g of KH₂PO₄, 1.58g of CaCl₂·H₂O and 2.45g of MgSO₄·7H₂O in 1 L of distilled water.

2.2.1.4 Na₂CO₃ solution

8g of Na₂CO₃ was added per 100 mL of distilled water and brought to the boiling point before being allowed to cool while being bubbled with 100% CO₂ (O₂-free). Na₂CO₃ solution was then sealed in CO₂-flushed serum vials with butyl rubber stoppers and autoclaved.

2.2.1.5 Reducing agent

To prepare reducing agent, the dissolved oxygen was removed from distilled water by boiling, then allowing it to cool while being bubbled with O₂-free N₂. L-cysteine·HCl·H₂O (1.25 g) was added to 100 mL of anoxic water under continuous N₂ gas flow. The pH of the mixture was then adjusted to 10, followed by adding 1.25 g of Na₂S·9H₂O to the L-cysteine solution. Reducing agent solution was sealed in N₂-flushed serum bottles with a butyl rubber stopper and crimp cap and subsequently autoclaved (Bryant *et al.*, 1961).

2.2.1.6 Selenite / Tungstate solution

The selenite / tungstate solution was prepared as described by (Tschech *et al.*, 1984). Briefly, 500 mg of NaOH, 3 mg of Na₂SeO₃·5H₂O and 4 mg of Na₂WO₄·2H₂O were added to 1 L of distilled water. After the components have dissolved, the solution was aliquoted into 250 mL screw cap bottles (200 mL per bottle) and autoclaved.

2.2.1.7 Trace element solution SL10

The trace element solution SL10 was made by dissolving components in the order listed in Table 2-6 to 1 L of distilled water. After the components were dissolved, the solution was aliquoted into 250 mL screw cap bottles (200 mL per bottle) and autoclaved (Widdel *et al.*, 1983).

Table 2-1. Trace element solution SL10 components.

Component	Volume or weight per L
HCl (7.7 M)	10 ml
FeCl ₂ ·4H ₂ O	1.5 g
CoCl ₂ ·6H ₂ O	190 mg
MnCl ₂ ·4H ₂ O	100 mg
ZnCl ₂	70 mg
H ₃ BO ₃	6 mg
Na ₂ MoO ₄ ·2H ₂ O	36 mg
NiCl ₂ ·6H ₂ O	24 mg
CuCl ₂ ·2H ₂ O	2 mg

2.2.1.8 Vitamin 10 concentrate

To prepare vitamin 10 concentrate, the dissolved oxygen was removed from distilled water by boiling, then allowing it to cool while being bubbled with O₂-free N₂. The components listed in Table 2-7 were dissolved in 1 L of anoxic water at room temperature under continuous N₂ gas flow (Kenters *et al.*, 2011). 100 mL aliquots were then passed through a filter (0.2 µm pore size) and then injected into a sterile nitrogen-flushed serum vial sealed with a butyl rubber stopper with a sterile syringe and needle.

Table 2-2. Vitamin 10 concentrate components.

Component	Weight per L
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

2.2.2 Media additives
2.2.2.1 Anaerobic glycerol solution

Composition of the anaerobic glycerol solution and order of component addition are given in Table 2-3. The mixture was brought to the boil by heating in a microwave oven and allowed to cool under the continuous flow of CO₂. Once cool 0.125g L.cysteine·HCl·H₂O was added per 500 mL and the glycerol solution transferred to serum bottles while being flushed with CO₂. The bottles were sealed with butyl rubber bungs and sealed with crimp caps before being autoclaved.

Table 2-3. Anaerobic glycerol solution components.

Component	Volume or weight^a
Salt solution A	85 mL
Glycerol	200 mL
dH ₂ O	130 mL
Salt solution 2B	85 mL
Resazurin solution 0.1% (w/v)	50 µL
NaHCO ₃	2.5 g

^a The total volume of anaerobic glycerol solution was 500 mL.

2.2.2.2 Rumen fluid collection for preparation of media

Samples of the whole rumen contents are collected from two hay-fed fistulated cows. Feed was withheld from the animals after 4 pm, and rumen contents collected at 9 -10 am the following morning. Rumen contents were filtered through a single layer of cotton cheesecloth with a mesh size of approx. 1 mm (Stockinette; Cirtex Industries Ltd., Thames, New Zealand) into a collection vessel and then fine particulate material was removed by centrifugation at $10,000 \times g$ for 20 min. The resulting supernatant (rumen fluid) was frozen at $-20\text{ }^{\circ}\text{C}$. Before use, the rumen fluid was thawed and centrifuged a second time at $10,000 \times g$ for 20 min.

2.2.2.3 Clarified rumen fluid for preparation of media

Thawed rumen fluid collected as described in previous section (2.2.2.2) was heated in a microwave until the point of boiling and then allowed to cool under a continuous flow of oxygen-free nitrogen gas for at least 10 min. The anoxic rumen fluid was then sealed in a nitrogen flushed serum bottle with a butyl rubber stopper and autoclaved. Once rumen fluid equilibrated to room temperature, 0.08 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1.63g per 100 ml) and 0.08 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.18g per 100 ml) was added, forming a heavy precipitate. The rumen fluid was then centrifuged at $25,500 \times g$ at $4\text{ }^{\circ}\text{C}$ for 20 min to remove the particulate material, resulting in clarified rumen fluid.

2.2.2.4 GenRFV – General Rumen Fluid-Vitamin mix

The GenRFV (Table 2-4) was prepared by adding the listed components to 100 mL of clarified rumen fluid and dissolved by heating until boiling in a microwave oven. The resulting mixture was made anoxic by being allowed to cool under a continuous flow of oxygen free nitrogen gas for 15 min. The mixture was passed through a filter (0.2 µm pore diameter) into a sterile nitrogen flushed serum vial sealed with a butyl rubber stopper. Finally, 2 mL of vitamin 10 concentrate solution (Table 2-7; 2.2.1.8) per 100 mL of mixture was added anoxically, using a sterile syringe and needle. The final growth media containing GenRFV were prepared by adding 0.5 mL of GenRFV to 9.5 mL of medium; this results in final medium concentrations: 5% (v/v) rumen fluid, 0.5 mM of cellobiose, 1 mM each of glucose, xylose, and arabinose, 5 mM lactate, 4 mM each of Ca²⁺ and Mg²⁺, and 1 g each of casamino acids, Bacto-peptone, and yeast extract per L.

Table 2-4. GenRFV solution components.

Component	Volume or weight
D-glucose (Labserv, Auckland, NZ)	0.36 g
D-cellobiose (Sigma-aldrich, Steinheim, Germany)	0.34 g
D-xylose (Sigma-aldrich, Steinheim, Germany)	0.30 g
L-arabinose (Sigma-aldrich, Steinheim, Germany)	0.30 g
Na L-lactate syrup (Applichem, Damstalt, Germany)	0.88 ml
Casamino acids (BD, Claix, France)	2 g
Bacto-peptone (BD, Claix, France)	2 g
Yeast extract (BD, Claix, France)	2 g
Rumen fluid	100 ml
Vitamin 10 concentrate solution ^a	2 ml

^aAdded to prepared anoxic mixture of all other components listed above.

2.2.2.5 2xS GenRFV - 2× Sugars General Rumen Fluid - Vitamin mix

2xS GenRFV was made by the same procedure as was GenRFV; except that the 2xS GenRFV contained two-fold as high concentration of D-glucose, D-cellobiose, D-xylose and L-arabinose (Table 2-5). The final growth media containing 2xS GenRFV were prepared by adding 0.5 mL of 2xS GenRFV to 9.5 mL of medium; this results in final medium concentrations: 5% (v/v) rumen fluid, 1.0 mM of cellobiose, 2 mM each of glucose, xylose, and arabinose, 5 mM lactate, 4 mM each of Ca²⁺ and Mg²⁺, and 1 g each of casamino acids, Bacto-peptone, and yeast extract per litre.

Table 2-5. 2xS GenRFV solution components.

Component	Volume or weight
D-glucose	0.72 g
D-cellobiose	0.68 g
D-xylose	0.60 g
L-arabinose	0.60 g
Na L-lactate syrup	0.88 ml
Casamino acids	2 g
Bacto-peptone	2 g
Yeast extract	2 g
Rumen fluid	100 ml
Vitamin 10 concentrate solution ^a	2 ml

^aAdded to prepared anoxic mixture of all other components listed above

2.2.2.6 GenV mix

GenV mix was modified from GenRFV mix with the clarified rumen fluid replaced with distilled water.

2.2.2.7 2xS GenV mix

2xS GenV mix was modified from 2xS GenRFV mix with the clarified rumen fluid replaced with distilled water.

2.2.2.8 Titanium (III) NTA solution

Titanium (III) NTA (nitrilotriacetic acid) solution was made as previously described by Moench *et al.*(1983). 1 L of distilled water was brought to the boil and allowed to cool under continuous flow of oxygen free nitrogen gas. 9.6 g of nitrilotriacetic acid (Sigma, Sreinheim, Germany) was added to 300 mL of the anaerobic water while under continuous nitrogen gas flow. The pH was adjusted to 9.0 with NaOH. To this mixture, 9.6 mL of 20% TiCl₃ solution (BDH, VWR International Ltd Poole, UK), kept under N₂, was slowly added, while maintaining the pH above 2.0 with Na₂CO₃ to avoid unwanted precipitates forming. The final pH was adjusted to 7.0 with anaerobic Na₂CO₃ solution and made up to the final volume of 500 mL with anaerobic water, before being transferred to a sealed N₂-flushed sterile serum vial through a sterile filter (0.22 µm pore size; Milipore) using a sterile syringe and needle. This results in final concentration of 25 mM Ti III and 100 mM NTA.

2.2.3 Preparation of isolation media

Media used for the isolation of bacteria from the plant-adherent fraction of rumen contents. Media were dispensed into Hungate tubes (16 mm diameter, 125 mm long; BellCo glass, Vineland, NJ, USA) with butyl rubber stoppers. The base media were supplemented with additives, as indicated in Table 2-1.

Table 2-6. Supplements added to isolation media.

Media	Additives ^a
RM02	GenRFV 0.5 mL to 9.5 mL of media
RM02 + agar	GenRFV 0.5 mL to 9.5 mL of media
RM02 + grass	GenRFV 0.5 mL to 9.5 mL of media
RM02 + sisal	GenRFV 0.5 mL to 9.5 mL of media
RM02 + pectin	GenRFV 0.5 mL to 9.5 mL of media
RM02 + Titanium	GenRFV 0.5 mL to 9.5 mL of media
BY	GenV 0.5 mL to 9.5 mL of media
CRF	GenV 0.5 mL to 9.5 mL of media
CRF + sisal	GenV 0.5 mL to 9.5 mL of media
98-5	none

^aPreparation of GenRFV and GenV are detailed in sections 2.2.4.4 and 2.2.4.6

2.2.4 Sub-culturing media

The media used for the maintenance of newly isolated bacteria are shown in Table 2-2. Media was dispensed into Hungate tubes with butyl rubber stoppers.

Table 2-7. Supplements added to sub-culturing media.

Media	Additives ^a
RM02	2xS GenRFV 0.5 mL to 9.5 mL of media
BY	2xS GenV 0.5 mL to 9.5 mL of media

^aPreparation of additives 2×S GenRFV and 2×S GenV are detailed in sections 2.2.4.5 and 2.2.4.7

2.2.5 Storage media

Bacterial cultures were stored anaerobically in short Hungate tubes with a butyl rubber stopper, containing 4.5 mL of BY medium (2.2.12) supplemented with 0.25 mL of 2xS GenV (2.2.2.7) and inoculated with 0.25 mL of overnight bacterial culture. Once the cultures reached the stationary phase (two – three days), 1.5 mL of anaerobic glycerol solution (2.2.2.1) was added before storing at $-85\text{ }^{\circ}\text{C}$.

2.2.6 RM02 medium

RM02 is a medium that mimics rumen mineral conditions for growing anaerobic microbes (Kenters *et al.*, 2011). Components listed in Table 2-8 were mixed and boiled, then allowed to cool in an ice bath, while being bubbled with CO_2 (O_2 -free). Once cooled to room temperature, 4.2 g of NaHCO_3 and 0.5g of L-cysteine $\cdot\text{HCl}\cdot\text{H}_2\text{O}$ were added per 950 mL of medium. 9.5 mL of the medium was then dispensed into Hungate tubes while being gassed with CO_2 and sealed with butyl rubber stoppers, resulting in a gas headspace in the tubes of 100% CO_2 . The tubes were then sterilised by autoclaving. Tubes were stored in the dark for a least 24 h before use. Additives (either GenRFV or 2xS GenRFV) were added as required (0.5 ml per tube) and pre-incubated for at least 12 h at $37\text{ }^{\circ}\text{C}$ before use.

Table 2-8. RM02 medium components.

Component ^a	Volume or weight
Distilled H_2O	950 ml
KH_2PO_4	1.4 g
$(\text{NH}_4)_2\text{SO}_4$	0.6 g
KCl	1.5 g
Trace element solution SL10	1 ml
Selenite / tungstate solution	1 ml
0.1% (w/v) resazurin solution	100 μL

2.2.7 Solid RM02 medium

Solid RM02 medium was prepared using the method described above for the liquid RM02 medium, except that bacteriological agar was added at 1.5% (w/v).

2.2.8 Grass-containing RM02 medium (RM02 + grass)

Grass-containing RM02 medium was prepared using the method described above for the standard RM02 medium, except that 0.5% (w/v) dried rye grass was added to it. To prepare the rye grass, fresh rye grass was harvested and desiccated in a kiln then ground to a powder with a coffee grinder.

2.2.9 Sisal-containing RM02 medium (RM02 + sisal)

Sisal-containing RM02 medium was prepared using the method described above for the standard RM02 medium, except that 2-4 cm of sisal string (Donaghys, Christchurch, NZ) was added per tube.

2.2.10 Pectin-containing RM02 medium (RM02 + pectin)

Pectin-containing RM02 medium was prepared using the method described above for the standard RM02 medium, except that 0.08% (w/v) of pectin was added per tube. Pectin isolated from apple was sourced from Sigma, Steinheim, Germany.

2.2.11 Titanium-containing RM02 medium (RM02 + titanium)

Titanium-containing RM02 medium was prepared using the method described above for the standard RM02 medium, except that 0.05 mL titanium (III) NTA solution (2.2.2.8) was added per tube, each of which contained 9.5 mL of RM02 medium.

2.2.12 BY medium

The components listed in Table 2-9 were mixed and boiled, then allowed to cool on ice, while being continuously bubbled with O₂-free CO₂. Once cooled to room temperature, 0.5 g of L-cysteine·HCl·H₂O were added per 1 L of medium. 9.5 mL of the medium was then dispensed into Hungate tubes while being gassed with CO₂; the tubes containing medium were sealed with butyl rubber stoppers, resulting in a gas headspace of 100% CO₂. The tubes were then sterilised by autoclaving. When needed 0.5 mL of additives (either GenV or 2xS GenV) were added to each tube and pre-incubated for at least 12 h at 37 °C before use.

Table 2-9. BY medium components.

Component	Volume or weight
Salt solution A	170 mL
Salt solution 2B	170 mL
Centrifuged rumen fluid	300 mL
Distilled water	350 mL
Trace element solution (SL10)	1 mL
NaHCO ₃	5.0 g
Resazurin solution 0.1% (w/v)	100 µL
Yeast extract	2.0 g

2.2.13 CRF medium

CRF medium (100% Clarified Rumen Fluid medium) was prepared from the rumen fluid collected as described in section 2.2.2.2, thawed and centrifuged 10,000 × g for 15 min. The rumen fluid was heated in a microwave until the point of boiling and then allowed to cool under a continuous flow of oxygen free nitrogen gas for at least 15 min. The anoxic rumen fluid was then dispensed into N₂-flushed serum bottles, which were sealed with a butyl rubber stopper and autoclaved. When cooled to room temperature, 0.815 g MgCl₂·6H₂O and 0.59 g CaCl₂·2H₂O was added per 1 L of rumen fluid. Precipitated particulate material was removed by centrifuging at 25,000 × g for 20 min. The rumen fluid was then made anoxic by boiling, followed by cooling in an ice

bath, while being gassed with O₂-free CO₂. Once cooled to room temperature, 4.2 g of NaHCO₃ and 0.5 g of L-cysteine·HCl·H₂O were added per 1 L of medium. 9.5 mL of the medium was then dispensed into Hungate tubes while being gassed with CO₂, and sealed with butyl rubber stoppers, resulting in a gas headspace of 100% CO₂. The tubes were then sterilised by autoclaving . GenV (0.5 mL) was added to each tube using a sterile syringe and needle as required. The tubes were pre-incubated for at least 12 h at 37 °C before use.

2.2.14 Sisal-containing CRF medium (CRF + sisal)

Sisal-containing CRF medium was prepared using the method described above for the standard CRF medium, except that 2-4 cm of sisal string (Donaghys, Christchurch, NZ) was added per tube.

2.2.15 Medium 98-5

Medium 98-5 has been modified from RGCA medium (rumen fluid, glucose, cellobiose, agar medium) as previously described by (Bryant *et al.*, 1961). The components listed in Table 2-10 were mixed and boiled, then allowed to cool while being continuously bubbled with O₂-free CO₂. 9.75 mL of the medium was then dispensed into Hungate tubes while being gassed with CO₂; the tubes were sealed with butyl rubber stoppers, leaving a gas headspace of 100% CO₂. The tubes were then sterilised by autoclaving. Before use, 0.25 mL of 8% Na₂CO₃ solution and 0.2 mL of reducing agent solution were added to each tube and were stored in the dark for at least 6 h. The addition of the 8% Na₂CO₃ solution and 0.2 mL of reducing agent solution changed the appearance of the medium from pale pink to colourless. Any tubes that remained pink or changed from colourless were discarded.

Table 2-10. Medium 98-5 components.

Component	Volume or weight per L
Mineral solution I	37.5 mL
Mineral solution II	37.5 mL
Resazurin 0.1% (w/v)	1 mL
Rumen fluid	400 mL
Glucose	0.25 g
Cellobiose	0.25 g
Soluble starch	0.5 g
Distilled water	500 mL

2.2.16 LB (Luria-Bertani) liquid and solid medium

LB liquid medium was prepared in aerobic conditions and contains 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract 1.0% (w/v) NaCl, with the pH adjusted to 7.0. LB solid medium was obtained by addition of 1.5% (w/v) bacteriological agar before autoclaving. The autoclaved bottles containing LB solution were allowed to cool to 55 °C in a water bath before adding antibiotics if required and pouring the medium into Petri dishes (plates).

2.3 Methods

2.3.1 Agarose gel electrophoresis

Agarose gels 1% (w/v) were made up in 1× TAE buffer (2.1.10) containing 2× final concentration of SYBR safe nucleic acid dye (Life Technologies, Carlsbad, USA). Amplified products were loaded into the wells with 20% (v/v) 10× green loading dye (2.1.4) (typically 4 µL of product and 1 µL of dye). A DNA size standard 1kb⁺ marker (Life Technologies, Carlsbad, USA) was loaded into the first and last well in each lane. Gels were run in 1× TAE buffer following the manufacturer's directions in either a super 120 tank or liberty fast gel tank (6MGel, fast agarose system, BioKeystone Co, California, USA) at 150V for 18 - 25 min. Bands were visualised using UV trans-

illumination and digitally photographed using the Gel Logic 200 imaging system (Eastman Kodak, New York, USA).

2.3.2 Animals and ethics approval

Rumen contents for media and optimisation trials for the cultivation experiment were taken from a fistulated Holstein, cow tag number 723, and a fistulated Romney cross sheep, tag number 5472, housed at AgResearch, Grasslands, New Zealand, and grazing on a rye-grass / clover pasture. Animal ethics permission was granted by the Grasslands Animal Ethics Committee permit number AE 12174.

Animals sampled for the cultivation and pyrosequencing work were at either Scott or Lye farms, Dairy NZ, Vaile Road, Hamilton, New Zealand. The animals used in this study were chosen from nine fistulated dairy cows that formed part of a normal production herd grazing *ad libitum* on a rye-grass / clover pasture. The herd at Dairy NZ were supplemented with pasture silage during the months when feed was limited, including the sampling times in May and August 2009 and also May 2010. The amount of silage offered was around 5-10% of the recommended daily intake per animal during these months. Animal ethics permission was granted by the Ruakura Animal Ethics Committee permit number AE 11483.

2.3.3 DAPI staining and cell counting

Samples were fixed in 4% PFA solution at a sample to PFA ratio of 1:3 before staining and counting. Dilutions of each sample were made in PBS and 8 μ L of each sample was spread evenly onto a 6 mm diameter well of a 10 well diagnostic microscope slide (Erie, Portsmouth, USA) and allowed to air dry. Samples were then dehydrated by soaking in solutions with increasing concentrations of ethanol, 50%, 80% and then 96% for 3 min each and then allowed to dry. Slides were then incubated in DAPI stain (2.1.1) for 10 min, with PBS for 10 min and then rinsed with distilled water. Once dry a small drop (just enough to cover the well) of vectashield anti-fade mounting medium (Vector Laboratories, California, USA) was applied to each well before applying the cover-slip and sealing with nail varnish. Samples were viewed at 1000 \times

magnification with oil under UV light using DM2500 microscope (Leica Microsystems, Wetzlar, Germany). A minimum of 50 fields per sample were counted for dilutions that had an average of ~60 cells per field. Each sample was counted in triplicate and an average was used to calculate the number of cells per sample.

2.3.4 DNA quantification

DNA was quantified using one or more of three methods

1. Using the Quant-iT dsDNA Broad-range (BR) Assay kit or for low DNA concentration the Quant-iT High sensitivity (HS) Assay kit (Life Technologies) as per manufacturer's instructions. These were measured on the Qubit® fluorometer (Life Technologies, Carlsbad, USA).
2. NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA)
3. Quantifying bands on a gel from a gel image using the Kodak ID 1.6 software.

2.3.5 General laboratory equipment

Samples were mixed with a vortex (PV1, Grant bio, Cambridge). Heating and incubation operations were carried out in a recirculating water-bath (Grant GB series, Cambridge) or an Accublock digital dry bath (Labnet International, Edison, NJ, USA). Autoclaving was done in a high pressure steam sterilizer ES-315 (TOMY TECH, USA). Cocoon Portable incubator was used to transport growing cultures (Dominion incubators Limited, Hamilton, NZ). The pH meter used in this work was a PHM62 pH Meter (Radiometer, Copenhagen, Denmark). N₂, CO₂ and H₂ gas were supplied by BOC gas (Auckland, NZ). Any traces of oxygen was removed from the gases by passing through a column packed with reduced copper filings, heated to 400 °C in an inline furnace. The copper filings were kept reduced by routinely flushing with hydrogen for several minutes. Work with anaerobic plates was carried out in an anaerobic glove box (Coy laboratory products, MI, USA) containing a 95% CO₂: 5% H₂ atmosphere. Several different centrifuges were used in this work described in Table 2-11.

Table 2-11. Centrifuge specifications.

Brand	Vessels spun	Max RCF × g (rotor used)	Temp control	Supplier
Spectrafuge Mini Centrifuge	0.6 mL tubes	2,000	No	Labnet (Edison, NJ USA)
Minispin personal microcentrifuge	1.5 mL and 2.0 mL tubes	16,000	No	Eppendorf (Hamburg, Germany)
MicroCL 17 Centrifuge	1.5 mL and 2.0 mL tubes	17,000	No	Thermo Fisher Scientific Inc.
Biofuge Fresco	1.5 mL tubes	16,000	Yes	Heraeus (Hanau, Germany)
Sigma 3-18K	15 mL, 50 mL falcon tubes Hungate tubes 96 well plates	4,248 (11180/13190) 3,500 2717 (11240)	Yes	SIGMA Laborzentrifugen GmbH (Germany)
Sorvall®Evolution RC	50 mL Oakridge tubes 250 mL Nalgene Tubes	48,000(SS34) 27,500 (SLC1500)	Yes	Thermo Fisher Scientific Inc.

2.3.6 Feed analysis

Feed samples were collected at each season sampling time from the pastures the animals were grazing. Pasture and silage analysis was carried out at feedTECH AgResearch, Palmerston North. The samples were dried to constant weight (until the weight stopped decreasing) at 65 °C and then ground to a particle size that could pass through a 1 mm sieve. Near-infrared reflectance spectroscopy (NIRS) was used to estimate: Crude Protein (CP), crude fat (lipid), Ash, Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF), Soluble sugars and starch – reducing sugars (SSS), *in vitro* DIGestibility (DIG), Organic Matter Digestibility (OMD) and Metabolisable Energy (ME) as a percentages of total dry matter, as described in Corson *et al.*, (1999).

2.3.7 Live/dead stain

Viability of cultures were assessed with *BacLight Live/Dead Bacteria Viability kit* (L13152 10 applicator sets) as per manufacturer's instructions

2.3.8 Most Probable Number of viable cells

Most probable number (MPN) of viable cells in the dilution series was calculated from the percentage of growth-positive tubes (Hurley *et al.*, 1983), using an online calculator (Curiale).

To calculate the MPN of cells per gram of rumen content, the following formula was used; $g = L \times f/e \times d/e \times b/c \times b/a$ where g = cells per gram of rumen content, where a = grams of rumen content sampled (~200 g), b = grams of rumen digesta in the sample, c = grams of rumen digesta sub-sampled for washing (~50g) d = grams of digesta left after the washes, e = grams of washed digesta sub-sampled for blending (~40g), f = volume of media used to blend washed digesta (400 mL) and L = MPN of cells per mL in the blended digesta fraction (MPN \times dilution factor)

The effect of culture media on MPN was assessed with one-way Analysis of variance (ANOVA), using the Genstat v13 software package (VSN International, Hemel Hempstead, UK) (Payne *et al.*, 2012). This was used to compare the means of the MPN values from the cultivation experiment. In addition, a permutation test was performed to validate the ANOVA. The influence that animal-to-animal variation had on MPN values was assessed with a linear mixed model using the Restricted Maximum Likelihood (REML) method.

2.3.9 Purification of PCR products

The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), following the manufacturer's instructions.

2.3.10 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was carried out at the Manawatu Microscopy and Imaging Centre (MMIC), Massey University, Palmerston North, New Zealand.

2.3.11 SEM sample preparation

Cultures for SEM were grown in RM02 + sisal medium. Initial sample preparations were carried out by Dr Adrian Cookson (AgResearch, Grasslands, NZ). Individual sisal string strands were washed 25 times, five times in each of five Falcon tubes containing 0.1 M phosphate buffered saline (pH 7.3) using ethanol flamed sterile forceps. After washing, excess diluent was removed from the sisal string by blotting with tissue and the sisal string was immersed in modified Karnovsky's fixative (2.1.9) for at least 24 h then washed three times for 15 min each in PBS. The samples were then transferred to the MMIC for further processing. Sisal string was dehydrated through incubation in a series of solutions with increasing concentrations of ethanol, 25%, 50%, 75%, 95% and 100%, for 15 min each. A final incubation step in 100% ethanol for one hour was carried out before samples were critical point dried using liquid CO₂ as the critical point fluid. The dried samples were mounted on aluminium SEM stubs on double-sided tape, and sputter coated with gold. Samples were studied and digital images saved with FEI Quanta 200 SEM.

2.3.12 Rumen sample collection

Digesta samples were collected from AgResearch and Dairy NZ animals that were grazing on a rye-grass clover pasture *ad libitum*. Samples were collected between 7- 9 am from each animal through a rumen cannula, sampling contents from many locations in the rumen to represent a good mix. The whole digesta was immediately placed in a CO₂-gassed bucket and transported back to the laboratory for processing. Subsamples for cultivation work were taken from the centre of the bucket to minimise the effect of oxygen on the sample.

2.3.13 Volatile fatty acids analysis

The rumen fluid (2 mL) was collected from each animal at each seasonal sampling; they were immediately frozen to -20 °C and stored at this temperature until the Volatile Fatty Acids (VFA) analysis was performed. The VFA concentrations were separated by high-performance liquid chromatography (HPLC; apparatus LC10AVP) coupled to simultaneous quantification using a RID 10A detector with the following parameters; Biorad aminex HPX-87H column (dimensions 300 × 7.8 mm), column temp 45 °C, Flow rate 0.8 mL per min, Injection volume 50 µL, with a Mobile phase 5 mM sulphuric acid.

2.4 Isolation experiments

2.4.1 Rumen content fractionation trial

In order to separate the plant-adherent fraction of the rumen contents from the planktonic fraction for use in cultivation, a fractionation method modified from (Larue *et al.*(2005) was tested using whole rumen contents from one cow (labelled 723) and one sheep (labelled 5472). This resulted in 14 fractions in total, illustrated in Figure 2-1. At each step the weight or volume of the sample was recorded and subsamples were taken for future DNA extraction and for cell counts. Subsamples for DNA analysis were immediately frozen and stored at -85 °C until processed. 2 g (or 2 mL) subsamples for cells counts were fixed in 6 mL of 4% PFA solution. The PFA-containing samples were allowed to fix for 12 h at 4 °C before two aliquots were processed for long-term storage. Each fixed sample was mixed thoroughly by vortexing and a 1 mL subsample was stored at -20°C. A second 1 mL subsample was centrifuged at 8000 × g for 5 min. The resulting pellet was resuspended in 1mL of 1× PBS buffer and then centrifuged a second time for 5 min at 8000 × g. The pellet was resuspended in 300 µL of 1 × PBS buffer and 300 µL of 100% ethanol before being stored at -20 °C.

2.4.1.1 Rumen content fractionation test - Cow (723)

All fractionation steps except centrifugation were carried out in glassware under a continuous flow of CO₂ gas. One litre of rumen contents was sampled from the animals

through a cannula into a CO₂ flushed container and taken directly to the lab for processing. The rumen contents samples were then squeezed through a double-layer cheesecloth. The liquid fraction was subjected to a slow spin to pellet digesta particles and protozoa at 150 × g for 1 min. The pellet was called the *large liquid fraction*. The supernatant was subsampled and then subjected to a fast spin to pellet microbial cells of 10,000 × g for 20min. The supernatant from this spin was discarded and the pellet, called the *small liquid fraction*, was resuspended in 6 mL of RM02 base (with no additives). Two times 50 g subsamples of squeezed digesta was washed by gently resuspending each in 200 mL RM02 base media (no additives) and stirring for 30 s. The mixture was then squeezed through a double-layer cheesecloth. The pooled liquid portion was subjected to a slow spin of 350 × g for 15 min to pellet any plant material which was discarded and then a fast spin of 10,000 × g for 20 min to harvest the microbial cells. This was called the *associated fraction* and was resuspended in 6 mL of RM02 base media (no additives). The washed digesta was resuspended in 200 mL of 0.15% (v/v) tween-80 and incubated on ice for 2.5 h to release adherent microbes from the plant material. This was squeezed through a double-layer cheesecloth and the liquid portion was subjected to a slow spin of 500 × g for 15 min to pellet any plant material which was discarded and then a fast spin of 10,000 × g for 20 min to harvest the microbial cells. This was called the *adherent fraction* and was resuspended in 6 mL of RM02 base media (no additives). The remaining Tween-80 treated digesta was called the *digesta fraction*.

2.4.1.2 Rumen contents fractionation test – Sheep (4572)

The sheep rumen contents fractionation was performed using a protocol outlined above, with the following modifications to the fractionation protocol: All of the squeezed digesta (~200 g from a 1 L sample) was fractionated rather than 2 × 50 g subsamples; furthermore, the volumes of RM02 base media (no additives) were increased to reflect the greater volume of the sample. RM02 (800 mL) was used to wash the digesta and 800 mL of 0.15% (v/v) tween-80 was used to dislodge adherent microbes from the plant material. The *small liquid fraction* was resuspended in 12 mL of RM02 base media and the associated pellet in 20 mL of RM02 base media.

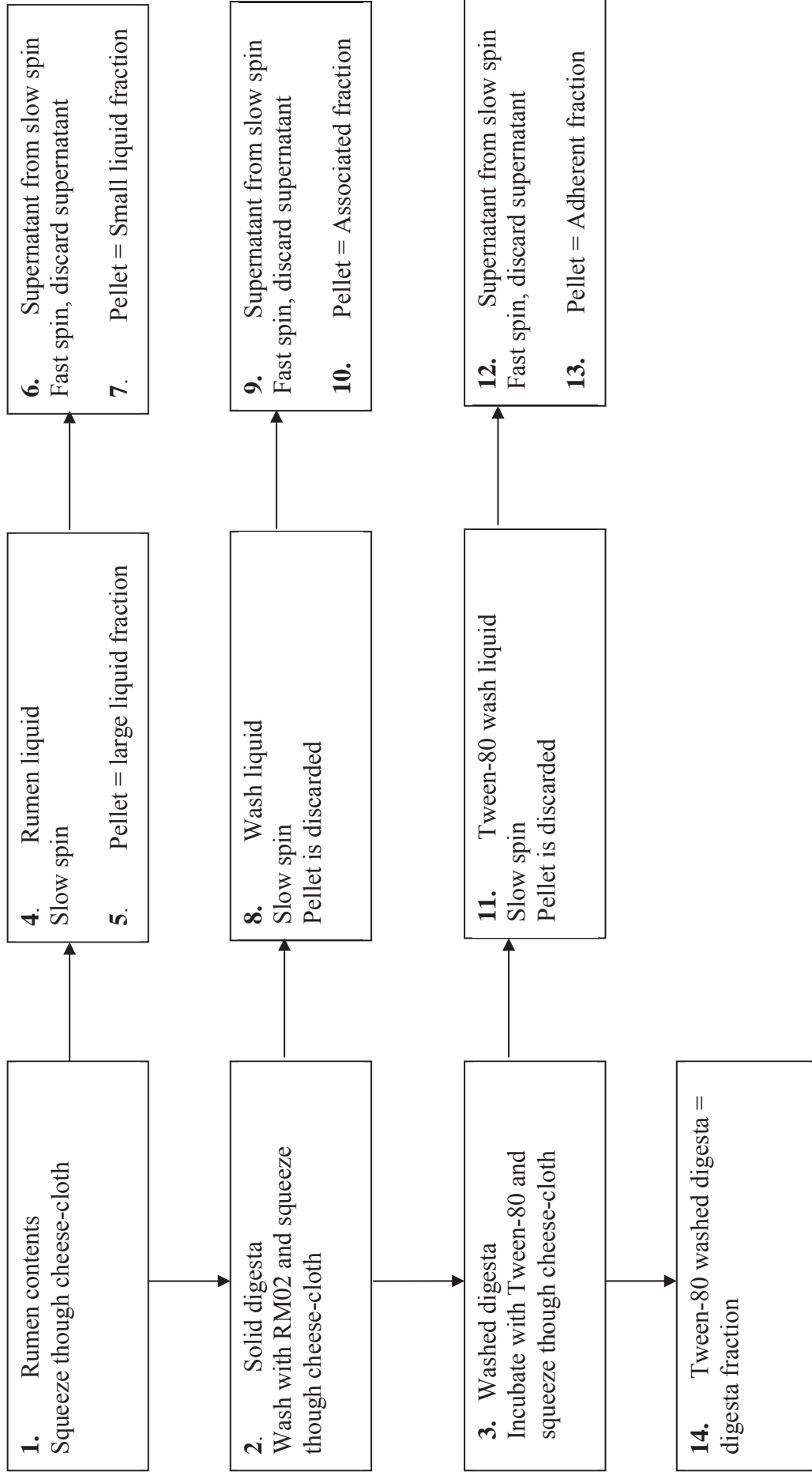


Figure 2-1. Procedure for separating plant-adherent rumen bacteria from rumen contents.

2.4.2 Optimisation of rumen content fractionation

2.4.2.1 Wash experiment 1

The number of buffer washes required to remove unattached bacteria from plant material in rumen contents was assessed. The rumen contents sample from cow 723 was fractionated (as described in 2.3.12). Under anoxic conditions, 200 g of rumen content was squeezed through a double layer of cheesecloth. Subsamples of 1 g or 1 mL of the liquid and solid fractions were taken for DNA extraction and cell counts. The solid rumen digesta (50 g) was washed nine times by gently resuspending the squeeze-drained solid digesta in 200 mL of RM02 base medium, followed by separation from the liquid fraction by squeezing again through a cheesecloth. The number of bacteria being washed off at each step was microscopically counted, also the bacteria still attached to the plant material after nine washes were counted. To assess the changes in community structure a subsample was taken from the liquid and solid fractions at each wash step for future DNA extraction and subsequent DGGE analysis and stored at -85 °C until processed.

2.4.2.2 Wash experiment 2

In this fractionation experiment, the effect of doubling the volume of digesta and buffer was assessed. As in wash experiment 1, the rumen contents were sampled from cow 723; a total of 200 g of rumen content was squeezed through a double-layer cheesecloth. The remaining solid rumen digesta (~100 g) were washed four times by gently resuspending in 400 mL of RM02 base medium, followed by squeezing through cheesecloth. The number of bacteria washed off at each step was microscopically counted. To assess the efficiency of cell counts on solid samples, a subsample of the four times washed digesta (40 g) was mixed with 360 mL of RM02 base medium and was disrupted in a Waring blender pulsed on high four times for 20 s with 30 s resting periods between and then microscopically counted. The number of bacteria still adhering to the plant material after four washes was also counted without the blending treatment.

2.4.3 Trial cultivation

A full test of the rumen content fractionation method to culture anaerobic plant-adherent bacteria was performed on the rumen contents from cow 723. Briefly, solid rumen digesta were separated from whole rumen contents and washed four times with RM02 base medium before being disrupted in a Waring blender. Blending times were optimised as described by Kenters *et al.*, (2011). All washing and blending steps were performed in anoxic conditions under a continuous flow of CO₂ gas. Serial dilutions of the blended digesta were made in order to obtain a range around a theoretical number of 0.2 viable cells/mL. Serial 10- and 100-fold dilutions were made down to 10⁷ fold. This dilution was then further diluted in a series of six, four-fold dilutions (labelled 1-6 in Figure 2-2). All dilutions were made in RM02 base media in sealed serum bottles with a sterile syringe and needle. A total of 0.1 mL of each dilution from the chosen range (1-6 shown below) were inoculated into 20 Hungate tubes containing RM02 + GenRFV and incubated at 37°C. Tubes that showed any turbidity were scored as positive for growth, subcultured into fresh RM02 media, examined microscopically and stored. Tubes were incubated for at least three weeks before being scored negative for growth. The pre-trial cultivation isolates were labelled with the letters DAZ, followed by the isolate number. A separate sample of whole rumen contents, called total fraction, was stored for subsequent DNA extraction and blended to enable cell counts. The pooled wash and rumen liquid fractions were called the combined wash fraction and were treated in the same manner as the total fraction.

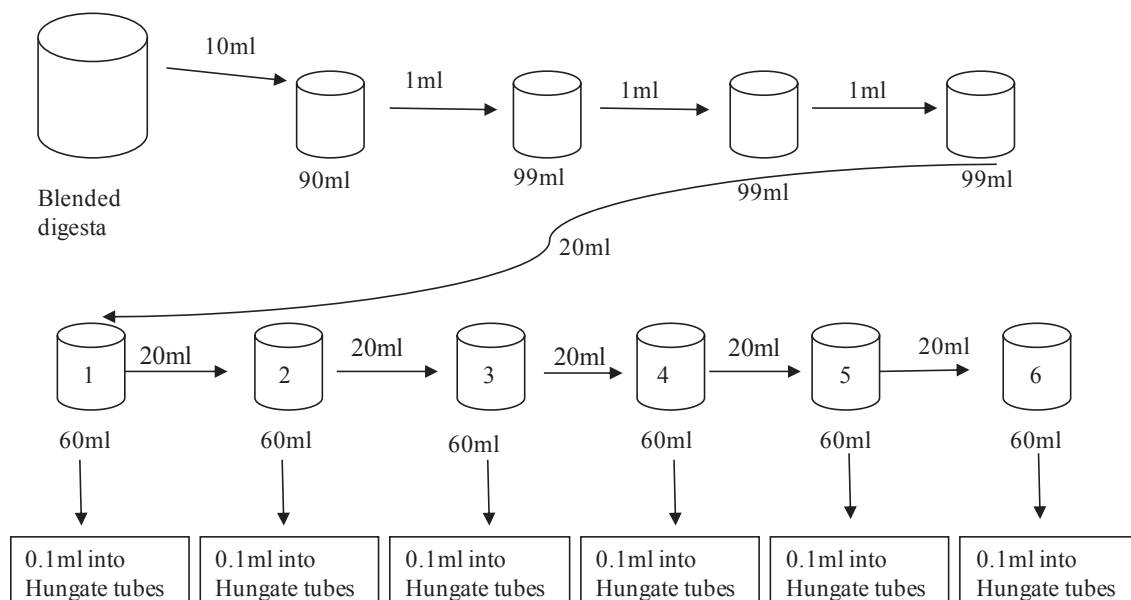
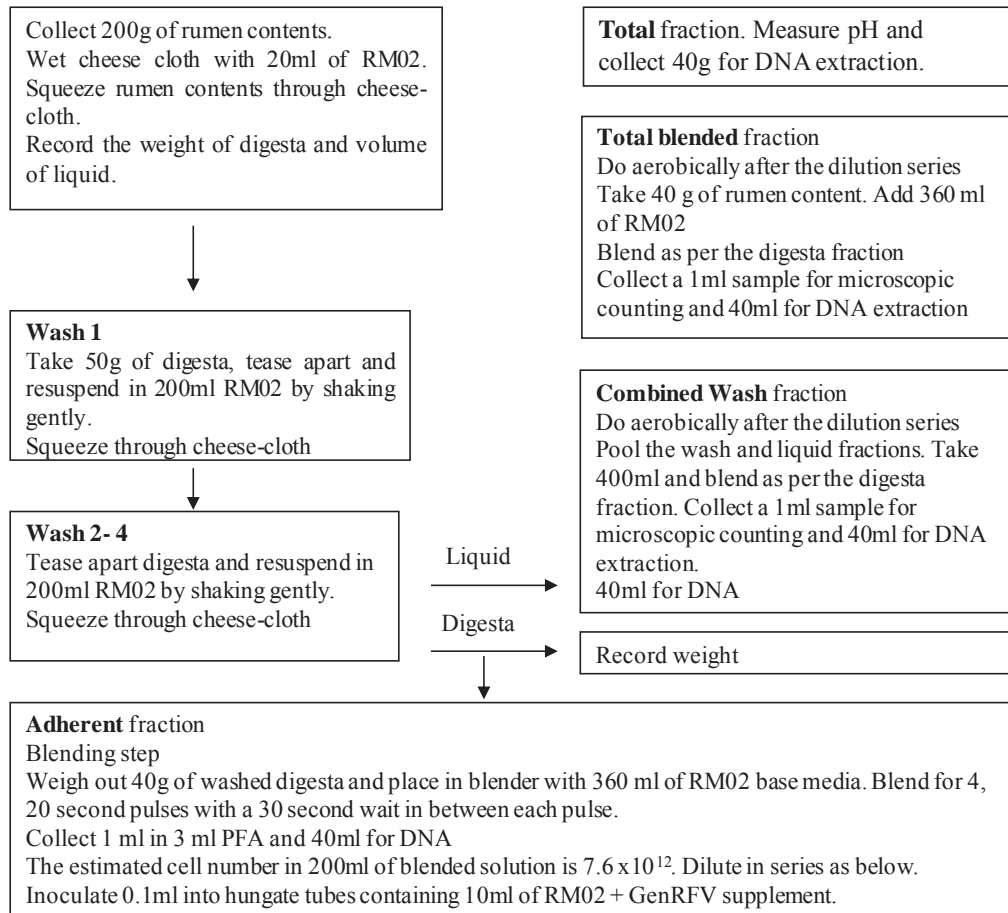


Figure 2-2. Optimised protocol for separating and cultivating plant-adherent bacteria from rumen contents.

2.4.4 Cultivation experiment

The cultivation experiment was conducted over the course of one year with five different sampling times, one in each season. The procedures for each sampling were similar. Details of animals that were sampled in each season are shown in Table 2-12. After the first sampling, cow 2225 (A) died and the reserve cow 3122 (E) was used. A new reserve cow 4109 (F) was selected but not sampled. Fractionating the sampled rumen content to isolate the plant-adherent fraction of bacteria at each sampling time followed the optimised procedure described above and depicted in Figure 2-2. Experiments differed from season to season in the dilutions chosen for inoculation and the number of RM02 Hungate tubes inoculated with each dilution. During the May (M) sampling, five four-fold dilutions were made (1-5 in Figure 2-2) and each was inoculated into 60 tubes per animal. For the August (A) sampling four dilutions were made (1-4 on Figure 2-2) and each inoculated into 60 or 90 tubes per animal. For the November (N), February (F) and second May (L) samplings four dilutions were made and three of those were inoculated (2-4 in Figure 2-2) and each inoculated into 60 tubes per animal. RM02 medium was used with all animals at all sampling times; however in addition to RM02 additional alternative media were used for samples from two animals at each sampling time (Table 2-13). For all of the alternative media, five dilutions were made per animal (1-5; Figure 2-2) and each dilution was inoculated into 20 tubes. For the M sampling no alternative media were trialled. For the A, N and L sampling dilutions from cows 3517 (B) and 4519 (D) were used in alternative media. For the February sampling dilutions from cows 4519 (D) and 3122 (E) were used in the alternative media. The inoculated culture tubes were incubated at 37 °C in a portable incubator during transport from Hamilton to Palmerston North. Upon arrival to the laboratory in Palmerston North they were transferred into a warm room heated to 39 °C. Tubes that showed any turbidity were scored as growth-positive, sub-cultured into fresh media, examined microscopically and stored. Tubes were incubated for at least four weeks before being scored negative for growth. Culture names were generated from their media type code letter/s followed by the sampling and animal code letters and then the dilution number.

Table 2-12. Cows sampled from DairyNZ, Hamilton.

Cow	Name	Number	May	Aug	Nov	Feb	May
A	Alice	2225	Yes ^a	Dead	Dead	Dead	Dead
B	Beyonce	3517	Yes	Yes	Yes	Yes	Yes
C	Cocoa	4110	Yes	Yes	Yes	Yes	Yes
D	Daisy	4519	Yes	Yes	Yes	Yes	Yes
E	Eleanore	3122	^b	Yes	Yes	Yes	Yes
F	Frances	4109					

^aYes, samples were taken.

^bBlank, no samples were taken

Table 2-13. Media types used at each sampling time.

Sample month	Media code	Media	Tubes per animal
April 2009 (trial)	Z	RM02	120
May 2009	M	RM02	300
August 2009	A	RM02	300
	YA	RM02 + agar	100
	YS	RM02 + sisal	100
	YR	RM02	100
	YG	RM02 + grass	100
November 2009	N	RM02	180
	XP	RM02 + pectin	100
	XB	BY	100
February 2010	F	RM02	180
	WC	CRF media	100
	WT	RM02 + titanium	100
May 2010	L	RM02	180
	V9	98-5	100
	VC	CRF + sisal	100

2.4.4.1 Preparation and storage of rumen samples for DNA extraction

Each rumen content sample from each cow was fractionated as described above (Figure 2-2). Four fractions were saved for DNA extraction: (i) the total fraction; (ii) total blended fraction; (iii) combined wash fraction; (iv) digesta fraction. The material for DNA extraction was immediately frozen and stored at -85°C . For the long-term storage, material destined for DNA extraction was lyophilised in a freeze drier (Cuddon, Blenheim, New Zealand) and then ground to a fine powder in a clean coffee grinder (Russel Hobbs, Manchester, England). Ground samples were stored in vacuum sealed bags at -85°C until DNA was extracted.

2.4.4.2 Samples for cell counts

Three rumen contents fractions from each cow were fixed for cell counts at each sampling time: (i) total blended fraction; (ii) combined wash fraction; (iii) digesta fraction (Figure 2-2). For fixing, 1 mL of each fraction was pipetted into a tube containing 3 mL of 4% PFA solution (giving a total of 4 mL fixed fraction) and incubated on ice for 3 h. A 1 mL aliquot of the fixed material was removed and immediately frozen at -20°C . A second 1 mL aliquot was centrifuged for 5 min at $9000 \times g$ to pellet microbial cells. The resulting pellet was resuspended in 1 mL of $1 \times$ PBS buffer and then centrifuged a second time for 5 min at $9000 \times g$. The pellet after second centrifugation was resuspended in 300 μL of $1 \times$ PBS buffer and 300 μL of 100% ethanol before being stored at -20°C .

2.5 Identification of cultures

2.5.1 Phase contrast microscopy

Agarose-coated slides were prepared by evenly pipetting 1.5 mL of molten 2% (w/v) agarose (Life Technologies, Carlsbad, USA) in distilled water over each microscope slide and allowing it to completely dry to a clear film. One or two drops (50 – 100 μL) of fresh culture was added to each slide (on the dried agarose side) and overlaid with a cover-slip. Cultures were examined on regular or agarose-coated slides at $100 \times$ magnification (with oil) using phase contrast (DM2500, Leica Microsystems,

Wetzlar, Germany). Images were captured digitally using the Leica application suite software.

2.5.2 DNA extraction from cultures

DNA was extracted from cultures using two methods described below.

1. 2 mL of culture was centrifuged to pellet the cells at $13,000 \times g$ for 5 min. The cell pellet was resuspended in 500 μL of sterile distilled water and spun again at $13,000 \times g$ for 5 min. The cell pellet was resuspended on 200 μL of 6% (w/v) Chelex 100 resin (InstaGene Matrix; Bio-Rad Laboratories Inc., Hercules, CA, USA) heated at 56 °C for 30 min, vortexed for 10 s, then heated at 100 °C for 8 min and vortexed again for 10 s. Cell debris and Chelex resin particles were removed by centrifuging at $11,000 \times g$ for 3 min and the supernatant transferred to a clean tube and stored at -20 °C until required.
2. Cells from 10 mL of culture were pelleted and resuspended in 100 μL of distilled water. DNA was extracted using ZR Fungal/Bacterial DNA miniprep kit (Zymo Research) following the manufacturer's directions. The high-speed cell disruption step was done in FastPrep FP120 cell disrupter (Bio101, Thermo-Savant, Qbiogene, Carlsbad, USA) 6.5 m/s for 40 s.

Method 1 was tried first for each culture. If the resulting DNA could not be successfully used to amplify a PCR product, then method 2 was used, starting from a fresh culture originating from the same isolate.

2.5.3 Purification of DNA from cultures

For extracted DNA that could not be amplified using the universal 16S rRNA gene primers, DNA was further dialysed to remove low molecular weight impurities. Nitrocellulose filter was used for dialysis in the following manner: drops of DNA extract (5 μL each) were placed on a nitrocellulose filter (0.025 μm pore size VSWP; Millipore) floating on distilled water and incubated at room temperature for 3 h.

Purified DNA was either used directly in a PCR reaction or stored at -20 °C until required.

2.5.4 16S rRNA gene sequencing for culture identification

Initially all isolates were subjected to partial sequencing of the 16S rRNA gene (V1-V3 region) for preliminary identification. This identification was used to select representative isolates for full-length sequencing of the 16S rRNA gene. Polymerase chain reaction (PCR) amplifications were performed in Hybaid Px2 thermal cyclers (ThermoElectron, Milford, MA, USA) or a DNA Engine® Peltier Thermal Cycler (Biorad, Hercules, CA, USA). A standard reaction mix listed in Table 2-14 was used with the amount of water added adjusted to bring the total volume of each reaction to 50 µL, depending on the volume of template DNA added. All constituents excluding the template DNA were added together and mixed well before being transferred to 0.2 mL thin walled PCR tubes (Eppendorf, Raylab New Zealand Ltd) or 96 well PCR plates (4titude, Wotton, Surrey, UK). After the addition of template DNA the mixture was vortexed for 5 s and quickly spun to return the contents to the bottom of the tubes or wells. The following PCR protocol was used: 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min then 72 °C for 1 min, followed by a final extension of 72 °C for 10 min.

Table 2-14. Components and their volumes in a typical 16S rRNA gene amplification PCR reaction for unknown rumen cultures.

Component	Volume per 1× reaction	Final concentration
DNA-free water (2.1.3)	34.8 - 37.8 μL	N/A ^a
10 × PCR buffer containing Mg^{++}	5 μL	1 x
50 mM MgCl_2	2 μL	2 mM
10 mM dNTP mixture (each)	1 μL	0.2 mM
Forward primer 27f (10 pmol/ μL)	1 μL	0.2 pmol/ μL
Reverse primer 1492r (10 pmol/ μL)	1 μL	0.2 pmol/ μL
Taq DNA polymerase (5 U/ μL)	0.2 μL	0.02 U/ μL
Template DNA	2 - 5 μL	N/A
Total volume	50 μL	N/A

^a N/A = not applicable

2.5.5 Primers for culture identification

Primers used for culture identification are listed in Table 2-15. Primer pair 27f and 1492r were used to amplify the near-complete 16S rRNA gene and primer 514r was used to sequence approximately the V1-V3 region for preliminary identification. Altogether, six primers: 27f, 1492r, 1382r, 1100r, 806r and 514r were used to sequence the near complete 16S rRNA gene. The primer pair 806r and 515f which were able to amplify some bacterial groups that were missed by other universal primers were used to try and identify difficult to PCR cultures.

Table 2-15. Primers used on unidentified rumen cultures.

Primer	Sequence	Reference
27f	GAG TTT GAT CMT GGC TCA G	(Sait <i>et al.</i> , 2002)
1492r	GGY TAC CTT GTT ACG ACT T	(Lane, 1991)
1100r	GGG TTG CGC TCG TTG	(Lane, 1991)
1382r	CGG TGT GTR CAA GGC CC	(Dorsch <i>et al.</i> , 1992)
806r	GGA CTA CVS GGG TAT CTA AT	(Bergmann <i>et al.</i> , 2011)
514r	CCG CGG CKG CTG GCA C	(Lane, 1991)
515f	GTG CCA GCM GCC GCG GTA A	(Bergmann <i>et al.</i> , 2011)

2.5.6 Sanger sequencing

PCR products were sent for sequencing to either the Massey Genome Sequencing Service (Massey University, Palmerston North, New Zealand) or Macrogen Inc. (Seoul, Republic of Korea). These services included fluorescent labelling of products using the BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit, subsequent removal of unincorporated fluorescent dideoxy NTPs by cleanup and precipitation of products, and capillary separation on an ABI3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) Results were received as ABI files and were viewed, edited and assembled using the Contig Express package of the Vector NTI suite (Vector NTI Advance 11, Life Technologies, Carlsbad, USA). Analysed ABI files were converted to fasta files to be used in further analyses.

2.6 Culture-independent community analysis

2.6.1 DNA extraction from rumen samples

DNA was extracted from ground freeze-dried rumen samples (see section 2.4.4.1) using two methods

1. Using the QIAamp Stool DNA mini kit (Qiagen, Hilden, Germany) starting from 50 mg of ground freeze-dried digesta samples and following the manufacturer's instructions.

2. Using a phenol-chloroform extraction with bead beating method (Henderson *et al.*, 2013). Description of DNA extraction buffers are shown in 2.6.1.1. Slight modifications were made to the method for around half of the samples to make it easier to get the required volume of supernatant after bead beating. The relative proportions of the buffers were kept the same. These differences are shown as version 1 (V1) and version 2 (V2). Around 30 mg (actual weight recorded) of freeze-dried rumen sample was weighed into a 2 mL screw cap tube containing 0.7 g of pre-baked 0.1 mm zirconium beads (Cat#11079012 Biospec Products, Bartlesville, USA). To this 375 μL (V1) or 450 μL (V2) of 120 mM NaPO_4 buffer, 125 μL (V1) or 150 μL (V2) of TNS solution and 500 μL (V1) or 600 μL (V2) of phenol/chloroform/isoamylalcohol (25:24:1) pH8 was added. Samples were disrupted in fast prep cell disrupter (Bio101, Thermosavant, Waltham, USA) for 45 s 6.5 m/s and immediately placed on ice. Tubes were then centrifuged at $13,000 \times g$ at 4 °C for 20 min. The aqueous phase (top layer; 400 μL) was transferred into a 2 mL tube and extracted with 400 μL of chloroform/isoamylalcohol (24:1) solution by inverting the tube. Tubes were centrifuged again at $13,000 \times g$ at 4 °C for 5 min. The aqueous phase (top layer; 350 μL) was transferred to a fresh tube and mixed with 700 μL of PEG solution to precipitate the DNA. The solution was homogenised by carefully inverting the tube five times. DNA was pelleted by spinning at $13,000 \times g$ at 4 °C for 60 min. The supernatant was carefully removed and pellet washed with 500 μL of chilled 70% ethanol and spun again for 10 min at $13,000 \times g$ at 4 °C. In V2 preparations, this ethanol wash step was repeated twice. The ethanol was carefully removed from the DNA pellet by pipetting, followed by drying of the pellet at 50 °C for at least 15 min. DNA was left to dissolve in 100 μL (V1) or 200 μL (V2) of EB buffer at 4 °C overnight.

2.6.1.1 DNA extraction buffers

120mM NaPO₄ buffer contains 112.87 mM Na₂HPO₄, 7.12 mM NaH₂PO₄, pH adjusted to 8.0 and filter-sterilized through a 0.2 µm pore size filter, then autoclaved.

TNS solution contains 500 mM Tris-HCl, pH adjusted to 8.0, 100 mM NaCl, 10% SDS (w/v) and filter-sterilized through a 0.2 µm pore size filter, then autoclaved.

PEG solution contains 30% (w/v) polyethylene glycol 6000 in 1.6 M NaCl, prepared with RNase free water, then autoclaved.

EB buffer contains 10 mM Tris-HCl, pH 8.5 prepared with RNase free water and filter-sterilized through a 0.2 µm pore size filter then autoclaved.

2.6.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed to monitor bacterial diversity in the rumen fractions and to test whether different experimental conditions affect the diversity seen in a rumen fraction. DGGE was performed on either a CBS scientific DGGE/TTGE gel system (C.B.S. Scientific Company, Del Mar, CA, USA) or InGeny phorU system, (Amundsenweg, The Netherlands). Polyacrylamide gels (7.5% w/v) with a denaturing gradient of 35% to 65% were run at 60 °C and 55 V for 16 h in 1 × TAE buffer. Denaturant solution (35%) was comprised of 7.35 g of urea, 7 mL of formamide, 9.38 mL of 40% acrylamide:bisacrylamide (37.5:1) solution, 0.5 mL 50 × TAE made up to 50 mL with distilled water. Denaturant solution (65%) was comprised of 13.65 g of urea, 13 mL of formamide, 9.38 mL of 40% acrylamide:bisacrylamide (37.5:1) solution, 0.5 mL 50 × TAE made up to 50 mL with distilled water. Ammonium persulfate (APS; 1/100 volume of 10% w/v solution) (Sigma, St Louis, USA) and TEMED (*N,N,N',N'*-tetramethylethylenediamine; 1/1000 volume of 100% solution; Merck, Darmstadt, Germany) were added to both denaturing solutions directly before casting the gel. Gels are loaded with normalised sample volumes (see below) alongside 5 µL of DGGE Marker I and/or DGGE maker II (Nippongene, Japan). Wells without marker or sample were loaded with 3µL of DGGE 2× Gel Loading Dye. Once run, the gels were rinsed in water for 10 min prior to being stained with 5 µL SYBR Gold (1/10,000 dilution; Life Technologies) in 1 L of water for 20 min and distained in 1 L of water for 2 h. The gels were viewed using UV transillumination and image captured with a Gel Logic 200

imaging system (Eastman Kodak, New York, USA). Images were taken with the same camera settings each time, with a shutter speed 1.6 s, Aperture f/5.6 and ISO 1000.

2.6.2.1 PCR for DGGE analysis

The 16S rRNA gene – encoding sequences were PCR-amplified in DNA Engine® Peltier Thermal Cycler (Biorad, Hercules, CA, USA) with the conditions listed in Figure 2-3. A standard reaction mix listed in Table 2-16 was used. During optimisation, the difference between two DNA polymerases, the HiFi platinum Taq (Life Technologies, Carlsbad, USA) and “regular” Taq polymerases (Roche Diagnostics, Auckland, New Zealand) were investigated. In the HiFi platinum Taq reactions, besides different enzyme, the specific buffer (10 × HiFi PCR Buffer; Life Technologies) and MgSO₄ (instead of MgCl₂) were used. The remaining components of the PCR reaction were the same as for regular Taq. Template DNA was measured by fluorometry using Qubit [Life Technologies; (2.3.4)] and then diluted to 4 ng/μL with 10 mM Tris before being used in the reaction.

Figure 2-3. Thermal cycler conditions for DGGE PCR

Initial Denaturation	94°C for 2 min		1 cycle
Denaturation	94°C for 30 sec	}	10 cycles
Annealing	62°C for 30 s -0.5°C/cycle		
Elongation	68°C for 30 sec		
Denaturation	94°C for 30 sec	}	14 cycles
Annealing	57°C for 30 s		
Elongation	68°C for 30 sec		
Final elongation	68°C for 15 min		1 cycle

Table 2-16. PCR components for DGGE analysis.

Component	Volume per reaction	Final concentrations
DNA-free water (2.1.3)	18.9 μ L	N/A
10 \times HiFi PCR buffer (Life Technologies, Carlsbad, USA)	2.5 μ L	1 \times
50 mM MgSO ₄	1 μ L	2 mM
10 mM dNTP mixture (each)	0.5 μ L	0.2 mM
Forward primer ^a - GC338f (10 pmol/ μ L)	0.5 μ L	0.2 pmol/ μ L
Reverse primer ^b - 515r (10 pmol/ μ L)	0.5 μ L	0.2 pmol/ μ L
HiFi platinumTaq (5 U/ μ L)(Life Technologies, Carlsbad, USA)	0.1 μ L	0.02 U/ μ L
Template DNA(4 ng/ μ L)	1 μ L	0.08 ng/ μ L
Total volume	25 μ L	N/A

^a **GC-338f** CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG

^b **515r** TT ACC GCG GCT GCT GGC AC

The PCR products were initially analysed by agarose gel electrophoresis to determine the size and amount. The amount of PCR product was quantified by densitometry of bands derived from a gel image using the Kodak ID 1.6 software. The average net intensity value was used to normalise the samples to the equivalent concentrations and load exactly the same amount of DNA for each sample. To prepare the PCR products for loading onto agarose or DGGE gels, one volume of dye (green loading dye for agarose gel electrophoresis or DGGE 2 \times Gel Loading Dye for DGGE gels) was added to four volumes of sample (6.25 μ L to 25 μ L of sample) and the PCR products were checked on an agarose gel (2.3.1).

2.6.2.2 Analysis of DGGE gels

The digital images of DGGE gels were analysed using the BioNumerics software program (Version 4.0; Applied Maths, Sint-Matens-Latem, Belgium). Sample

fingerprint patterns were normalised relative to the lanes containing the marker. Differences between the patterns were analysed using the Pearsons correlation; relatedness of the DGGE patterns/profiles were represented by a tree, using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) method. The initial DGGE and profile analysis that were used to choose the animals for the main sampling experiment were performed by Dong Li (AgResearch) in BioNumerics (Version 5.0) using Jaccard Coefficient clustering method and UPGMA.

To test for differences between samples, band-matching was performed in BioNumerics and normalized band intensities were exported and the differences between treatments assessed by Adonis (Anderson, 2001) and visualised with multidimensional scaling plots (MDS) using the R Project for Statistical Computing (<http://www.r-project.org/>) an open source programming language for statistical computing and graphics.

2.6.3 Clone library construction

A small clone library was constructed from the March sampling, cow A (MA) digesta sample. DNA was extracted from 50 mg of ground freeze-dried washed digesta fraction using the QIAamp Stool DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA (1 μ L) was used as a template for a 25 μ L PCR reaction. A standard reaction mix listed in Table 2-17 was used. The reactions were cycled for 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min then 72 °C for 1 min followed by a final extension of 72 °C for 10 min. The PCRs were done in triplicate for each DNA sample and visualised by agarose gel electrophoresis (1% w/v agarose). The amplicon replicates were then pooled, combined with 20% (v/v) of 10 \times green loading dye and added to a single well of a 0.8% (w/v) agarose gel containing a final concentration of 2 \times SYBR® Safe DNA stain. The PCR product was visualised on a transilluminator and excised from the gel with a sterile scalpel. DNA was extracted from the gel slice using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) following the manufacturers' instructions. Purified PCR products (4 μ L) were ligated into the pCR 2.1 TOPO cloning vector (TOPO TA cloning kit, Life Technologies, Carlsbad, USA), following the

manufacturer's instructions and then chemically transformed into One Shot TOP10 competent cells (Life Technologies, Carlsbad, USA) following the manufacturer's instructions. Transformed cells were plated onto pre-warmed LB agar plates (2.2.16) containing 50 µg/mL of ampicillin and 40 µL of 40 mg/mL X-gal spread onto each plate; the plates were incubated overnight at 37°C. White or light-blue colonies were deemed positive. They were colony purified by streaking onto a LB agar plate containing ampicillin only (no X-gal).

Table 2-17. Clone library PCR components.

Component	Volume per 1× reaction	Final concentration
DNA-free water (2.1.3)	18.9 µL	N/A ^a
10× PCR buffer containing Mg ⁺⁺	2.5 µL	1 x
50 mM MgCl ₂	1 µL	2 mM
10 mM dNTP mixture (each)	0.5 µL	0.2 mM
Forward primer 27F(10 pmol/µL)	0.5 µL	0.2 pmol/µL
Reverse primer 514R (10 pmol/µL)	0.5 µL	0.2 pmol/µL
Taq DNA polymerase (5 U/µL)	0.1 µL	0.02 U/µL
Template DNA	1 µL	N/A
Total volume	25 µL	N/A

^a N/A = not applicable

2.6.3.1 Colony PCR

To obtain crude DNA extract, colonies of transformed *E. coli* were resuspended in 50 µL of DNA-free water and lysed by boiling cells for 10 min; 1 µL of this crude extract was used per PCR reaction. Alternatively, a small amount of colony (toothpick scrape) was resuspended directly in the pre-set PCR reactions. Colony PCR amplifications were performed in Thermo Hybaid Px2 thermal cyclers (ThermoElectron, Milford, MA, USA), using the reaction mix listed in Table 2-18. All constituents were added together and mixed well before being transferred to 96 well

PCR plates. After the addition of template DNA the mixture was vortexed for 5 s and quickly spun to return the contents to the bottom of the tubes or wells. The reactions were cycled for 94 °C for 1 min followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s then 72 °C for 1 min followed by a final extension of 72 °C for 7 min. The primer pair, GEM2987F, CCC AGT CAC GAC GTT GTA AAA CG (Life Technologies, Carlsbad, USA) and TOP168R, ATG TTG TGT GGA ATT GTG AGC GG (Life Technologies, Carlsbad, USA) was used to amplify the cloned fragment and M13R, CAG GAA ACA GCT ATG AC (Life Technologies, Carlsbad, USA) used for sequencing the amplified insert. The PCR products were separated by agarose gel electrophoresis (1% w/v agarose) and visualised by staining using the SYBR® safe stain. The PCR products of expected size were sequenced (Macrogen Inc.; Seoul, Republic of Korea).

Table 2-18. Colony PCR components.

Component	Volume per 1× reaction	Final concentration
DNA-free water (2.1.3)	32.5 µL	N/A ^a
10× PCR buffer containing Mg ⁺⁺	5 µL	1 x
50 mM MgCl ₂	1 µL	1 mM
2 mM dNTP mixture (each)	10 µL	0.4 mM
Forward primer GEM2987F(10 pmol/µL)	0.5 µL	0.1 pmol/µL
Reverse primer TOP168R (10 pmol/µL)	0.5 µL	0.1 pmol/µL
Taq DNA polymerase (5 U/µL)	0.5 µL	0.05 U/µL
Template DNA	0 - 1 µL	N/A
Total volume	50 µL	N/A

^a N/A = not applicable

2.6.3.2 Analysis of clones

Clone sequences were compared to their nearest cultured relative using algorithm NAST (Near Alignment Space Termination) (DeSantis *et al.*, 2006a) within an online tool, Greengenes (DeSantis *et al.*, 2006b). The NAST algorithm aligns the submitted

sequence to a 16S rRNA gene database, Greengenes (<http://greengenes.lbl.gov>). The NAST algorithm first aligned the submitted sequences in the Greengenes 7,682-character format, then compared to the prokMSA to find near-neighbours using Simrank. Sequence divergence from near-neighbours was calculated using the DNAML option of DNADIST (PHYLIP package). The Lane mask (Lane, 1991) was used to restrict calculations to 1,287 conserved columns (lanes) of aligned characters. Phylogenetic groups were assigned to the sequences using the online classifier tool on RDP (<http://rdp.cme.msu.edu/>).

2.6.4 Pyrosequencing

Pyrosequencing was carried out by MacroGen Inc with a Roche 454 Genome Sequencer FLX Instrument, using GS FLX Titanium chemistry (454 GS FLX). One half a plate of unidirectional sequencing was carried out; the templates were V1-V3 PCR-amplified portion of the 16S rRNA gene. Bar-coding of each sample was used in a multiplex approach to obtain individual data for each rumen/sample/season.

2.6.4.1 Pyrosequencing sample preparation

DNA was extracted from rumen samples as described in 2.6.1 using the phenol/chloroform bead beating method. The integrity of each DNA extract was checked on an agarose gel. DNA quality was checked with the Nanodrop and quantified by fluorometry using “Qubit” fluorometer (Life Technologies, DS DNA kit). DNA was diluted to 4 ng/μL to use in PCR amplification for generation of the pyrosequencing template. The PCR reaction components are listed in Table 2-19 and the PCR program was as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 45 s. The final elongation was at 68 °C for 20 min. Forward primers were designed as amplicon fusion primers with a directional GS FLX titanium primer including, in 5' to 3' direction, a four base pair key sequence, followed by the Multiplex Identifier (MID) sequence (barcode) and the template-specific primer. The reverse primer lacks the MID sequence. Primers are listed in Table 2-20. A negative control (no template DNA) using the forward primer with its own barcode

(MID sequence) was amplified alongside the samples and included in the sequencing mix. To investigate the primer bias, one sample was amplified twice, each with different barcoded (MID sequence) primers. The PCR was conducted in triplicate on three separate runs and the PCR products were pooled. The pooled individual samples were analysed by agarose gel electrophoresis, stained with ethidium bromide and viewed using UV transillumination. The gel image was captured with a Gel Logic 200 imaging system and then quantified from the gel image using Kodak gel doc software. The equivalent amounts of all samples were combined. The pooled PCR product from all samples was subjected to preparative agarose gel electrophoresis (1.1% agarose) pre-stained with $2 \times$ SYBR safe. A total of 1 mL of the pooled PCR reaction was loaded into two large wells and separated at 1 V/cm for 30 min. The bands (~500 bp) were visualised on a safe light viewer box and excised from the gel with a sterile scalpel. DNA was extracted from the gel slices using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) following the manufacturer's instructions.

Table 2-19. Pyrosequencing PCR components.

Component	Volume per 1 × reaction	Final concentration
DNA-free water (2.1.3)	38.8 µL	N/A
10 × HiFi PCR buffer (Life Technologies, Carlsbad, USA)	5 µL	1 X
50 mM MgSO ₄	2 µL	2 mM
10 mM dNTP mixture (each)	1 µL	0.2 mM
Forward primer ^a - (20 pmol/µL)	1 µL	0.4 pmol/µL
Reverse primer- B514*R (20 pmol/µL)	1 µL	0.4 pmol/µL
HiFi platinumTaq (5 U/µL)	0.2 µL	0.02 U/ µL
Template DNA(4 ng/µL)	1 µL	0.08 ng/µL
Total volume	50 µL	N/A

^a Each forward primer has a different barcode, one per sample

Table 2-20. Barcoded primers for pyrosequencing PCR.

Primer	Sample	Primer sequence
Afd1 *MID55	AB	CGT ATC GCC TCC CTC GCG CCA TCA GAT CTA CGC TGG AGT TTG ATC MTG GCT CAG
Afd1 *MID56	AC	CGT ATC GCC TCC CTC GCG CCA TCA GCT CGC ATA CGG AGT TTG ATC MTG GCT CAG
Afd1 *MID57	AD	CGT ATC GCC TCC CTC GCG CCA TCA GAC AGA CAC GTG AGT TTG ATC MTG GCT CAG
Afd1 *MID58	AE	CGT ATC GCC TCC CTC GCG CCA TCA GCA CGA TCT ACG AGT TTG ATC MTG GCT CAG
Afd1 *MID59	NB	CGT ATC GCC TCC CTC GCG CCA TCA GAG CGC TCA GTG AGT TTG ATC MTG GCT CAG
Afd1 *MID60	NC	CGT ATC GCC TCC CTC GCG CCA TCA GCG CTA GTG TAG AGT TTG ATC MTG GCT CAG
Afd1 *MID61	ND	CGT ATC GCC TCC CTC GCG CCA TCA GGT AGT ACA TGG AGT TTG ATC MTG GCT CAG
Afd1 *MID62	NE	CGT ATC GCC TCC CTC GCG CCA TCA GTG AGT GTC ACG AGT TTG ATC MTG GCT CAG
Afd1 *MID63	FB	CGT ATC GCC TCC CTC GCG CCA TCA GGA GCA CTA GCG AGT TTG ATC MTG GCT CAG
Afd1 *MID64	FC	CGT ATC GCC TCC CTC GCG CCA TCA GTA GCA CGC GAG AGT TTG ATC MTG GCT CAG
Afd1 *MID65	FD	CGT ATC GCC TCC CTC GCG CCA TCA GGC TGA GTC ACG AGT TTG ATC MTG GCT CAG
Afd1 *MID66	FE	CGT ATC GCC TCC CTC GCG CCA TCA GTC TGA CAC TCG AGT TTG ATC MTG GCT CAG
Afd1 *MID67	LB	CGT ATC GCC TCC CTC GCG CCA TCA GGT CTG CTC AGG AGT TTG ATC MTG GCT CAG
Afd1 *MID68	LC	CGT ATC GCC TCC CTC GCG CCA TCA GTG CGA CTG ATG AGT TTG ATC MTG GCT CAG
Afd1 *MID69	LD	CGT ATC GCC TCC CTC GCG CCA TCA GGA CGA TGC ATG AGT TTG ATC MTG GCT CAG
Afd1 *MID70	LE	CGT ATC GCC TCC CTC GCG CCA TCA GTA CGC AGC TGG AGT TTG ATC MTG GCT CAG
Afd1 *MID71	MA	CGT ATC GCC TCC CTC GCG CCA TCA GGC GTG TAT GCG AGT TTG ATC MTG GCT CAG
Afd1 *MID72	MB	CGT ATC GCC TCC CTC GCG CCA TCA GTC GCG CTC TAG AGT TTG ATC MTG GCT CAG
Afd1 *MID73	MC	CGT ATC GCC TCC CTC GCG CCA TCA GAC GTA CTC ACG AGT TTG ATC MTG GCT CAG
Afd1 *MID74	MD	CGT ATC GCC TCC CTC GCG CCA TCA GCA GTG CTC TCG AGT TTG ATC MTG GCT CAG
Afd1 *MID54	-ve	CGT ATC GCC TCC CTC GCG CCA TCA GAG ATG ATA GCG AGT TTG ATC MTG GCT CAG
Afd1 *MID51	FD	CGT ATC GCC TCC CTC GCG CCA TCA GAC GCT ATC GAG AGT TTG ATC MTG GCT CAG
B514*R		CTA TGC GCC TTG CCA GCC CGC TCA GCC GCG GCT GCT GGC AC

2.7 Phylogenetic analysis

The near full-length sequence of 16S RNA gene from the isolates was determined using Sanger sequencing, whereas the sequence of the uncultivated samples from the rumen was determined by pyrosequencing (454 GS FLX). With the ultimate task of phylogenetically characterising the cultivated isolates and comparing them to the uncultured rumen samples, separate and combined pipelines were established by combining a number of software packages and resources as described below.

2.7.1 Computer software used in phylogenetic analysis

Computer software and web software used for sequence analysis is shown in Table 2-21.

Table 2-21. Software programs.

Program	Web site	Reference
Mega4 and Mega5	http://www.megasoftware.net	(Kumar <i>et al.</i> , 2008; Tamura <i>et al.</i> , 2007; Tamura <i>et al.</i> , 2011)
Vector NTI Advance [®] 11	http://www.invitrogen.com	(Lu <i>et al.</i> , 2004)
QIIME	http://qiime.sourceforge.net	(Caporaso <i>et al.</i> , 2010b; Kuczynski <i>et al.</i> , 2011)
Greengenes	http://greengenes.lbl.gov	(DeSantis <i>et al.</i> , 2006b)
ARB	www.arb-home.de	(Ludwig <i>et al.</i> , 2004)
RDP	http://rdp.cme.msu.edu	(Cole <i>et al.</i> , 2009; Wang <i>et al.</i> , 2007)
ITOL	http://itol.embl.de	(Letunic <i>et al.</i> , 2007, 2011)
RAXML7.0.4	http://www.exelixis-lab.org	(Stamatakis, 2006)
CD-HIT-OTU	http://weizhong-lab.ucsd.edu/cd-hit-otu	(Li <i>et al.</i> , 2012b)

2.7.2 Checking and correcting isolate sequences

Complete 16S rRNA gene sequences were obtained as ABI files and were initially viewed, manually corrected and the primer sequence was removed using the Contig Express programme of the Vector NTI advance package (Life Technology). For the partial (V1-V3 region) of the 16S rRNA gene sequences, the sequence reads were assessed for quality (no greater than 2% ambiguous base calls) and exported to FASTA file format for analysis using other software. The complete 16S rRNA gene sequences were assembled and assessed for quality (no greater than 1% ambiguous base calls and >1300 bp long) and the consensus sequence for each isolate was exported to FASTA file format for analysis by other software.

2.7.3 Elimination of PCR-mediated chimeras

Assembled full-length isolate sequences were checked for the presence of chimeric PCR products using the Bellerophon program (Huber *et al.*, 2004). Any putative chimeric sequences identified by Bellerophon were then confirmed individually by using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) to compare their 16S rRNA gene sequence to their closest cultured relatives in the NCBI nucleotide database (<http://blast.ncbi.nlm.nih.gov>).

2.7.4 Phylogenetic analysis of partial (V1-V3) isolate sequences

The phylogenetic positions of the new rumen isolates were assessed with three complementary methods on the partial 16S rRNA gene sequence reads (V1-V3). First, isolates a presumptive genus association using the BLAST algorithm to compare their partial 16S rRNA gene sequence to their closest cultured relatives in the Ribosomal Database Project (RDP) (265493 sequences were downloaded from RDP on 23/09/2011) (Maidak *et al.*, 2001). Second, the isolate sequences were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline v1.4.0 with the Greengenes database (release gg_otus_4feb2011) as a taxonomic reference as described in 2.7.6.

Finally, phylogenetic and molecular evolutionary analyses of the partial 16S rRNA gene sequences were conducted using MEGA version 5. Nucleotide sequences were globally aligned using ClustalW with a gap-opening penalty of 40 and a gap extension penalty of 20, using the IUB DNA matrix and a transition weight of 0.5. Phylogenetic trees were constructed using the Neighbour Joining method, with the Jukes Cantor model or p-distances, both with pair-wise deletions of gaps. Maximum likelihood trees were constructed with the Jukes Cantor model. Bootstrap analysis with 1000 iterations was used to assess the statistical strength of the branch positions. The 16S rRNA gene sequence from *Aquifex aeolicus* VF5 (GenBank accession number AE000657) was used as an out-group for the tree.

2.7.5 Clean-up of pyro-sequence reads

Pyrosequence reads were filtered for quality and assigned to starting samples by their nucleotide barcodes using the QIIME pipeline. Pyrosequences were quality filtered by applying a quality score check with a sliding window (50 bases) along the length of the sequence. The sequence was truncated downstream from the first position in the window in which the average quality score dropped below Q27. The truncated sequences were further examined to determine whether they still met the minimum length requirements of 400 bases. Reads shorter than 400 bases were discarded. Reads containing ambiguous bases or mismatches in the primer sequence were also discarded.

2.7.6 QIIME analysis

Taxonomic and phylogenetic profiles of the partial isolate sequences were analysed alone and in combination with pyrosequence using QIIME pipeline v1.4.0 and v1.5.0 respectively. Operational taxonomic units (OTUs) were chosen based on the similarity within the reads using UCLUST and a cut-off of 96% and a representative sequence (most abundant) was selected for each OTU. Representative sequences were aligned using PyNAST (Caporaso *et al.*, 2010a) as the alignment method. For each representative sequence, a taxonomic identity was assigned using the RDP classification system with a confidence value of 0.8 and the Greengenes database for reference

(release gg_otus_4feb2011 downloaded from <http://greengenes.lbl.gov>). The alignments were filtered before being used to create a phylogenetic tree using the FastTree method. The QIIME summary of the OTUs was presented in a table. Taxonomy summary chart and tables are derived from the OTU table, allowing visualisation of the overall taxonomic structure of the cultivable rumen fraction. Alpha diversity within the isolates was calculated and rarefaction curves were derived by computing the alpha diversity matrix from a rarefied OTU table. Diversity matrices were calculated to compare the types of communities based on the taxonomic and phylogenetic assignments. The differences were visualised using UPGMA and Principal Coordinate Analysis (PCoA) plots.

2.7.7 Pyrosequence analysis, CD-HIT-OTU pipeline

Quality pre-processed pyrosequence reads (see 2.7.5) were parsed through the CD-HIT-OTU pipeline which removes noise and chimeric reads followed by clustering of the denoised reads (<http://weizhong-lab.ucsd.edu/cd-hit-otu>). The pipeline performed the following steps; (1) Low quality reads were filtered out and reads with extra long tails were trimmed. (2) Filtered reads were clustered at 100% identity using the CD-HIT-DUP clustering algorithm (3) Chimeric clusters were identified and removed. (4) Secondary clusters were recruited into primary clusters. (5) Noise sequences cluster size were calculated and were removed. (6) The remaining reads from non-chimeric clusters were reclustered at 96% similarity level. The CD-HIT-OTU generated clusters were imported into the QIIME pipeline where phylogeny was assigned and OTU tables generated as described in section 2.7.6.

2.7.8 Choosing representative isolate sequences

To determine the identity of the isolates with greater precision using near full-length 16S rRNA gene sequences a selection of isolates representing the full diversity was chosen. The isolate sequence files were assigned a unique identifier, merged with the pyrosequence files and then analysed using the QIIME pipeline (version 1.3) as described in section 2.7.6. OTUs were generated (at the cut-off of 96% similarity) and

representative sequences of each OTU were chosen as described above. The representative sequences for each OTU that contained at least one isolate and an equal number of the largest OTUs that did not contain an isolate were exported as a fasta file and information about the OTU (number of pyrosequences and isolate sequences it contained) was added to the sequence name label. The fasta file containing renamed representative sequences was analysed in combination with all of the isolate 16S rRNA gene sequences (V1-V3) in Mega version 4, thus information about the OTUs size and taxonomic assignments could be used to guide the selection. Nucleotide sequences were globally aligned using ClustalW and the following parameters: a gap opening penalty of 40, a gap extension penalty of 20, the IUB DNA matrix and a transition weight of 0.5. Phylogenetic trees were constructed using the neighbour joining method, with the p-distances and pair wise deletions of gaps. The 16S rRNA gene sequence from *Aquifex aeolicus* VF5 (GenBank accession number AE000657) was used as an out-group for the tree. The resulting tree was used to confirm the QIIME OTU clusters and pick representative isolate sequences to be subjected to full-length 16S rRNA gene sequencing. To determine the distance matrix generated during the construction of the phylogenetic tree in Mega, it was reordered so that the sequences appeared in the same order as the sequences in the tree (using a PERL script shown in appendix 2, written by Eric Altermann, AgResearch). Clusters of sequences that were $\geq 96\%$ similar were visualised from the distance matrix in Microsoft excel. Each cluster was given a name and recorded in a master spreadsheet. At least one representative isolate was randomly selected from each cluster. Two or more isolates were selected from large, diverse or interesting clusters. A cluster was considered to be an interesting cluster if it contained at least one isolate sequence and either a representative sequence that was from a large OTU or was from part of the tree that was under-represented by isolate sequences.

2.7.9 Phylogenetic analysis of full-length isolate sequences

Fasta files of near full-length (>1300 bp) 16S rRNA gene sequences of isolates were phylogenetically determined relative to full-length 16S rRNA gene sequences of the SILVA database (SSUref 108 released 31/08/11) using the ARB software package. The newly imported isolate sequences were aligned with the sequences of their closest

relatives and the alignments were manually checked and corrected. An evolutionary distance dendrogram was constructed using two different methods. 1) Neighbour joining (NJ) tree algorithm with the Jukes Cantor substitution model and the bacterial position variability filter constructed within ARB. 2) A maximum likelihood (ML) tree constructed in RAXML-VI-HPC (version 7.0.4) (Stamatakis, 2006) with the General Time Reversible (GTRGAMMA) substitution model and 500 bootstrap replicates. ML trees were then imported into ARB for viewing.

2.7.1 Statistical analysis

Pyrosequences were analysed with two nonparametric multivariate statistical tests, Adonis(Anderson, 2001) and ANOSIM, performed in QIIME using the compare categories script on the weighted and unweighted Unifrac matrices.

2.7.2 Isolate sequence and pyrosequence comparisons

To determine the success of the cultivation three datasets were analysed in combination. 1) Denoised V1-V3 16S rRNA gene pyrosequences were taken from the CD-HIT-OTU pipeline before the final clustering step at 96% similarity (see section 2.7.7). 2) partial (V1-V3) 16S rRNA gene sequence reads from the isolates (see section 2.7.2) 3) 345848 good quality >1200bp bacterial isolate sequences were downloaded from RDP website (<http://rdp.cme.msu.edu>) to form the 'All RDP isolate' dataset. The pyrosequence dataset was combined with the isolate sequences or both the isolate sequences and the All RDP isolate dataset. Both combined datasets were analysed in QIIME as described in section 2.7.6.

Chapter 3 Cultivation method development

3.1 Introduction

The rumen harbours a complex ecosystem of micro-organisms that work in unison with the animal to break down fibrous plant material. Many of these microbes have yet to be obtained in pure culture and their roles within the rumen remain unknown. In New Zealand, dairy cows eat half a million tonnes of forage each day. New Zealand pasture is typically made up of a mix of ryegrass and clover, which contains a large proportion of fibre (Holden *et al.*, 1994; Kusmartono *et al.*, 1997; Waghorn *et al.*, 2004). Ruminants do not produce enzymes for degrading fibre and are only successful at using the fibrous components of forage plants as a significant food source because of the microbiota residing within the rumen. The ruminant provides a suitable habitat for the microbes to reside, while the microbes in return ferment plant matter to products that are able to be used by the host. Importantly, the ruminant regulates the system by excluding oxygen and controlling the passage rate, so that a partial fermentation of the feed results.

The rumen bacteria are primarily responsible for the breakdown of plant material. In particular, bacteria that attach to the plant fibres are important for fibre degradation (Koike *et al.*, 2003b; Weimer *et al.*, 1993). For practical reasons, much of the isolation work has concentrated on cultivating from the rumen fluid, ignoring what is attached to rumen solids, protozoa and the epithelial wall of the rumen. This is often on the assumption that the fluid associated bacteria are representative of those attached to the fibre. However, in recent years, it has become apparent that different bacterial communities are found within the different fractions in the rumen, attached to plant material, planktonic (free in the fluid), associated with the rumen epithelium, and attached to the surface of protozoa (Koike *et al.*, 2003b; Kong *et al.*, 2010; McAllister *et al.*, 1994). This may be a contributing factor to the high number of uncultivated species detected in the rumen. In this study, the focus was on the feed-attached community, on the assumption that the bacteria that constitute this community are different and play the major role in plant fibre fermentation in the rumen.

3.2 Rumen content fractionation test

A protocol reported by Larue *et al.*, (2005) was modified to separate bacteria tightly adhered to the feed material from planktonic and loosely adherent bacteria in rumen contents (total rumen digesta). This method was initially tested with rumen contents sampled from one cow (labelled 723) and one sheep (labelled 4572). To obtain the plant-adherent fraction, rumen contents were squeezed through a double layer of cheesecloth and the solid digesta were washed by resuspending in a RM02 base medium before being squeezed through cheesecloth again. The digesta were then resuspended in RM02 base medium containing 0.15% v/v Tween-80 and incubated on ice for 2.5 h to release the attached bacteria. The released bacteria were then collected by squeezing through cheesecloth and concentrated by centrifugation to obtain a cell pellet. At each step, a sample was taken to count the bacteria, thus following how many bacteria were being removed at each step. Microscopic cell counts were used to estimate the number of cells in each fraction. Each sample was accurately diluted until there were about 60 cells per field, the number of cells counted in 50 fields, and the total number of cells in the original sample calculated (Figure 3-1). The number of bacterial cells released from the adherent fraction of the plant material was very low, ~1.5% of the total, while around 15-30% of the total cells still remained attached to the plant material. Plant material fragments from the rumen samples before any treatment and after washes and Tween-80 incubation were examined microscopically and both were completely covered in adherent bacteria. Figure 3-2 shows a typical view from an untreated rumen sample.

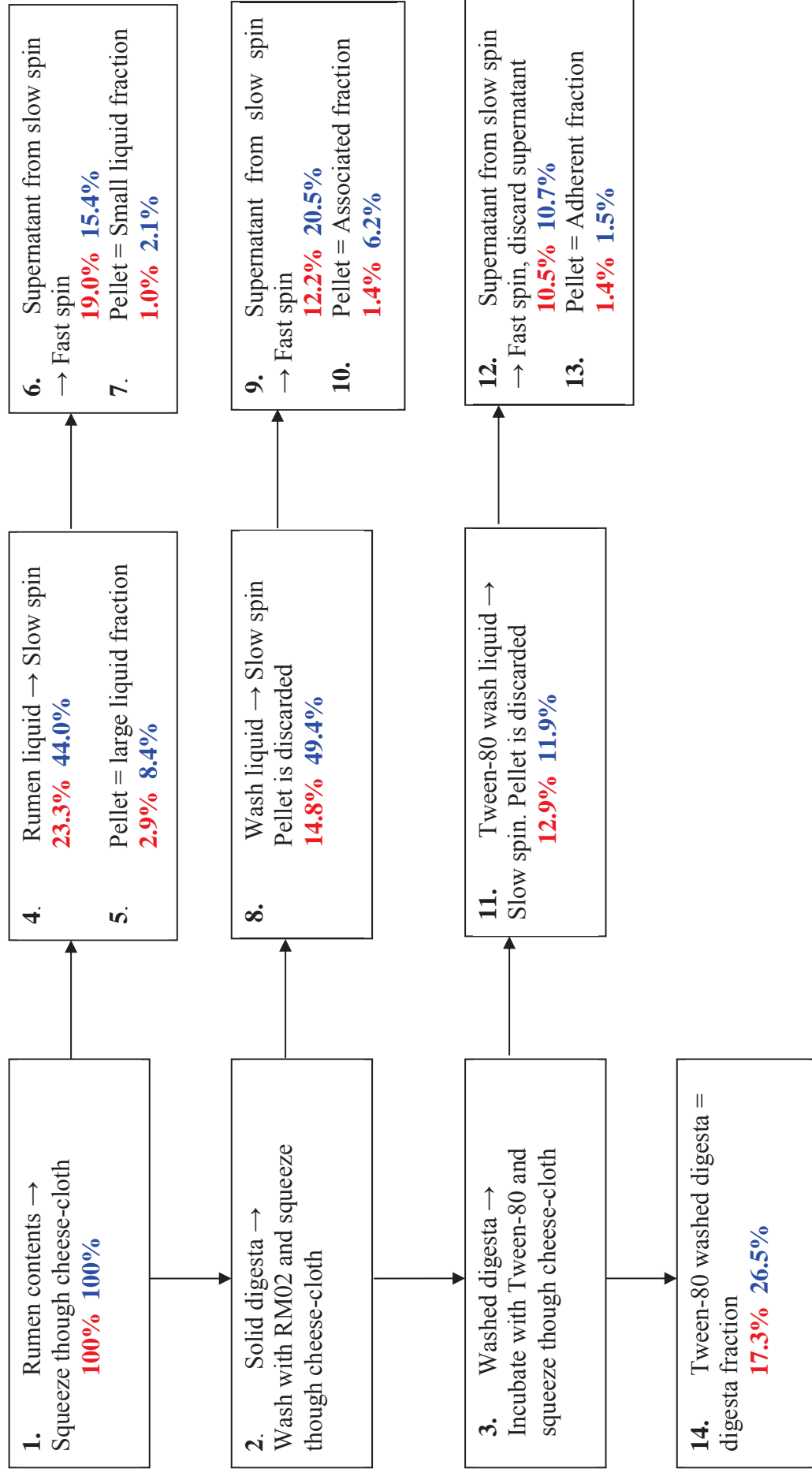


Figure 3-1. Estimated distribution of bacterial cells in rumen fractions. Estimates are calculated as a percentage of the bacteria counted in the total rumen contents from a cow (723) shown in red and a sheep (4572) shown in blue. Due to losses during processing fractions do not add up to 100%

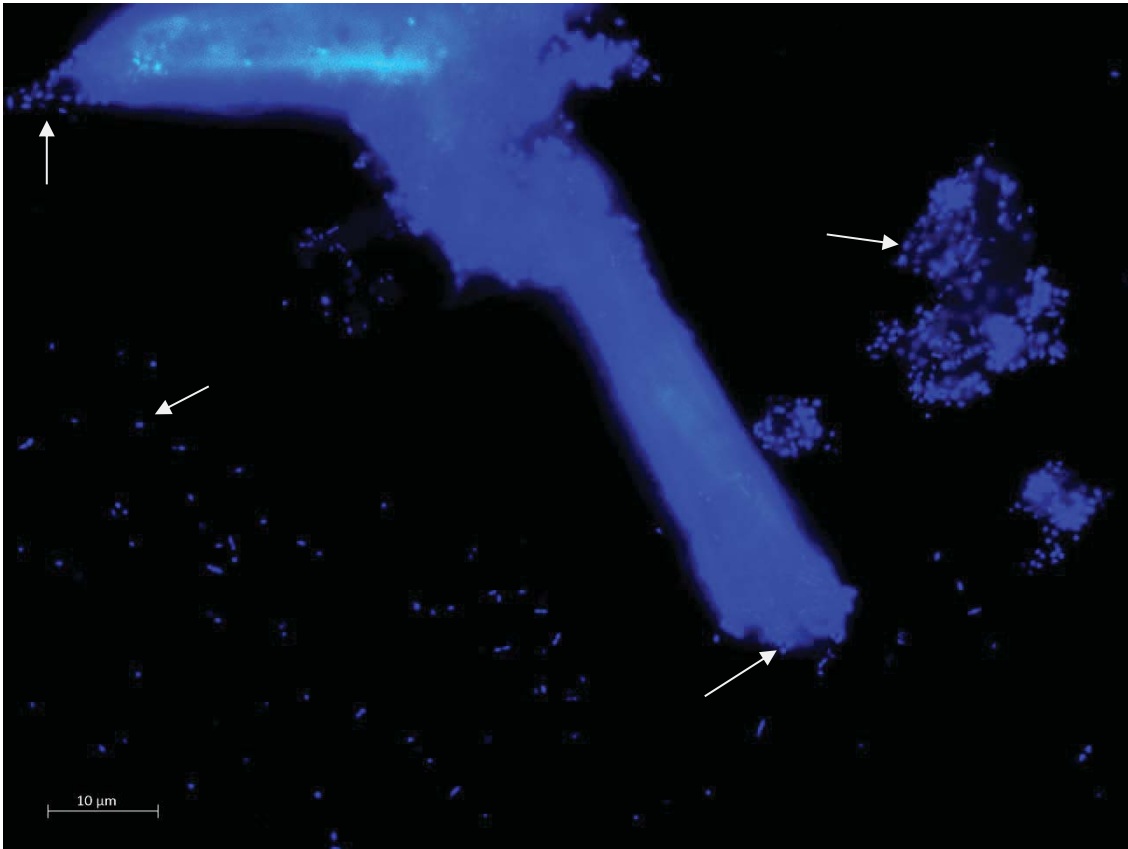


Figure 3-2. DAPI-stained micro-organisms attached to plant material in rumen contents from cow 723. Bacteria appear as small cells away from the plant material and as clumps on the plant material. A few examples are indicated with an arrow.

3.3 Optimisation of rumen content fractionation

A new method to separate bacteria that are tightly adhered to the feed material from planktonic and loosely adherent bacteria in rumen contents (total rumen digesta) was investigated. In the following sections two separate experiments to determine the amount of washing needed are described. Rumen contents were sampled from one cow (labelled 723) for each experiment.

3.3.1 Wash experiment 1

This experiment was performed to ascertain how many washes were required to remove unbound and loosely bound micro-organisms from feed material sampled from the rumen. Rumen digesta (50 g) were washed nine times by gently resuspending in

RM02 base medium, followed each time by squeezing through cheesecloth. The number of micro-organisms being washed off at each step was counted by microscopy (Figure 3-3). There were more micro-organisms released in the first wash than from the liquid (rumen fluid) fraction of the initial rumen sample. After four washes, the number of micro-organisms being washed off remained constant (Figure 3-3) and so four washes was chosen for subsequent experiments. Many (24.3%) micro-organisms were still attached to the digesta even after nine washes. One millilitre samples of each of the washes were centrifuged in a 2-mL tube to estimate the pellet size. The pellets were dark green containing small fragments of plant material and no obvious difference in pellet size was noticed for washes 1-6. Washes 7-9 had very little pelleted material, and the initial liquid fraction produced the largest pellet.

The composition of the bacterial community still attached to the feed material and being washed off at each wash step was assessed using DGGE. For comparison, DGGE analysis was also performed on DNA extracted from two grass samples: one a sample of sterile ryegrass (SG) and the other a cut mixture of grasses from the paddock the animals were grazing (NG). PCR amplification of the V3 region of 16S rRNA genes (Figure 3-4) produced bands of similar intensity except for DNA extracted from washes 6, 7 and 8, which were less intense, and wash 9, which was very faint. While attempts were made to standardise the amounts of DNA used at all steps this was not always possible or accurate at low DNA concentrations. This particularly affected wash samples 6-9. Overall the DGGE banding patterns (Figure 3-5) were very similar for all the rumen samples, but less intense for wash samples 6, 7 and 8. Wash sample 9 had very low DNA concentration (data not shown) and produced a very faint PCR band and corresponding faint DGGE profile. Consequently, this sample was omitted from the clustering analysis (Figure 3-6).

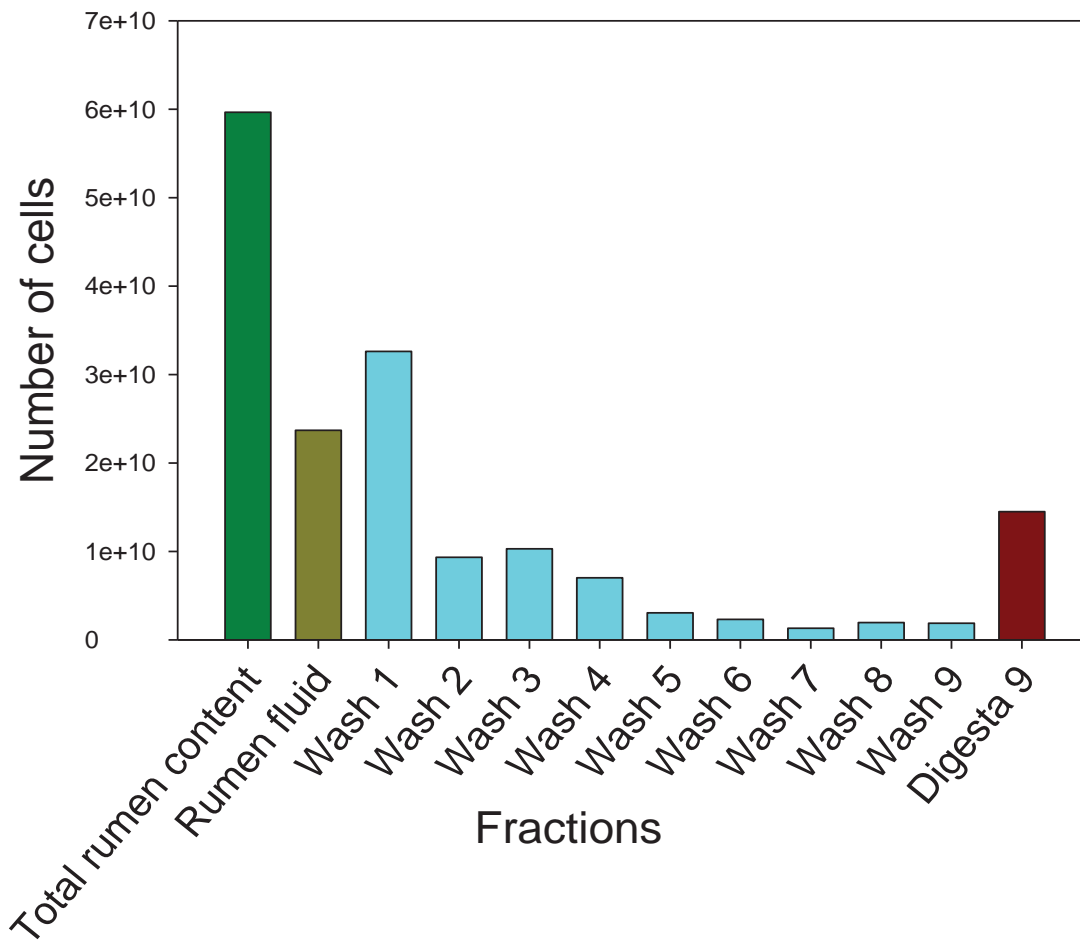


Figure 3-3. Microbial cell numbers during fractionation of rumen contents. The number of micro-organisms (per g of initial digesta) remaining in rumen fractions or washed off rumen contents were counted by microscopy. Fractions: Total rumen content = unprocessed rumen content, Rumens fluid = the rumen fluid separated from the solid digesta by squeezing through a cheesecloth, Washes 1-9 = liquid fraction from each wash, Digesta 9 = solid digesta remaining after nine washes. Each fraction was prepared once and only one count was obtained per fraction.

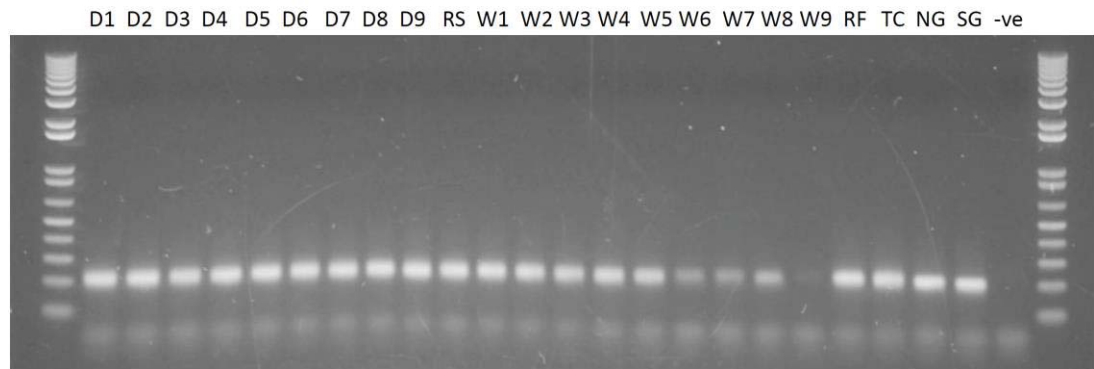


Figure 3-4. PCR amplification using DGGE primers targeting bacterial 16S rRNA genes (V3 region) in DNA extracted from rumen fractions: D1-D9 are the rumen digesta after 1-9 washes, RS = rumen solids before any washes, W1-W9 = the liquid fraction from each wash, RF = rumen fluid (the liquid fraction of rumen contents), TC = total rumen content, NG = normal paddock grass and SG = sterile ryegrass. -ve = negative control lane. The marker in the first and final lanes is 1 kb+ marker (Invitrogen).

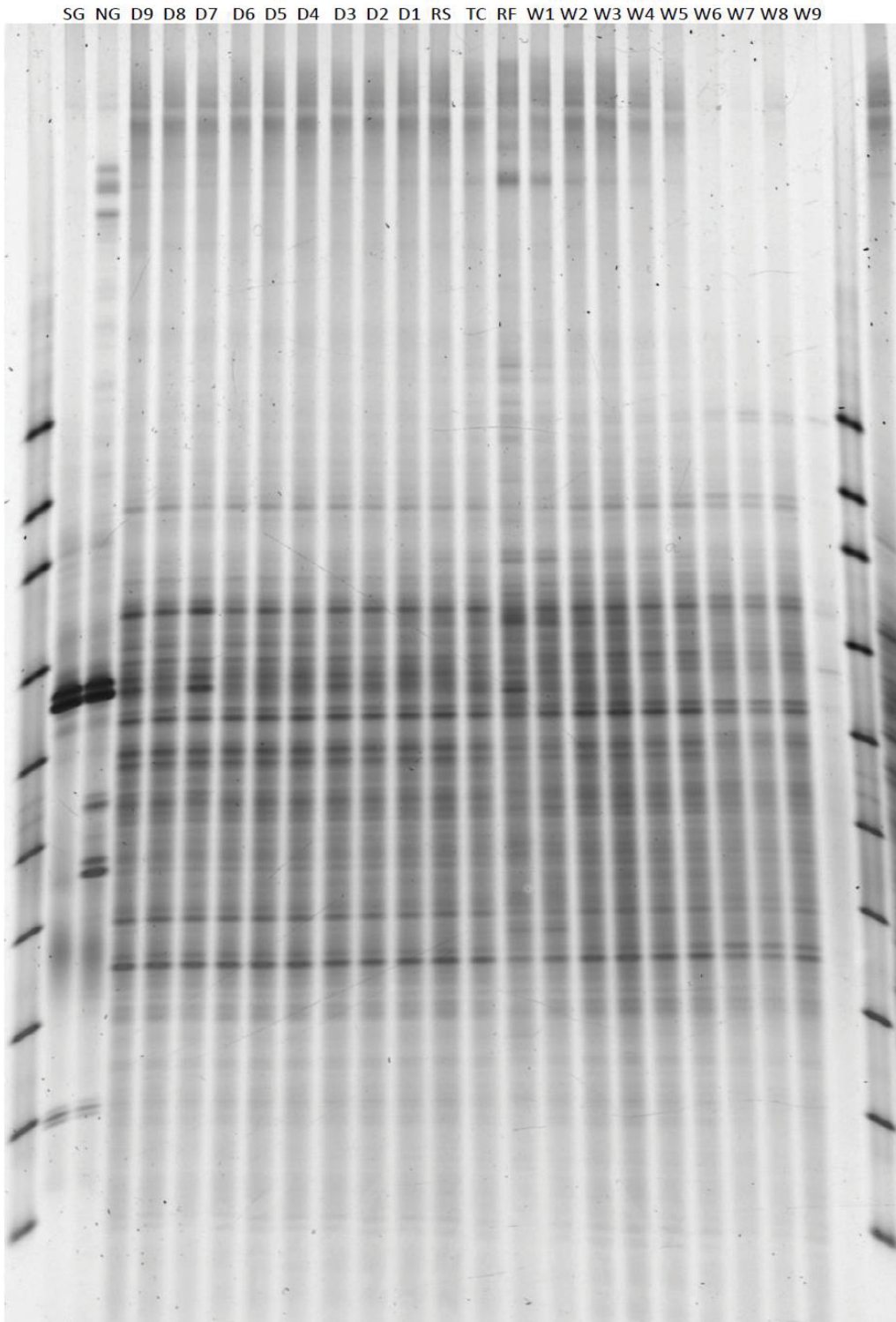


Figure 3-5. DGGE fingerprints of bacterial 16S rRNA genes (V3 region) from successively washed rumen fractions. Sterile ryegrass (SG) and normal paddock grass (NG). D1- D9 are the rumen digesta after 1-9 washes and RS = rumen solids before any washes. TC = total rumen content. RF = rumen fluid (the liquid fraction of rumen contents) and W1-W9 = liquid fraction from each wash. The marker in the first and final lanes is DGGE MII (Invitrogen)

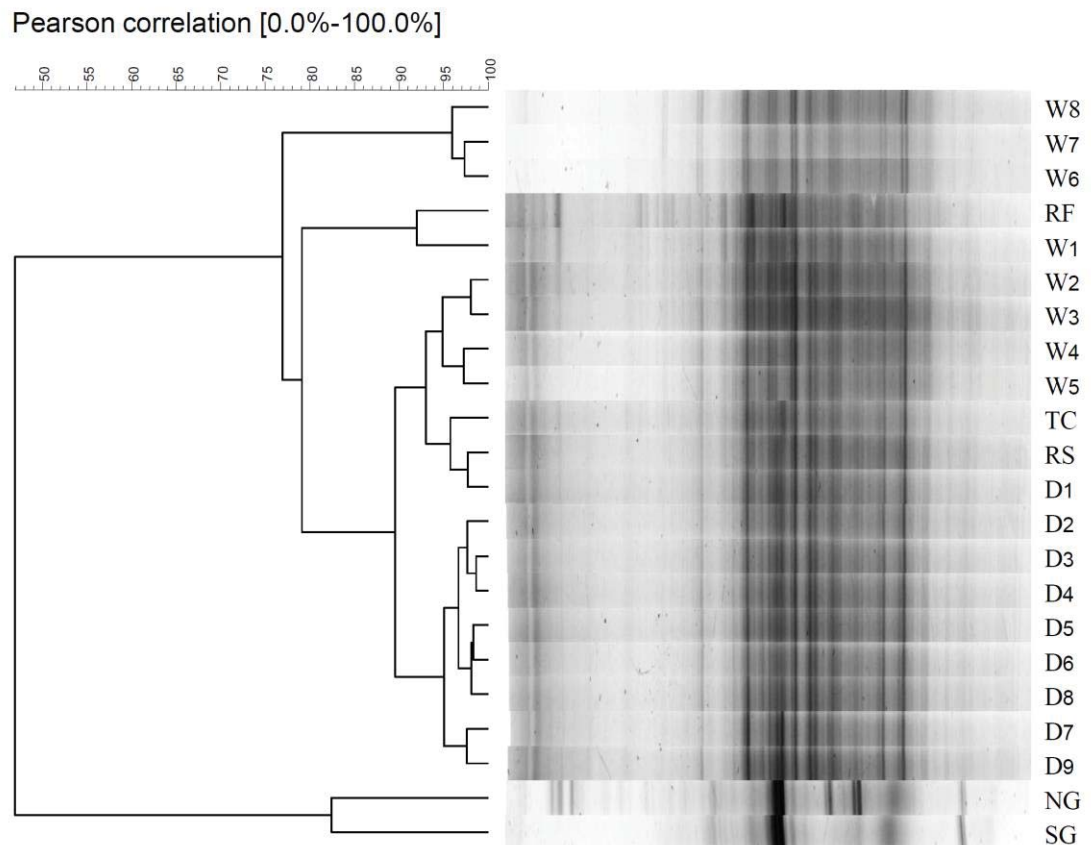


Figure 3-6. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) generated from DNA extracted from rumen fractions with successive washes. Differences between the patterns were analysed using the Pearson correlation and pattern relatedness is shown by branch length using the UPGMA method. Rumen fractions are: SG = Sterile ryegrass, NG = normal paddock grass, D1- D9 are the rumen digesta after 1-9 washes and RS = rumen solids before any washes. TC = total rumen content. RF = rumen fluid (the liquid fraction of rumen contents) and W1-W9 = liquid fraction from each wash.

3.3.2 Wash experiment 2

This experiment was performed to assess the effect of processing a larger amount of digesta with larger volumes of wash buffer using only four washes. The effect that mechanical blending had on the cell counts in the resulting fractions was also determined. Rumen digesta (~100 g) were washed four times in 400 mL RM02 base medium, followed each time by squeezing through cheesecloth. The number of micro-organisms being washed off at each step was microscopically counted (Figure 3-7). Figure 3-8 compares the counted microbial cells in the fractions from wash experiments 1 and 2. The total number of micro-organisms counted was 6.0×10^{10} /g digesta in wash experiment 1 and 1.2×10^{11} /g digesta in wash experiment 2. The number of micro-organisms remaining after the washes was also higher in wash experiment 2 compared to wash experiment 1 (5.0×10^{10} and 1.5×10^{10} /g digesta respectively). 75.8% of the microbial cells were found in the digesta before any washes and 41.8% were still adherent to the feed material after four washes. The number of micro-organisms dislodged in the first wash in experiment 2 was lower than the first wash in experiment 1 but was similar in washes 2-4. Changing the volume of wash buffer did not reduce the number of washes need to remove loose and non-adherent cells from the feed material. Later experiments were carried out with 50 g of digesta and 200 mL wash volumes.

To assess the efficiency of counting cells adherent to solid particles in samples, a subsample of the digesta that had been washed four times was disrupted in a Waring blender and compared to a parallel subsample that was blended. The number of micro-organisms counted in the sample was greater (1.6 - 3.3 times) when the sample was blended before counting (Figure 3-9). Because of this, further cell counts on rumen digesta fractions by microscopy were performed on blended samples. As a control, fresh plant material from the paddock the animals were grazing was blended in a similar manner to the rumen digesta and examined microscopically. Nothing was stained that could be mistaken for a microbial cell in the samples examined.

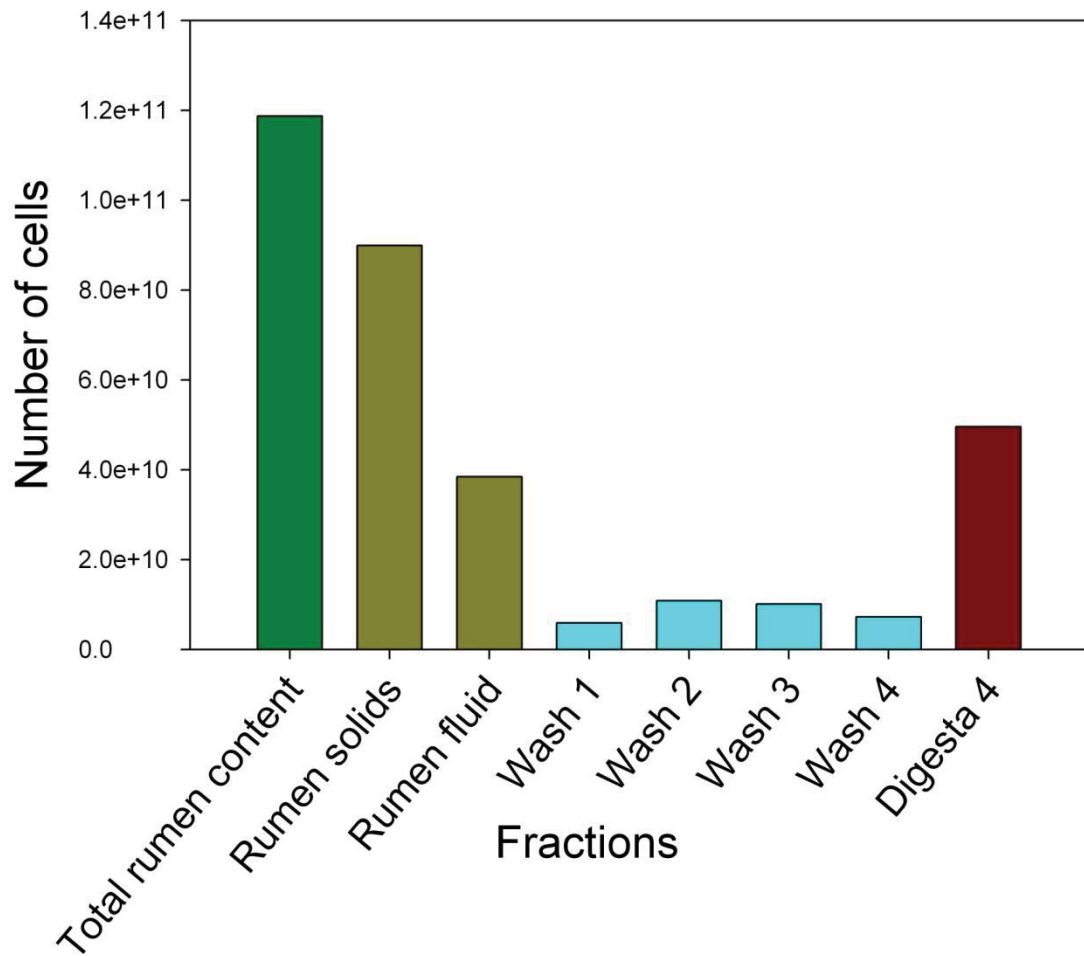


Figure 3-7. Microbial cell numbers during fractionation of the rumen contents. The number of micro-organisms (per g of digesta) remaining in rumen fractions or washed off rumen contents were counted by microscopy. Total rumen content = unprocessed rumen content, Rumen solids and Rumen fluid = the rumen fluid separated from the solid digesta by squeezing through cheesecloth, Washes 1-4 = liquid fraction from each wash, Digesta 4 = solid digesta remaining after four washes. Each fraction was prepared once and only one count was obtained per fraction.

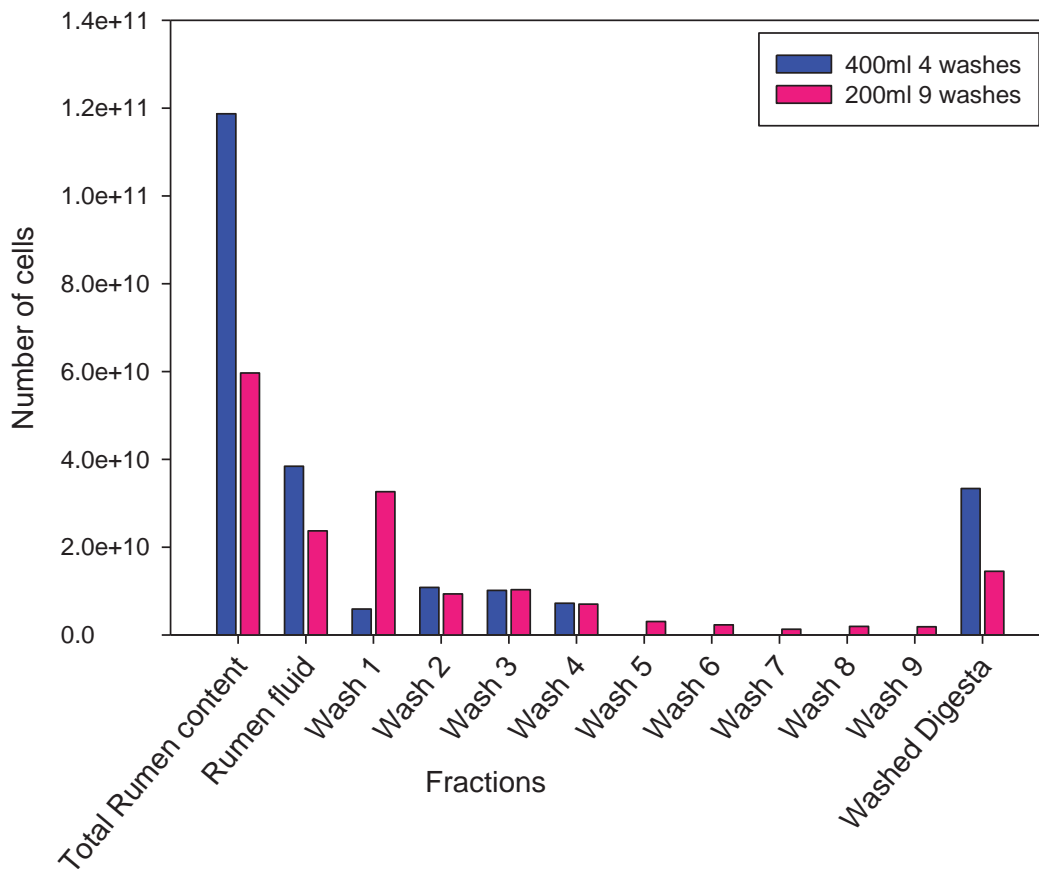


Figure 3-8. Comparison of the numbers of micro-organisms (per g of digesta) counted in each fraction when using either 200 mL of medium for 50 g or 400 mL of medium for 100 g to wash the digesta. Total rumen content = unprocessed rumen content, Rumen fluid = the rumen fluid separated from the solid digesta by squeezing through cheesecloth, Washes 1-9 = liquid fraction from each wash, Washed digesta = solid digesta remaining after four or nine washes.

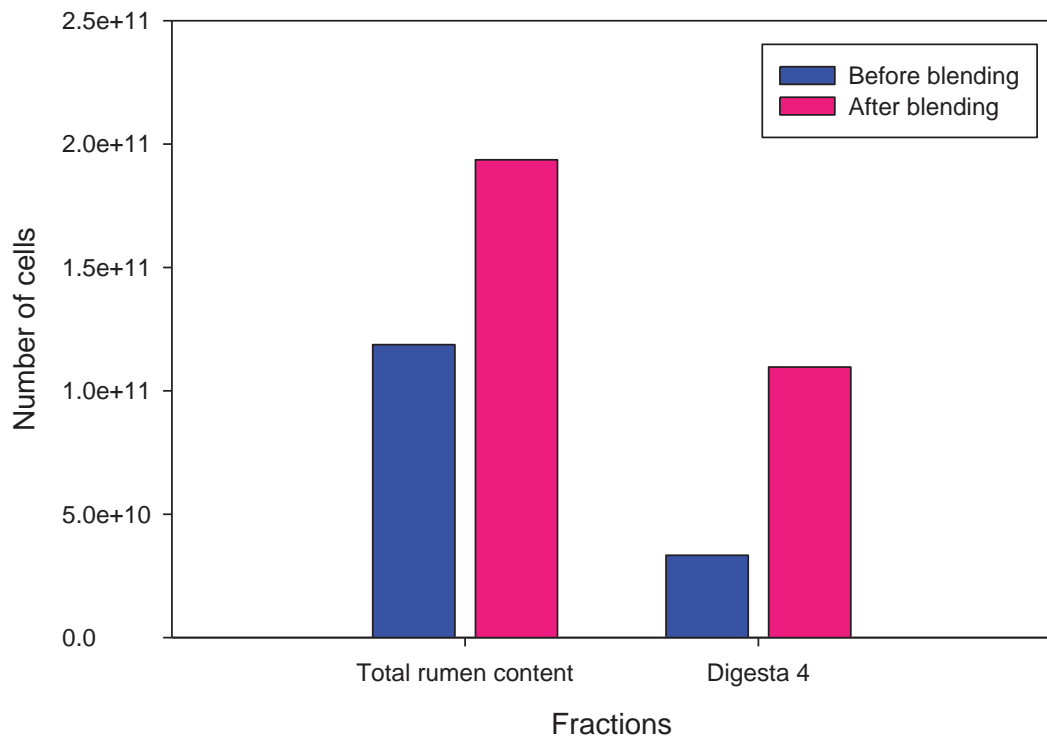


Figure 3-9. The number of microscopically counted micro-organisms in rumen digesta (per g of digesta), before and after blending of otherwise identical subsamples. Total rumen content = unprocessed rumen content, Digesta 4 = solid digesta remaining after four washes.

3.4 Trial cultivation

3.4.1 Estimating the inoculum size

Appropriate dilutions were estimated to obtain a target of 0.2 viable cells per tube as inoculum (which should result in growth in 20% of the inoculated tubes). This target was chosen using dilution theory to result in about 90% of growth positive tubes having come from a single viable cell (Button *et al.*, 1993). There were 1.1×10^{11} bacterial cells per gram of digesta in wash experiment 2. Starting from this assumption, 40 g of washed digesta was blended in 400 mL of anoxic RM02 base media. Serial dilutions then were made to dilute this inoculum 250,000,000 times. From the final dilution, a

further series (six in total) of smaller dilutions (each four fold) was used to inoculate 20 Hungate tubes each. These tubes would receive 28 to 0.03 cells each. However, not all of these cells would be viable. The culturability of bacteria on this media using blended ovine whole rumen content was between 7.6 and 21.2% (Kenters *et al.*, 2011). Using an estimate of the culturability of the counted cells of 10%, the tubes inoculated from the middle of the range (dilution 3) would then receive close to the target of 0.2 cells per tube (Figure 3-10). Because the actual culturability is likely to be different from 10%, using a range of dilutions increases the likelihood of capturing the dilution where the tubes receive 0.2 cells each.

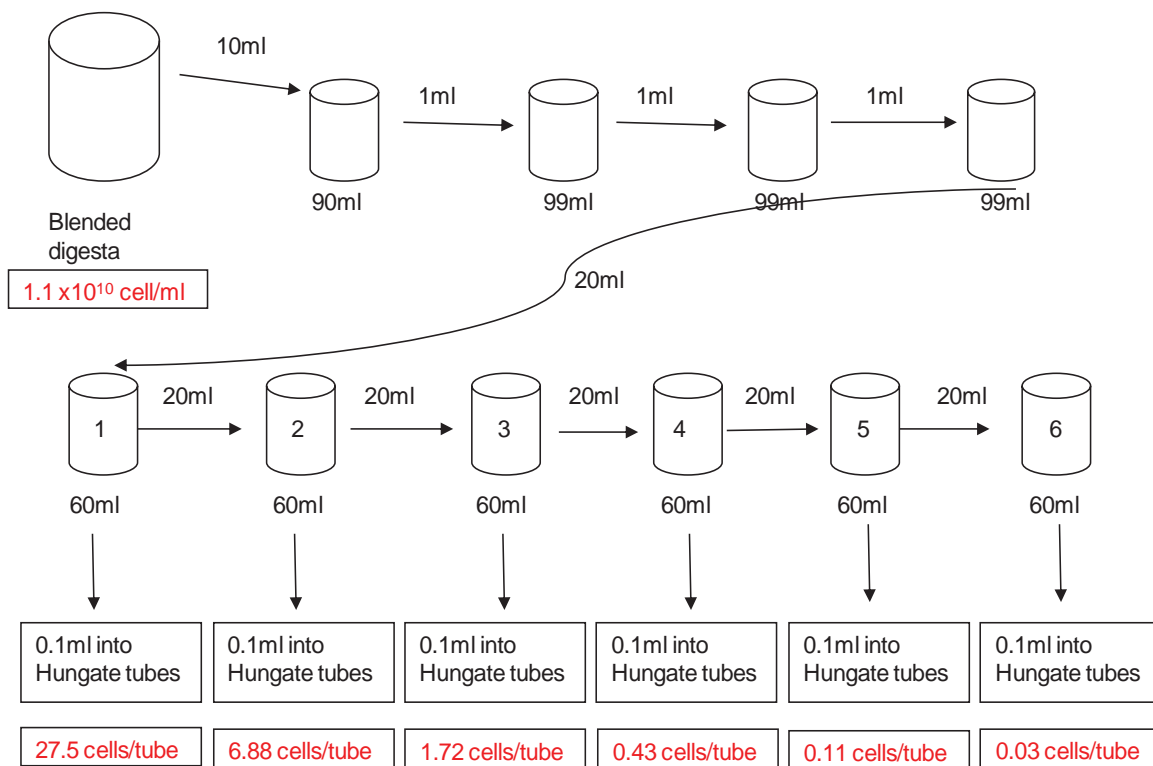


Figure 3-10. Estimated numbers of cells that will be inoculated into each tube from serially diluted blended, washed rumen digesta.

3.4.2 Estimating percentage of single cells

Inoculating tubes with single viable cells relies on being able to successfully detach the adherent cells from the plant material. Small clumps of plant material from the blending may have many cells still attached which would be inoculated together into a culture tube. The blended digesta sample was examined, counting bacteria that were found singly and those that were still attached to plant fragments or each other in clumps. An average of 480 clumps were each counted in triplicate samples (Figure 3-11). The clumps ranged from singletons to 47 cells, each of which is considered to be a possible viable unit. The average clump size was 1.7 cells after blending. 46.2% of the cells were singletons but these made up 74.0% of the potential viable units. Without blending, it was difficult to count accurately because the clumps of cells were often very large and often only half of the surface was visible.

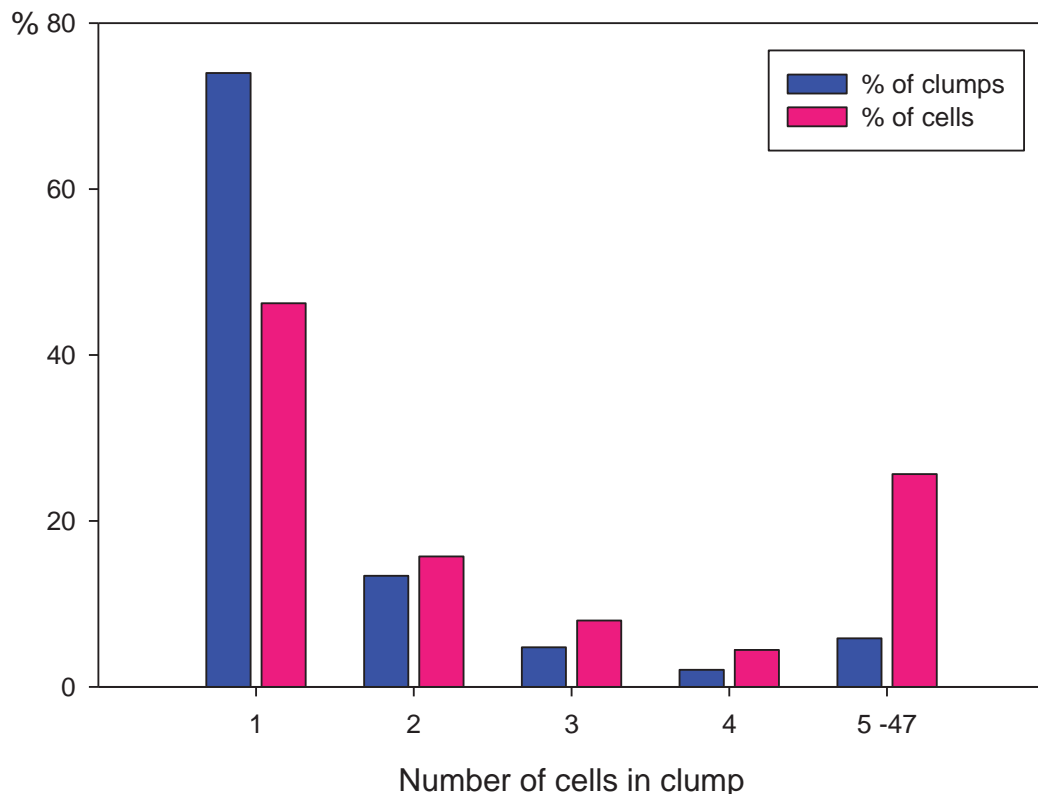


Figure 3-11. Number of cells observed as singletons, in pairs or in clumps with three, four or more cells in washed rumen digesta treated by blending. The pink bars represent the percentage of cells occurring in each category, and the blue bars represent the percentage of potential viable units (clumps) in each category.

3.4.3 Trial cultivation test

A small scale cultivation was performed to test the new fractionation method. Rumen contents were taken from a fistulated Friesian cow (cow 723) grazing on a ryegrass and clover pasture at Grasslands, Palmerston North. The blended adherent fraction was diluted as in Figure 3-10, and 20 tubes of medium were inoculated at each of six dilutions. Assuming similar numbers to those estimated above, this should have resulted in ~0.03 to 30 cells being inoculated per tube. Over 70% of the tubes that grew, did so after three days of incubation, but some took up to 14 days. No more growth positive tubes were observed after 14 days of incubation. The most probable number (MPN) of viable cells calculated from the growth positive tubes was 6.6×10^9 (lower confidence limit 4.3×10^9 , upper confidence limit 9.9×10^9) of viable cells per gram of rumen content (Table 3-1). The number of cells counted in the blended washed digesta fraction was 1.5×10^{11} /mL of rumen content and the culturability was calculated at 4.46%. Tubes from dilutions two and three received close to the optimal 0.2 cells per tube (Table 3-2). All growth positive tubes were sub-cultured and microscopically examined. 24 cultures were obtained, 21 appearing to be of uniform cell morphology and hence possibly clonal. The cultures were labelled with the letters DAZ, followed by the unique isolate number.

Table 3-1. Comparison of microbial counts from blended rumen fractions.

	Wash 2	Trial cultivation
Number of micro-organisms in whole rumen content (cells per g of rumen content)	1.9×10^{11}	NC ^a
Number of micro-organisms in washed digesta fraction (cells per g of rumen content)	8.3×10^{10}	1.5×10^{11}
MPN per g of rumen content ^b	NA ^c	6.6×10^9
Culturability ^d	NA ^c	4.5%
Percentage of adherent cells. ^e	43.0%	NC ^a

^a No cell counts were made on blended total rumen content. ^bMPN (Most Probable Number) was calculated from the percentage of growth positive tubes. ^c NA, no attempt at cultivation was made in wash experiment 2. ^dCulturability was calculated by dividing the MPN by the total number of cells counted. ^e Percentage of adherent cells was calculated by dividing the number of cells in the adherent fraction by the total number of cells.

Table 3-2. Growth of cultures in trial cultivation experiment on rumen contents from cow 723.

Dilution ^a	Number of cells inoculated per tube	Number of growth positive tubes after incubation for different times				% of growth positive tubes
		3 days	5 days	10 days	14 days	
1	31.42	12	14	15	15	75
2	7.86	3	5	6	6	30
3	1.97	1	1	1	2	10
4	0.49	0	0	0	0	0
5	0.13	0	0	0	0	0
6	0.03	1	1	1	1	5
Total		17	21	23	24	20

^a Dilutions 1-6 as per Figure 3-10

3.4.4 Identification of isolates

The 24 cultures obtained from the trial cultivation experiment from cow 723 were identified by comparing their partial 16S rRNA gene sequences with sequences from known isolates (summarised in Table 3-3). Three isolates appeared to be of mixed cell types, but only one of these (DAZ6001) produced sequence with a mixed trace. Two isolates (DAZ1008 and DAZ1014) did not produce a PCR product. These three isolates were eliminated from the further analyses. The remaining isolates fell into three bacterial phyla, but nearly all the isolates were in the phylum *Firmicutes*. One isolate (DAZ1007) was in the phylum *Spirochaetes*, and one (DAZ2005) was in the phylum *Actinobacteria*. 85.7% of the isolates were >97% similar to a previously cultured isolate. Three isolates (DAZ1006, DAZ1015 and DAZ2002) appear to be members of as-yet undescribed species. A further four isolates (DAZ1003, DAZ1010, DAZ2004 and DAZ3001) were similar to a non-validly name isolate.

Table 3-3. Descriptions of 24 isolates obtained from the trial cultivation experiment.

Isolate	Single cell type	Description	PCR product	Top BLASTN match to an isolate Genus and species (if named); isolate; GenBank accession for 16S rRNA gene	Percentage match
DAZ1001	yes	Chains of rods	yes	<i>Butyrivibrio fibrisolvens</i> ; NCDO 2221; X89970	99.60%
DAZ1002	uncertain	Oval cells, singly, in pairs or small chains	yes	<i>Streptococcus equinus</i> ; BP5-36; AB563266	99.80%
DAZ1003	yes	Rods in short chains	yes	Unnamed rumen bacterium; 3/9293-14A; DQ392995	98.40%
DAZ1004	yes	Chains of rods	yes	<i>Pseudobutyrvibrio ruminis</i> ; pC-XS2; AF202260	98.00%
DAZ1005	yes	Small crescent	yes	<i>Pseudobutyrvibrio ruminis</i> ; L4; AY699285	98.00%
DAZ1006	yes	Thin rods in short chains	yes	<i>Pseudobutyrvibrio ruminis</i> ; pC-XS7; AF202262	93.90%
DAZ1007	yes	Spirals and spheroplasts	yes	<i>Treponema bryantii</i> ; DSM 1788; FR749895	98.00%
DAZ1008	yes	Straight long rods	no		
DAZ1009	yes	Chains of ovals	yes	<i>Pseudobutyrvibrio ruminis</i> ; pC-XS7; AF202262	98.40%
DAZ1010	yes	Long straight rods	yes	Unnamed rumen bacterium; NK4A142; GU324402	99.10%
DAZ1011	no	Rods and cocci	yes	<i>Butyrivibrio hungatei</i> ; JK614; GU121459	99.60%
DAZ1012	no	Long thin rods with the occasional thick rod	yes	<i>Pseudobutyrvibrio ruminis</i> ; pC-XS7; AF202262	98.00%
DAZ1013	yes	Chains of rods	yes	<i>Butyrivibrio fibrisolvens</i> ; OB251; U77341	97.40%
DAZ1014	yes	Small and large spheres	no		
DAZ1015	yes	Chains of cocci	yes	<i>Ruminococcus flavefaciens</i> ; C1a; AM915271	94.40%
DAZ2001	yes	Rods in chains (large clumps)	yes	<i>Butyrivibrio fibrisolvens</i> ; L8; AY699274	98.10%
DAZ2002	yes	Rods in short chains	yes	<i>Butyrivibrio fibrisolvens</i> ; L8; AY699274	90.60%
DAZ2003	yes	Short fat rods	yes	<i>Lactobacillus vitulinus</i> ; TB-A09; AB425916	100.00%
DAZ2004	yes	Short fat rods	yes	Unnamed rumen bacterium; 7/94-26; DQ393006	97.40%
DAZ2005	yes	Irregular fat rods	yes	<i>Propionibacterium acnes</i> ; 1570; JF277163	100.00%
DAZ2006	yes	Thin rods in clumps	yes	<i>Pseudobutyrvibrio ruminis</i> ; pC-XS7; AF202262	98.20%
DAZ3001	yes	Rods in short chains	yes	Unnamed rumen bacterium; E2; DQ393023	98.90%
DAZ3002	yes	Short rods in chains	yes	<i>Pseudobutyrvibrio ruminis</i> ; L4; AY699285	98.70%
DAZ6001	no	Short rods and cocci	yes/mixed		

3.5 Discussion

Bacteria that attach to ingested feed material in the rumen are known to play an important role in fibre degradation (McAllister *et al.*, 1994; Michalet-Doreau *et al.*, 2001). Members of this community may have been overlooked because previous cultivation work has often used rumen fluid as the inoculation source. The assumption may have been that the rumen fluid was representative of the whole rumen community, or that it was more practical to sample and dilute the fluid rather than the bulky digesta which is not able to easily be inoculated directly into isolation media. Recently, the plant-adherent fraction of the rumen microbial community has been examined in order to gain a greater understanding of the organisms responsible for fibre digestion (Kong *et al.*, 2010; Larue *et al.*, 2005; Tajima *et al.*, 1999). These studies showed there were differences in the community composition of feed adherent and planktonic rumen fractions.

The isolation method and the media type used influence which bacteria are isolated. Some species such as *Butyrivibrio* sp., can be routinely isolated from rumen samples, while many others remain uncultured (Kopečný *et al.*, 2003). The bias created by culturing the 'easy to grow' bacteria should be addressed in order to obtain representatives of each species present in the rumen. One way to reduce the cultivation bias is to inoculate growth media with a single viable cell, thus giving each cell a chance to grow without competition. This can be achieved by cell sorting (Czechowska *et al.*, 2008; Nebe-von-Caron *et al.*, 2000) to give highly reproducible distributions of cells, or it can be done by diluting cells so that the probability of only one single viable cell being introduced into a culture vessel is high. However the use of cell sorting provides technical challenges and must be done while keeping the cells under anaerobic conditions or the cells would no longer be viable. To the best of my knowledge this has not yet been demonstrated. Using dilution theory, a suitable point where it is likely that the inoculum contains only one cell can be calculated (Button *et al.*, 1993). By inoculating many culture vessels (e.g. tubes) with this inoculum, many cultures that are each derived from a single cell can be grown. As the number of cells in the inoculum is reduced, the probability of growth being initiated from a single cell increases. If the average inoculum size is 1, greater than 50% of the resultant cultures are estimated to

arise from more than one viable cell but all of the inoculated tubes will be growth positive. If the average inoculum size is <1 , not all the inoculated tubes will receive a viable cells but those that do are more likely to receive just one viable cell. Here a target of 0.2 viable cells per tube was selected, which should result in 20% of the vessels being growth positive. This is a compromise, to maximise the number of cultures that arise from a single cells while still obtaining enough cultures. While only 20% of the culture vessels will grow, over 90% of these are expected to have arisen from a single cell. (Figure 1-5)

The direct microscopic counts of rumen micro-organisms greatly exceed the cultivable counts (Hungate, 1966). This has been observed for many other microbial systems (Hugenholtz, 2002; Vartoukian *et al.*, 2010) and is referred to as the great plate count anomaly (Staley *et al.*, 1985). A contributing factor to low percentage of cultivated species is the difficulty of sufficiently replicating the complex rumen environment. Medium RM02 has been designed to better mimic the physico-chemical conditions prevailing in the rumen. This medium, in combination with diluting the inocula, was successfully used to isolate bacteria belonging to putative new genera from the rumen contents of pasture grazing sheep (Kenters *et al.*, 2011). RM02 has additional benefits of being nearly colourless and free of any particulate matter (introduced into most rumen fluid containing media). This allows even weak growth to be visible, enabling bacteria that grow poorly in laboratory conditions to be recovered. Additionally, dilution in liquid media eliminates the need to use labour intensive methods like roll tubes. Isolates of interest can be identified first, then purity ensured by subsequent application of roll tube or plating methods to derive truly clonal cultures. The aim of the work presented in this chapter was to find a suitable method to separate the plant-adherent micro-organisms from the rest and culture them.

The rumen content fractionation test was based on a protocol modified from Larue *et al.* (2005) who reported a method to separate parts of the rumen microbial community into three fractions: planktonic, and either weakly or tightly plant-adherent. Solid plant material was separated from the rumen liquid and washed to remove weakly attached micro-organisms. In the final step the plant-adherent micro-organisms were separated from the washed plant material by incubating with Tween-80. However, this method failed to release most of the adherent micro-organisms from the plant material. The

number of micro-organisms released was only around 1.5% of the total population and may not be representative of the adherent population. 15-30% of the total population of micro-organisms was still attached to the plant material after Tween-80 treatment. Microscopic observation of plant material before and after incubating with Tween-80 confirmed that micro-organisms were still attached after the Tween-80 treatment. The adherent population was apparently very tightly attached to the feed material. In an attempt to overcome this, a physical disruption method was explored. This might dislodge the micro-organisms or disrupt the feed material, both of which would result in more finely-divided viable units (particles that could result in microbial growth).

In order to determine how many washes were needed to remove planktonic and loosely attached micro-organisms, a sample of rumen digesta was serially washed nine times. The micro-organisms that remained attached to, and that were washed off, the feed material were examined microscopically and with DGGE fingerprint analysis. Microscopic counts revealed most of the microbial cells were removed with the first wash of the feed material. The number of cells removed declined strongly with further washes, with very few being removed after four washes. A barely detectable amount of DNA was extracted from material removed by the 9th wash. Even after nine washes, some 25% of the micro-organisms were still attached to the digesta, suggesting that continued washing will not remove all the adherent cells. As increasing the volume of the wash medium and digesta did not improve the method (numbers of micro-organisms removed in washes 2-4 were similar) volumes of 50 g of digesta and 200 mL of wash media were chosen for later experiments.

From wash experiment 1 it was determined between 5 and 10% of the adherent population of micro-organisms were removed with each wash step. These may be micro-organisms that are continuously being washed off or freed from the matrix of feed material or that are attached to the small fragments of digesta that pass through the cheesecloth and into the wash. DGGE profiles of bacterial 16S rRNA genes generated by PCR amplification from rumen digesta and cells washed off the rumen digesta in nine successive washes showed a remarkable similarity in all the rumen fractions. Initially, the unwashed digesta community (RS) and digesta after just one wash (D1) appear like the total rumen community (TC). After the second wash, the plant-adherent community (D2-D9) can be distinguished from the wash communities and the total

community, although the differences are modest. The profiles of bacteria present in washes 2-5 were very similar to each other and more similar to the total community (TC) than profiles from wash 1 (W1) and the rumen fluid (RF). These presumably represent the loosely adherent population (with many members in common with the adherent population) while the rumen fluid (RF) and wash 1 (W1) represent the planktonic population and washed digesta (D2-D9) represents the plant-adherent community. Even though the amount of DNA loaded onto the gel was normalised DGGE profiles from wash 6-8 were less intense and clustered away from the rest of the washes. This corresponds with a decrease in cell pellet size in these washes corresponding with a fainter PCR product. Although these may be genuine differences, there is a possibility they could be an artefact due to PCR bias at low DNA template concentrations (Chandler *et al.*, 1997). It should be noted DGGE analysis is able to show major community members but some subtle differences in community structure may have been beyond the detection limit of DGGE (DNA extracted from 10^1 - 10^8 CFU of bacteria per mL) (Bester *et al.*, 2010; Ercolini, 2004).

There were obvious differences in the DGGE profile from the rumen fluid (RF) component of the rumen contents and some of the unique bands are seen to a lesser extent in wash 1(W1). From the subsequent washes (W2-W5) the community more closely resembles the total community (TC) and the unwashed digesta (RS) than the rumen fluid (RF). This indicates the planktonic and adherent communities could be separated with just one wash. However, there is a danger if the bacteria in the planktonic fraction are more readily cultivable in liquid media. They could then be over represented in the resulting isolate collection, and so a more rigorous removal of planktonic cells trapped in the feed material is warranted. Also, more washes are needed to remove the loosely associated fraction from the tightly plant-adherent fraction. The DGGE profiles of the washed digesta after 2-9 washes were very similar to each other, showing that, as more bacteria are washed off, the community does not change. This was also seen in a study that examined initial bacterial colonisation of plant material, where more vigorous washing of the plant material removed more cells but did not change the attached communities (Edwards *et al.*, 2007). To be certain that only the plant-adherent fraction was being cultured, four washes with anaerobic medium was chosen. After this, the number of bacteria being removed with each wash was minimal.

Universal bacterial primers can also amplify chloroplast DNA from plant material, confounding the interpretation of DGGE profiles (Dent *et al.*, 2004). Primer 338f matches chloroplast 16S rRNA genes (Edwards *et al.*, 2007). The primer 338f used in this work had the addition of a GC clamp for DGGE analysis, and it was not known if this pair (338f and 515r) would also amplify chloroplast DNA. Bands were amplified from DNA extracted from both sterile ryegrass and normal paddock plant material (comprised of a mix of grass and herbs) using these universal bacterial primers for the V3 region. The DGGE profiles from these plant materials were much less diverse than the rumen sample DGGE profiles. Two very strong bands were seen in both samples and bands at the corresponding position were seen in the digesta samples, particularly after seven washes. This indicated that plant DNA was contributing to the banding profiles generated of the rumen samples and these bands became more prevalent as more bacteria were removed. However, this did not alter the interpretation of the wash experiments, as there were only a few bands associated with the plant material compared to the many in the rumen samples.

Initial microbial cell counts were made by microscopy on solid plant digesta, but these were technically difficult due to the large plant fragments (up to 4 cm). Representative portions of the sample could not be taken as large fragments would not fit in the 8-mm diameter wells of the counting slide. Within a well, the distribution of micro-organisms was heterogeneous, with hundreds of micro-organisms attached to large plant fragments and other views with very few micro-organisms. For large plant fragments, only half of the attached micro-organisms were visible using the microscopy, with the other half being obscured by the feed material itself. Blending the samples disrupted the feed material and enabled more accurate cell counts that were 1.6 - 3.3 times higher. The total, fluid and adherent fractions were blended before cell counts were performed. The blending procedure was very effective at breaking up cell clumps. After blending the digesta sample, 77.8% of cell clumps were single cells. The average clump size was 1.7 cells, which affected the dilutions required to obtain an average inoculum of 0.2 cells and also affects the requirement for a single cell inoculum. For the purpose of dilution, a small clump of cells is treated as a single unit and will be introduced into a culture tube together. This may increase the number of mixed cultures expected if there are more than one cell type in the clump and more than one cell

initiates growth. In the trial cultivation experiment, the dilutions that received close to 0.2 viable cells per tube (dilutions 2 and 3) yielded no obviously mixed cultures, so on this small scale it appears that the expected number of mixed cultures (~10%) was not exceeded.

The proportion of micro-organisms associated with plant material (adherent and loosely-adherent) can be 70-80% of microbial organic matter (Craig *et al.*, 1987). Using a cell separation method based on that of Larue *et al.* (2005), the percentage of micro-organisms in the adherent fraction was estimated at 15-30%. In wash experiment 1, 25% were still adherent after nine washes and in wash experiment 2, 42% were still adherent after four washes. However 75.8% of bacteria were associated with the feed material before the digesta was washed. Direct microscopic counts of blended total rumen contents were 1.9×10^{11} cells /g of content. This is higher than some reported values of $1-10 \times 10^{10}$ /mL, but these were expressed per mL of content and as it is not reported how the samples were treated before counts were made (Hungate, 1966; Waghorn *et al.*, 2007).

The number of dilutions needed to reach a target of 0.2 viable cells inoculated per tube (= 20% of tubes being growth positive after incubation) was estimated assuming that the culturability of the total blended digesta was around 10%. A series of close dilutions were used to inoculate 0.03 - 31.42 cells (not all will be viable) into each tube, so even though the culturability for the trial cultivation was calculated at 4.46%, just over half the estimated value of 10%, the target of 0.2 viable cells was still achieved. The dilutions series used as inoculum in the trial cultivation experiment encompassed the point where 20% of the tubes grew (between dilution 2 and 3). All of the growth positive tubes were sub-cultured. Even though dilution 1 with 75% growth positive tubes was likely to have more tubes in which growth initiated from more than one viable cell, only three tubes from dilution 1 appeared to have two distinct cell types. From all dilutions in the trial cultivation experiment, 87.5% of the resulting cultures appeared to be clonal, although the cultures would still need to be grown on solid media and a single colony picked to be sure they are pure. The aim for this work was to isolate, identify and analyse the bacteria first, then to select which members warrant further investigation and then confirm their purity. The rationale behind this was that a

community analysis would be made on many isolates without the difficult and time consuming purification steps, which can be performed only on the isolates of interest.

A variety of isolates were obtained in the trial cultivation experiment. These isolates were from three phyla: *Spirochaetes*, *Actinobacteria* and *Firmicutes*. The majority of the isolates were *Firmicutes*, which are commonly isolated from the rumen. Even from this small number of isolates, three appear to be novel (with 16S rRNA genes <97% similar to those from known cultures), demonstrating that previously uncultured bacteria can be easily obtained using this isolation method. Several known ruminal bacteria species were also isolated including: *Butyrivibrio fibrisolvens*, *Pseudobutyrvibrio ruminis*, *Treponema bryantii* and *Butyrivibrio hungatei*.

Chapter 4 Isolation

4.1 Introduction

Since Hungate developed techniques suitable for routine cultivation of anaerobic bacteria from the rumen (Hungate, 1947), hundreds of bacteria have been isolated and characterised (Kong *et al.*, 2010). Krause and Russell (1996) surveyed the literature and found that only 22 species of predominant ruminal bacteria had been described. However, these only represent a fraction of what has been detected by molecular surveys, in one recent example by Kim *et al.* (2011c) found 88 bacterial genera and six archaeal genera therefore it is considered that the species diversity of the rumen environment is far greater than what was originally thought (Firkins *et al.*, 2006). With the use of molecular techniques, especially the use of the 16S rRNA gene as a phylogenetic marker the estimated number of bacterial species in the rumen has greatly increased indicating the majority of bacterial species have yet to be isolated and classified (Edwards *et al.*, 2004; Kim *et al.*, 2011c; Kong *et al.*, 2010; Tajima *et al.*, 1999). Previous studies have resulted in tens to the low hundreds of new isolates (Bladen *et al.*, 1961; Caldwell *et al.*, 1966; Koike *et al.*, 2010). Previous experience with soil bacteria has shown that, in addition to using suitable media, increasing the number of isolates is important for increasing the diversity of isolates obtained (Janssen, 2008). This is because some groups of bacteria appear to initiate growth in microbiological media only rarely, decreasing their abundance among cultured isolates compared to their abundance in the source sample. To apply the same approach to the rumen this study aimed to isolate over 1000 bacteria to improve the chances of culturing some of the uncultured majority.

A method to isolate plant-adherent rumen bacteria was described in Chapter 3. This method has the advantages of not requiring specialised equipment beyond what is need for culturing anaerobes and being quick as well as being proven to be successful in isolating new genera. Dilution theory is used to calculate the point where the inoculum is diluted until it is likely that a resulting culture would have arisen from a single cell. By separating the inoculum to increase the likelihood that cultures received only a single viable cell, the bias towards isolating only the bacteria that grow most rapidly in

laboratory media may be reduced. This is used in conjunction with a liquid medium that mimics rumen conditions to isolate cultures that are likely to be clonal. Once grown and microscopically examined, cultures of one cell type are presumptively pure. The cultures can be identified by sequencing their 16S rRNA genes, and then isolates which warrant further investigation can be cultured to ensure purity by dilution or by plating. The rationale behind this is that an initial analysis can be made on many isolates without the difficult and time consuming purification steps, which will be performed only on the isolates of interest.

The overall aim of the study described in this chapter was to culture previously uncultured rumen bacteria and to obtain a better representation of the plant-adherent community. After that, the phylogenetic placement of representative isolates was determined in more detail (see Chapter 6). Because the total rumen bacterial diversity seen in pasture-grazing dairy cows may not be seen in one animal sampled just once, multiple animals, sampling times and isolation media were used. By carrying out the isolations a number of times, the limitations of the experimenter's capacity to handle a great many cultures at one time was overcome. To capture the rumen bacterial diversity potentially present in grazing cows typical of the New Zealand herd, samples were taken from four representative animals (the selection of which is described in the previous chapter), in accordance with the suggestion of Bryant (1959) that repeat samples be taken over time to see patterns of change in the rumen communities. The chemical composition of pasture changes through the seasons (Parker *et al.*, 1996) and changes in diet are known to cause changes in the rumen communities (Kong *et al.*, 2010; Stiverson *et al.*, 2011; Tajima *et al.*, 2001). In this study, each animal was sampled five times over the course of a year, once in each season, with the fifth sample replicating the first one year later. To the best of my knowledge, this is the first time a cultivation strategy has run for over a year. To be able to compare the diversity of isolates from different animals and seasons, a common medium (RM02) was used for each sample times (Kenters *et al.*, 2011). Besides RM02, additional media were used at four of the five sample times in an attempt to capture bacteria that may have growth requirements not met by RM02. Because it was not expected that the number of isolates would be large enough to detect differences between animals, seasons, or media, unless these differences were very obvious, samples of rumen contents were also retained to

analyse changes using culture-independent molecular biological methods (see Chapter 5). Aside from deep analysis of the rumen microbial community composition, these molecular analyses were used to determine the effectiveness of the cultivation strategy (see Chapter 6).

4.2 Experimental design

4.2.1 Cultivation

The method for isolating plant-adherent bacteria was discussed in detail in Chapter 3 of this thesis. Rumen contents were sampled from four cows five times (one in each season) over the course of a year (Table 2-12). The plant-adherent fraction was obtained by washing the digesta (4 times) with basal RM02 medium, followed by squeezing through cheesecloth. The retained fraction was then blended to release the microbial cells from the plant material. The blended adherent fraction was diluted (Figure 3-10), and inoculated into tubes of culture medium. Assuming that the number of micro-organisms in the blended digesta fraction was similar to that obtained in the cultivation trial (Chapter 3), this should have resulted, over the complete range of dilutions, in ~0.03 to 30 cells being inoculated per tube. If the culturability is also similar, then in the middle of the dilution range each tube is likely to receive close to 0.2 viable cells. Growth was assessed as visible turbidity at approximately weekly intervals. The cultures were incubated for at least four weeks before being considered growth negative. Bacterial cells present in growth positive tubes were microscopically assessed for uniformity and type of cell morphology, and photographed for future reference (not shown). Cultures that appeared to be of more than one cell type were discarded. DNA was extracted from the putative pure cultures and stored at -20 °C for future analysis. Two tubes of each culture were grown in BY medium and anoxic glycerol was added before freezing at -85 °C.

4.3 Animal selection for the main sampling experiment

The objective of the animal selection was to obtain rumen samples from animals that were representative of the national dairy herd. The ruminally fistulated Friesian dairy cows used in this experiment were kept at Dairy NZ's Lye and Scott Farms (Vaile Road, Hamilton, New Zealand). From the nine available animals, six were selected

(four for sampling and two in reserve). Unfortunately, cow A (2225) died after the first sampling, and the reserve cow E (3122) was used as its replacement. The second reserve cow F (4109) was not used for any analyses. The age of the cows and their milk production were taken into account when selecting the cows to be sampled. At least one animal born in each of the years 2002, 2003 and 2004 were selected, and the individuals with the highest and lowest milk yields were also selected (Table 4-1). DNA for DGGE was extracted from freeze dried rumen contents from all nine animals and DGGE of the V3 region of the 16S rRNA gene showed remarkable similarity between all of the cows. The six animals selected spanned the diversity observed among the nine animals considered (Figure 4-1). Whole rumen contents from the cows selected were pooled, and the solid digesta separated and washed four times using the same method as the trial cultivation to obtain the washed digesta fraction. Cell counts done on this fraction were comparable to those obtained in wash experiment two and the trial cultivation, at 1.9×10^{11} /g of digesta or 2.7×10^{11} /g of rumen content.

Table 4-1. Milk production values of the nine animals under consideration for sampling.

Cow ID	Date of birth	Milk yield L/day ^a	Milk solids kg ^b	Selected animal code
2225	6/08/2002	16.1	303.31	A
2628	13/07/2002	15.4	382.66	
3122	29/06/2003	17.3	341.93	E
3517	16/07/2003	17.1	360.94	B
3950	20/07/2003	13.1	364.62	
4109	8/07/2004	13.6	329.17	F
4110	10/07/2004	11.6	320.4	C
4512	15/07/2004	13.2	341.68	
4519	22/07/2004	13.1	345.14	D

^a Average daily milk yield over seven days taken from 13-02-2009.

^b Total milk solids (fat + protein) collected for the lactation up until 20-02-2009.

Data obtained from personal communications with Dr Garry Waghorn, DairyNZ, Hamilton, NZ.

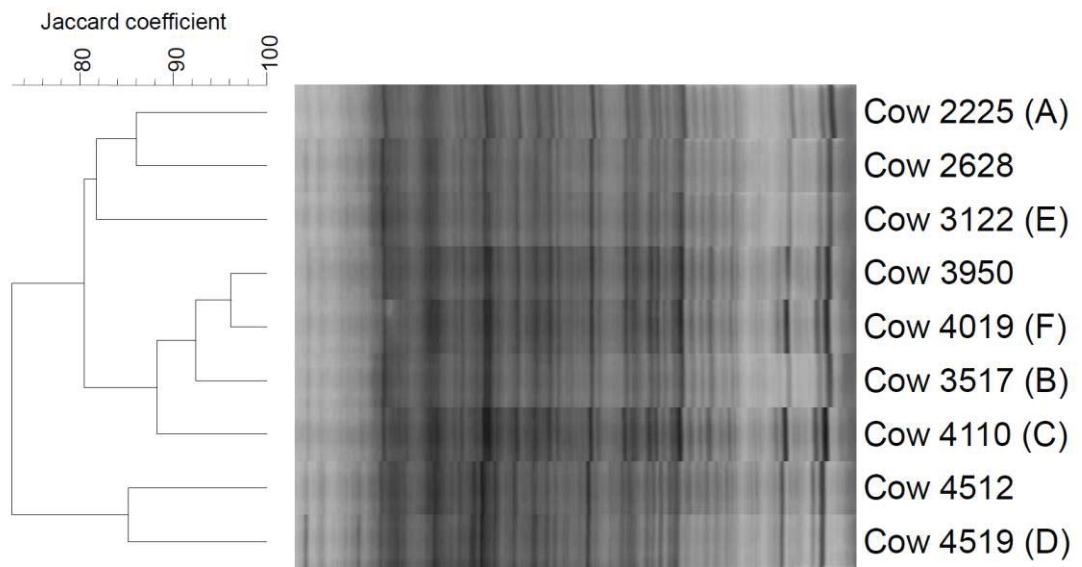


Figure 4-1. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) generated from DNA extracted from rumen contents of nine animals under consideration for sampling. Differences between the patterns were analysed using the Jaccard correlation and pattern relatedness is shown by branch length using the UPGMA method. DGGE gel courtesy of Dong Li (AgResearch, Grasslands, NZ)

4.3.1 Sampling and media

All culture tubes containing the RM02 medium were coded according to the month of the sampling:

M – May 2009

A – August 2009

N – November 2009

F – February 2010

L – May 2010 (last)

Media other than RM02 are collectively referred to as the ‘other’ media and RM02 as the ‘main’ medium. All media were prepared using the same anaerobic techniques and (with the exception of 98-5) supplemented with a sugar and vitamin substrate mix containing; D-glucose, D-cellobiose, D-xylose, L-arabinose, Na L-lactate, casamino acids, bacto peptone, yeast extract and vitamin mix. For RM02 and RM02-based media, this mix was dissolved in clarified rumen fluid and designated GenRFV. For the media already containing rumen fluid (BY and clarified rumen fluid), the mix was dissolved in water. The sample code for the type of media and sampling month is summarised in Table 4-2.

At the August 2009 sampling, three other media were compared with RM02. These media were designed to test whether adding a solid surface for bacteria to attach to would increase their culturability. The nomenclature for these ‘other’ media was as follows:

YA – RM02 medium with the addition of 0.5% w/v bacteriological agar

YG – RM02 medium with the addition of 0.5% w/v ground dried ryegrass

YS – RM02 medium with the addition of 2-5 cm of sisal string

YR – RM02 medium (control)

A control of RM02 medium was included, using the same dilution range and number of tubes inoculated as the ‘other’ media. With YA medium, the agar set at bottom of the tube while the top remained liquid. Growth of bacteria was often observed at the solid-liquid interface.

At the November sampling, two new media were used. These media were selected to explore whether culturability and isolate diversity could be increased using a more

complex medium and one with different substrates. The nomenclature for these ‘other’ media was as follows:

XB – BY medium

XP – RM02 medium with the addition of 0.08% w/v pectin (from apple)

At the February sampling, two new media were used. The nomenclature for these ‘other’ media was as follows:

WC – CRF medium, clarified rumen fluid (100%)

WT – RM02 medium with the addition of titanium (III) NTA solution

Many of the WT media tubes changed colour from clear to pale yellow over 2-6 weeks of incubation and a loose white precipitate formed. In some batches, but not others, WC medium formed a dark brown precipitate. These batches were not used for the isolation experiments in case poor growth was obscured.

At the May 2010 sampling two new media were used. The nomenclature for these ‘other’ media was as follows:

V9 – Medium 98-5

VC – CRF+sisal, Clarified rumen fluid (100%) with the addition of sisal string

Each combination of sampling month, animal and medium was treated as a separate cultivation experiment.

4.3.2 Cultivation dilution range

The blended plant-adherent fraction was diluted before being inoculated into culture media, to obtain an inoculum with a theoretical 0.2 viable cells per tube (which should give rise to growth in 20% of the inoculated tubes). A series of 4-fold dilutions were used to inoculate the growth media. As it was established which dilutions consistently produced the expected number of growth-positive tubes, fewer dilutions were needed to obtain the same number of cultures in RM02 medium (Table 4-2). During the first May sampling, 60 tubes were inoculated for each of five dilutions of the inoculum from each animal. For the August sampling, 60 or 90 tubes were inoculated for each of four dilutions of the inoculum from each animal. For the November, February, and final May samplings, 60 tubes were inoculated for each of three dilutions of the inoculum from each animal. The previously untested ‘other’ media had the

potential to increase or decrease the culturability, so the larger range of five dilutions (20 tubes each) of the inoculum from each per animal was used. For each sampling, a dilution that covered the targeted percentage (20%) of growth-positive tubes was obtained (Table 4-3). The most probable number (MPN) was calculated from the numbers of growth positive tubes in each dilution. A summary showing the average of the MPN values and their associated confidence intervals for each medium type are shown in Figure 4-2. Tubes of a dilution set that received close to the theoretical 0.2 viable cells per tube were selected for subculture.

4.4 Results

4.4.1 Sequencing controls

All of the media used to isolate bacteria contained rumen fluid, and may have contained intact DNA derived from lysed bacteria and present in the filtered rumen fluid that could potentially be amplified by PCR. DNA extractions were performed on each of the uninoculated media batches, purified using the filter drop method (2.5.3) and tested with the universal bacterial PCR primers used for isolate identification. Putative DNA was extracted from each type of uninoculated medium using both the Chelex 100 resin method and the ZR Fungal/Bacterial DNA miniprep kit, as described in the methods section (2.5.2). The new cultures were generally sub-cultured into RM02 or BY media, regardless of which medium they were isolated on, although a few cultures would only grow on the isolation medium. Therefore, most of the DNA extractions from the new isolates were performed on RM02 or BY media. To reflect this, seven tubes of RM02 medium and seven tubes of BY medium underwent extraction and sequencing, while only two tubes of each other type of media were investigated in the same way. In addition to this, batches of medium or individual tubes that were atypical were subjected to PCR analysis, even though these were not used for isolation or sub-culturing. The PCR was performed at least twice for each control. No PCR product was consistently amplified (in both reactions) from any of the media controls. One of the RM02 controls produced a weak band, but this result was not reproducible and the

resulting sequence from the PCR product was of very low quality without a clearly readable sequence.

One tube of BY media developed a precipitate and had turned pink, indicating the presence of oxygen and so suggesting that the rubber seal had failed. A strong PCR product was obtained from this tube of medium. The sequence of this product had a BLASTN identity of 89.9% to *Acinetobacter* sp. Ld3 (GenBank accession HQ659186). None of the isolates that underwent sequencing were similar to or even in the same phylum as this sequence.

4.4.2 Most probable number (MPN)

Linear mixed model analysis using Restricted Maximum Likelihood (REML) method was performed in order to assess the level of cow to cow variability of MPN estimates. The cow to cow variability was much smaller (2 orders of magnitude) than the sample variability, and hence was not contributing to the differences in MPN. The MPN values were averaged for each medium type and analysed with one way analysis of variance (ANOVA). The type of medium used for culturing plant-adherent bacteria had an effect on the number of bacteria culturable from the rumen samples as determined by MPN ($p < 0.001$), and this was confirmed with permutation analysis ($p = 0.02$). Overall, the data indicated little evidence of difference among MPN values from RM02 medium (ANOVA; $p = 0.365$). MPN values from the 'other' media types had larger error ranges (determined by the upper and lower confidence limits) except for the control YR (RM02) and WT media (Figure 4-2). The RM02 medium with addition of sisal string (YS) and clarified rumen fluid medium (CRF) had the highest MPN values with both animals sampled. Compared to the control YR treatment, four treatments (YS, WC, VC and V9) had significantly higher average MPN values as determined by Least Significant Difference (LSD) at a significance level of 0.05.

Table 4-2. Effect of medium type on most probable number (MPN).

Season	Sample month	Medium	Sample code	No. of culture tubes	No. of dilutions inoculated	Average MPN per g of rumen content ^a
Autumn	April 2009	RM02	Z	120	6	6.61×10 ⁹ (4.25×10 ⁹ , 9.92×10 ⁹)
Late Autumn	May 2009	RM02	M	300	5	6.33×10 ⁹ (4.97×10 ⁹ , 8.21×10 ⁹)
	August 2009	RM02	A	300	4	1.06×10 ¹⁰ (8.64×10 ⁹ , 1.34×10 ¹⁰)
Late winter	August 2009	RM02 + agar	YA	100	5	1.31×10 ¹⁰ (8.69×10 ⁹ , 1.99×10 ¹⁰)
	August 2009	RM02 + sisal	YS	100	5	2.66×10 ¹⁰ (1.77×10 ¹⁰ , 3.99×10 ¹⁰) ^b
	August 2009	RM02	YR	100	5	7.96×10 ⁹ (5.30×10 ⁹ , 1.2×10 ¹⁰)
	August 2009	RM02 + grass	YG	100	5	9.70×10 ⁹ (6.22×10 ⁹ , 1.44×10 ¹⁰)
Late spring	November 2009	RM02	N	180	3	7.86×10 ⁹ (5.44×10 ⁹ , 1.14×10 ¹⁰)
	November 2009	RM02 + pectin	XP	100	5	7.84×10 ⁹ (5.01×10 ⁹ , 1.17×10 ¹⁰)
	November 2009	BY	XB	100	5	1.26×10 ¹⁰ (8.71×10 ⁹ , 1.92×10 ¹⁰)
	February 2010	RM02	F	180	3	8.14×10 ⁹ (5.64×10 ⁹ , 1.16×10 ¹⁰)
Late summer	February 2010	CRF medium	WC	100	5	2.39×10 ¹⁰ (1.58×10 ¹⁰ , 3.58×10 ¹⁰) ^b
	February 2010	RM02 + titanium	WT	100	5	8.47×10 ⁹ (5.79×10 ⁹ , 1.28×10 ¹⁰)
	May 2010	RM02	L	180	3	8.32×10 ⁹ (5.87×10 ⁹ , 1.2×10 ¹⁰)
Late autumn	May 2010	98-5	V9	100	5	1.86×10 ¹⁰ (1.13×10 ¹⁰ , 2.54×10 ¹⁰) ^b
	May 2010	CRF + sisal	VC	100	5	1.54×10 ¹⁰ (1.03×10 ¹⁰ , 2.31×10 ¹⁰) ^b

^a The most probable number (MPN) was an average from 2 animals (YA, YS, YR, YG, XP, XB, WC, WT, V9 and VC) or four animals (M, A, N, F and L). The Z trial cultivation was from just one animal. Numbers in the brackets refer to the averaged lower and upper confidence limits (95% confidence interval). ^b treatments whose mean MPN value was in excess of one LSD above the control (YR) at significance level 0.05.

Table 4-3. Percentage of growth-positive tubes in different experiments.

Medium and sample month	Cow	Cultures obtained	Percentage of growth positive tubes in each dilution				
			Dilution	Dilution	Dilution	Dilution	Dilution
			1	2	3	4	5
M - (May)	A	37	73.3	38.3	25.0	6.7	0.0
M - (May)	B	33	78.3	45.0	11.7	1.7	0.0
M - (May)	C	25	73.3	40.0	8.3	5.0	0.0
M - (May)	D	15	71.7	26.7	3.3	5.0	0.0
A - (Aug)	B	42	81.7	35.6	11.1	1.7	
A - (Aug)	C	59	96.7	48.9	11.1	8.3	
A - (Aug)	D	33	80.0	21.1	14.4	1.7	
A - (Aug)	E	56	90.0	38.9	21.1	3.3	
YR - RM02 (Aug)	B	11	80.0	35.0	10.0	5.0	5.0
YA - agar (Aug)	B	13	100.0	45.0	20.0	0.0	0.0
YG - grass (Aug)	B	11	90.0	40.0	15.0	0.0	0.0
YS - sisal (Aug)	B	30	100.0	40.0	50.0	55.0	20.0
YR - RM02 (Aug)	D	6	80.0	25.0	5.0	0.0	5.0
YA - agar (Aug)	D	8	95.0	30.0	20.0	0.0	0.0
YG - grass (Aug)	D	8	90.0	20.0	15.0	0.0	0.0
YS - sisal (Aug)	D	30	85.0	75.0	50.0	15.0	0.0
N - (Nov)	B	22		26.7	11.7	3.3	
N - (Nov)	C	25		35.0	13.3	1.7	
N - (Nov)	D	27		35.0	13.3	1.7	
N - (Nov)	E	32		35.0	16.7	1.7	
XP - pectin (Nov)	B	20	75.0	25.0	5.0	15.0	0.0
XB - BY (Nov)	B	20	80.0	45.0	20.0	5.0	0.0
XP - pectin (Nov)	D	21	95.0	35.0	30.0	5.0	10.0
XB - BY (Nov)	D	30	90.0	60.0	15.0	20.0	15.0
F - (Feb)	B	34		33.3	23.3	0.0	
F - (Feb)	C	22		30.0	1.7	5.0	

Medium and sample month	Cow	Cultures obtained	Percentage of growth positive tubes in each dilution					
			Dilution	Dilution	Dilution	Dilution	Dilution	
			1	2	3	4	5	
F - (Feb) ^a	C	24		48.1				
F - (Feb)	D	13		30.0	8.3	0.0		
F - (Feb)	E	32		38.3	11.7	8.3		
WC - CRF (Feb)	D	18	100.0	55.0	35.0	10.0	0.0	
WT - titanium (Feb)	D	5	90.0	10.0	15.0	0.0	0.0	
WC - CRF (Feb)	E	20	100.0	75.0	30.0	5.0	0.0	
WT - titanium (Feb)	E	14	80.0	45.0	20.0	0.0	5.0	
L - (May)	B	26		35.0	13.3	5.0		
L - (May)	C	38		43.3	18.3	1.7		
L - (May)	D	22		31.7	8.3	0.0		
L - (May)	E	26		36.7	6.7	1.7		
V9 - 98-5 (May)	B	14	75.0	45.0	30.0	0.0	0.0	
VC - CRF + sisal (May)	B	34	90.0	70.0	10.0	5.0	0.0	
V9 - 98-5 (May)	D	24	100.0	55.0	20.0	30.0	0.0	
VC - CRF + sisal (May)	D	33	100.0	45.0	20.0	5.0	0.0	
1013^b			<i>87.1^c</i>	<i>39.7^c</i>	<i>16.8^c</i>	<i>5.8^c</i>	<i>2.5^c</i>	

^a Extra tubes were inoculated in the February sampling from cow C, dilution 2.

^b Total number of cultures obtained (after successful subculture), excluding cultures obtained from the trial cultivation (Z).

^c Average of the percentage of growth-positive tubes for this dilution.

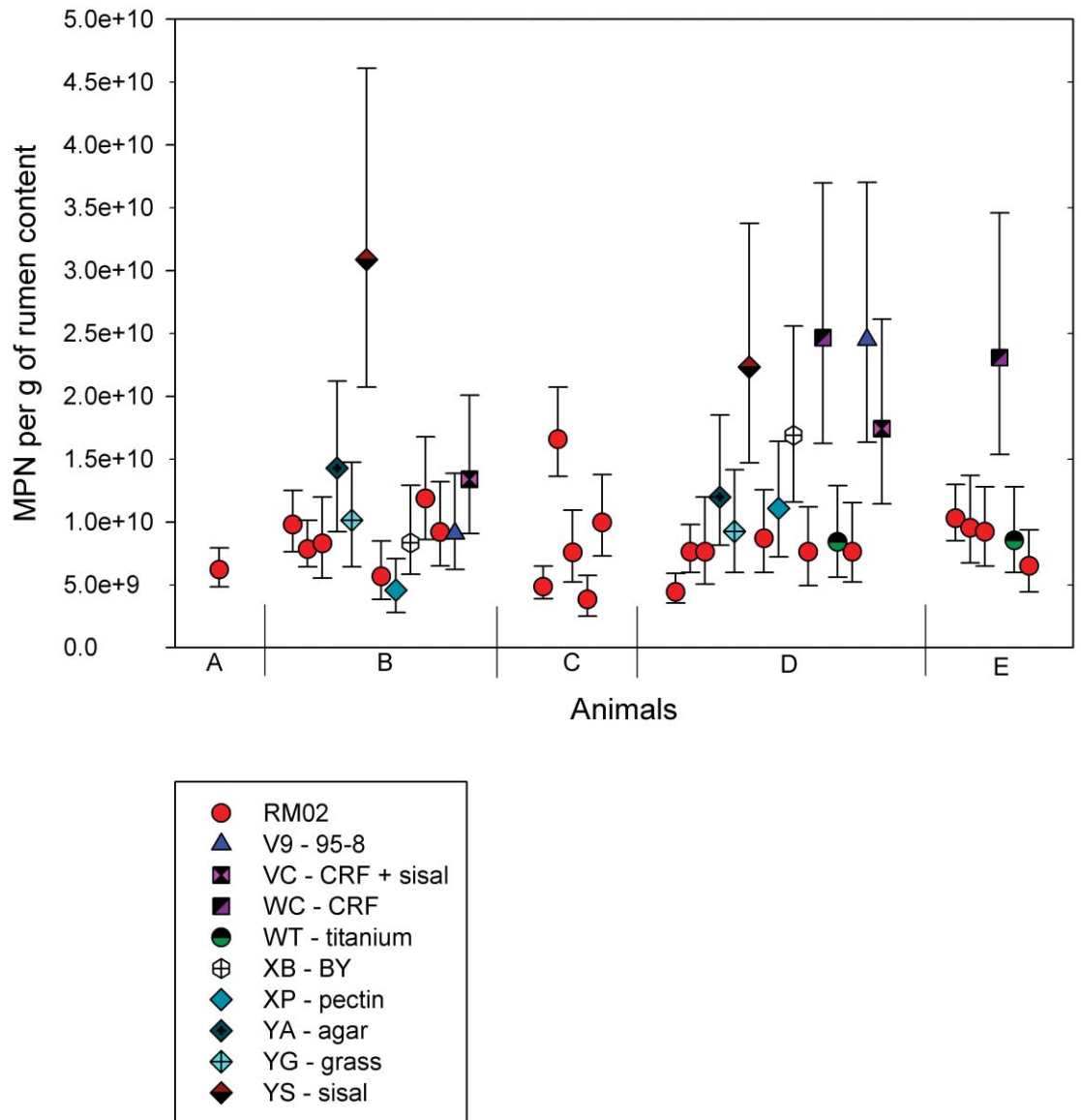


Figure 4-2. Most probable numbers (MPNs) of viable bacteria in the plant-adherent fraction per g of rumen content. These were calculated from a dilution series inoculated into either RM02 or other media from five cows sampled over five time points. Cow A died after the first sampling. The key to the types of media used is shown below the graph. Error bars reflect the upper and lower confidence limits calculated for the MPN.

4.4.3 Culturability

Microscopic bacterial counts were performed on the total and plant-adherent fraction from the May samples within four weeks of sampling. The calculated culturability was similar to that obtained in the cultivation trial (Z), ranging from 3.7% - 5.6% of total plant-adherent cells. The percentage of adherent cells was 67.0% - 84.0% in each of the cows A, C and D.

Table 4-4. Estimates of culturability and adherent cells in rumen content.

Sample month	Cow	MPN per g of rumen content	Plant-adherent cells per g of rumen content	Total cells per g of rumen content	Culturability ^a	Percentage of adherent cells ^b
Z - Trial	723	6.6×10^9	1.5×10^{11}	NC ^c	4.5%	NC ^c
M - May	A	6.2×10^9	1.2×10^{11}	1.4×10^{11}	5.4%	84.0
M - May	B	9.8×10^9	1.7×10^{11}	NC ^c	5.6%	NC ^c
M - May	C	4.9×10^9	1.3×10^{11}	1.9×10^{11}	3.9%	67.0
M - May	D	4.4×10^9	1.2×10^{11}	1.5×10^{11}	3.7%	79.8

^a Culturability was calculated by dividing the MPN by the total number of cells counted.

^b Percentage of adherent cells was calculated by dividing the number of cells in the adherent fraction by the total number of cells.

^c NC, not calculated (microscopic counts from the total rumen contents were not obtained for this sample).

4.4.4 Isolates

The total number of cultures obtained after all five samplings plus the trial cultivation was 1037. A summary of the numbers of cultures from inoculation through to identification by comparative 16S rRNA gene sequence analysis is shown in Figure 4-3. The cultures present in growth-positive tubes were microscopically assessed for cell morphology, the DNA from each was isolated and the 16S rRNA gene sequence was analysed. The descriptions for each of the 828 isolates for which good quality 16S rRNA gene sequence was determined are shown in (Table A1). Over 95% of the cultures appeared to contain cells of only a single cell morphology when examined microscopically. Among all subcultured samples, there was a very large diversity of cell

types (Figure 4-4), suggesting that a wide diversity of bacterial groups had been isolated. Only 4.43% of tubes that grew after subculture appeared to contain cells of more than one type, and these were excluded from further analysis.

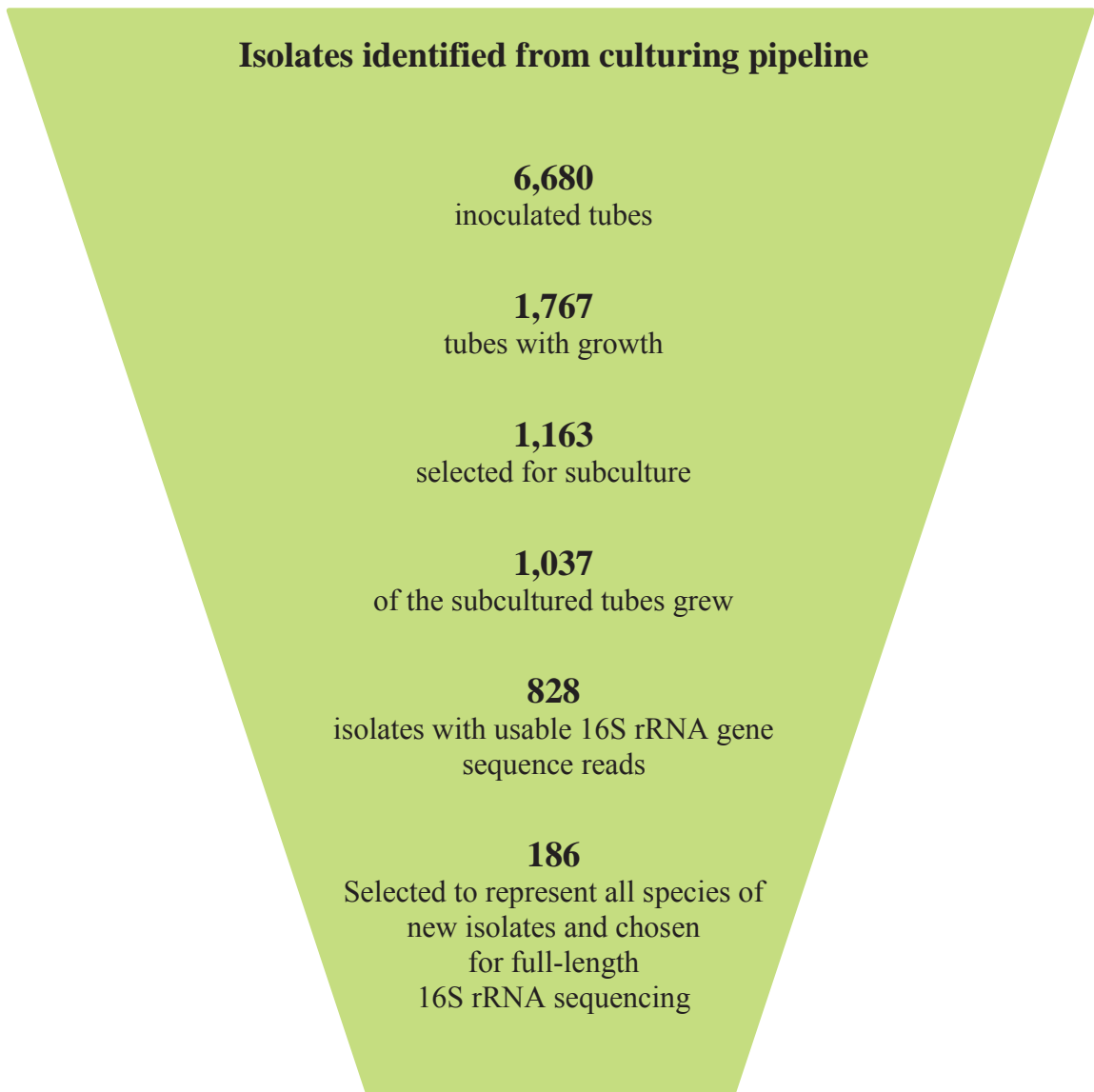


Figure 4-3. Summary of the numbers of cultures from inoculation through to identification by comparative gene sequence analysis. Cultures were selected for subculture if they arose from dilutions that were likely to have originated from a single cell i.e. dilutions where 100% of the inoculated cultures grew were not selected.

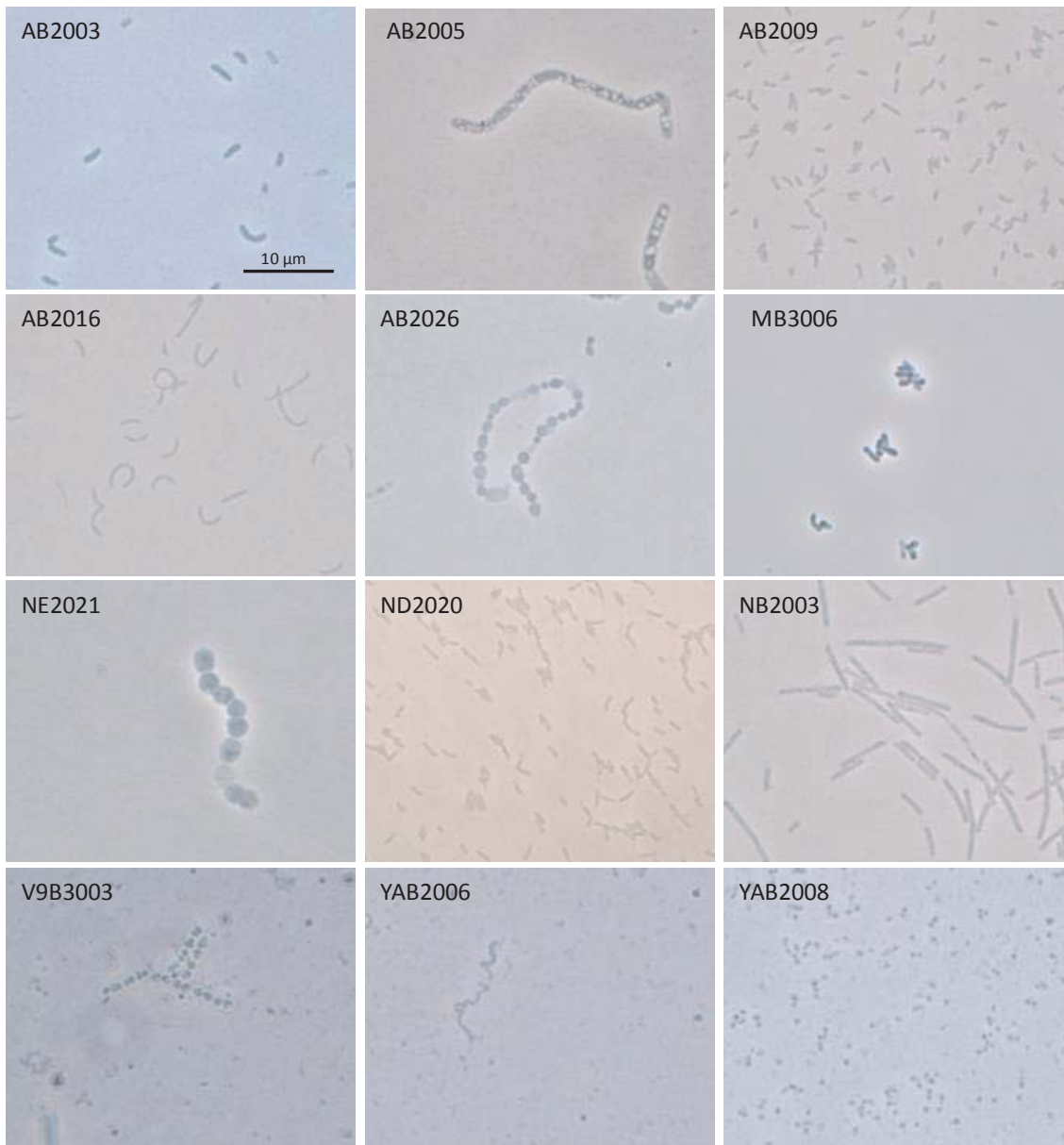


Figure 4-4. Examples of different cell morphologies found in the isolates. The scale bar represents 10 µm and applies to all panels. All isolates except AB2003 were identified and are listed in appendix 1.

4.4.5 Isolates that were omitted from further analysis

A total of 1037 cultures (89.2% of the initial number of cultures) grew when subcultured the first time (Figure 4-3). From the 1037 cultures obtained, usable 16S rRNA gene sequence reads were obtained for 828 (79.8%) of the isolates. Furthermore, if the percentage of initial cultures that were identifiable by their 16S rRNA genes is examined in relation to the isolation medium, a smaller number (63.2% of isolates) cultured from the 'other' media types, whereas sequences of as much as 88.5% of isolates cultured from RM02 were usable (good quality).

No usable 16S rRNA gene sequences were obtained for 209 cultures because: they failed to grow after the first subculture, the culture appeared to contain more than one cell type when examined microscopically, the growth was so poor that sufficient DNA for subsequent PCR could not be extracted from the culture, the extracted DNA would not form a PCR product after at least three attempts, the 16S rRNA gene sequence trace was mixed, indicating that the culture was mixed, the 16S rRNA gene sequence trace was of poor quality after at least two separate sequencing attempts or if there was no usable PCR product from the first DNA extraction and the culture could not be revived from its frozen stock after two attempts. 828 (71.2%) of 1,163 growth-positive tubes that were selected for subculture resulted in an identified putatively pure isolate. The percentage of identified isolates was not the same for all isolation media (Table 4-5). The percentage of isolates successfully identified on each medium type were: RM02, 88.5% (values for individual rumen samples ranged from 67.6 – 100%); all 'other' media combined 63.2%; V9, 57.9%; VC, 50.7%; WC, 86.8%; WT, 94.7%; XB, 66%; XP, 70.7%; YA, 71.4%; YR, 84.2%; and YS, 38.3%.

YS medium is RM02 medium containing a piece of sisal string. This medium had the highest MPN values and the highest percentage of cultures excluded from the analysis (61.7%). All of the excluded cultures appeared as a hazy growth around the sisal string and did not settle to the bottom of the culture tube. When the culture medium was centrifuged, an opaque jelly-like pellet formed. Microscopic observation revealed small (<1 μm diameter) cocci embedded in a granular extracellular matrix. These cell-like structures were much smaller than any of the verified isolates. Attempts to subculture these cultures were unsuccessful. DNA was extracted from some original cultures, but failed to produce a product with PCR using several combinations of

primers for the bacterial 16S rRNA gene (27f, 1492r, 806r and 515f) and also with universal primers for fungi and methanogens. The amount of DNA extracted from many of these cultures was very low, below the detection limit for measuring DNA using either the NanoDrop or Qubit methods (see methods 2.3.4). Scanning electron microscopy (SEM) was performed to help determine whether the cocci in these cultures were potentially micro-organisms (Figure 4-5). Culture YSB4012 was incubated for six weeks in YS medium alongside a control of just YS medium. Growth was not visible in the incubated tube but the sisal string appeared degraded compared to the control on the SEM, and $<1 \mu\text{m}$ diameter spherical structures were adhered to the string surface (see picture B Figure 4-5). Many of the spheres appeared to be in the process of dividing. Slightly larger oval-shaped structures were found on the sisal string in the control cultures, although these were much fewer in number over the whole sisal strand. Most of these structures were irregular in shape and not uniform in size. Some of the structures are continuous with the surface of the sisal string (see bottom left of picture A Figure 4-5). It is possible the cell-like structures were already attached to the sisal string before being incorporated into the YS medium or are in fact part of the string. A live-dead staining procedure was used to see if the cultures were potentially alive. The results were inconclusive. No “live” cocci were observed, but the original cultures were over two months old and no growth was visible in the subcultures. Finally, a newly-made uninoculated batch of YS medium developed a fuzzy haze around the sisal string in around 10% of the tubes, similar in appearance to that which was first seen in the cultures, suggesting that these may just be artefacts of the sisal string. The sisal string used in this study may not be homogeneous in its chemical composition along the length of the string, explaining why only some of the YS medium tubes formed this hazy opaque material. Microscopic and SEM observations indicated there was development of small round cell like structures in inoculated YS medium, but these could not be proven to be living micro-organisms by 16S rRNA gene analysis or live-dead staining. Even if they were not an artefact of the sisal string, the original cultures could not be sub-cultured, and so were omitted from the analysis.

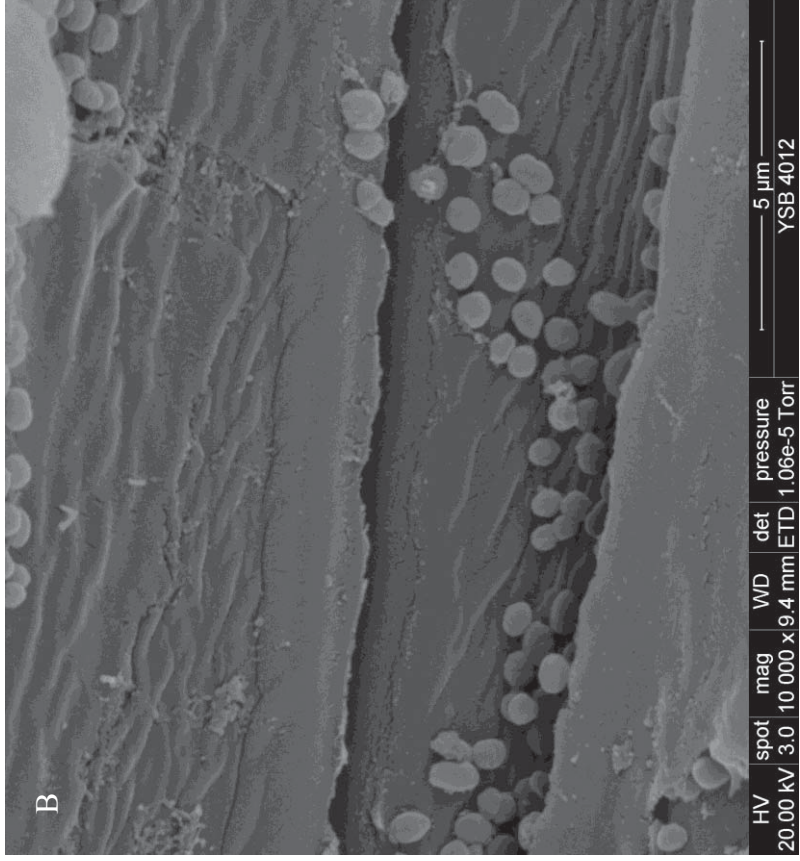
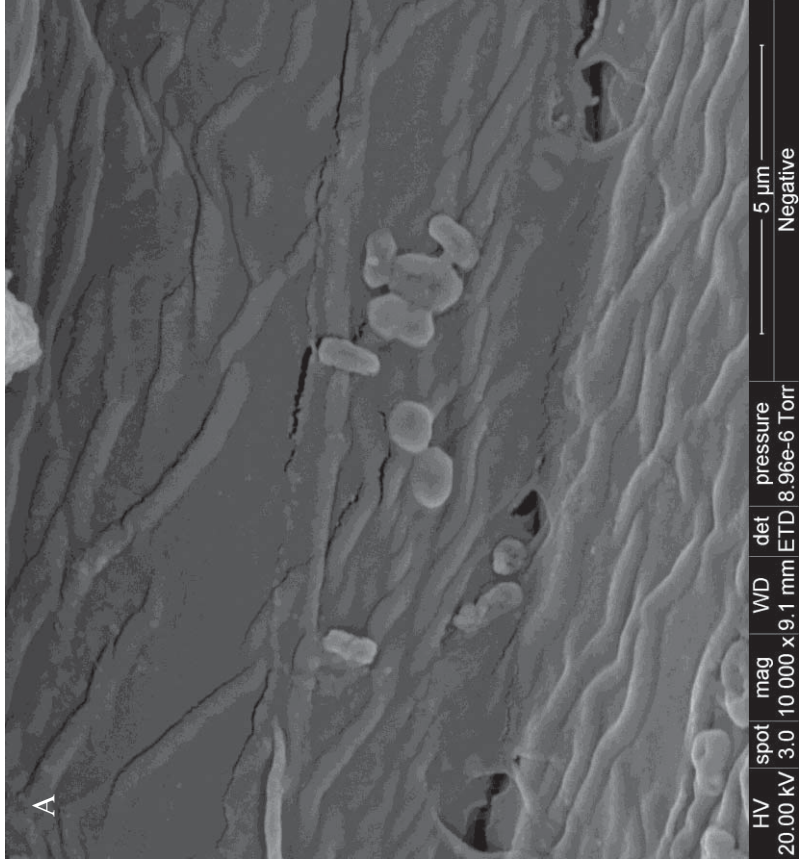


Figure 4-5. Scanning electron micrograph images of a strand of sisal string. A is a strand of sisal string from uninoculated YS medium viewed at 10 000× magnification. B is a strand of sisal string from YS medium inoculated with YSB4012 at viewed at 10 000× magnification.

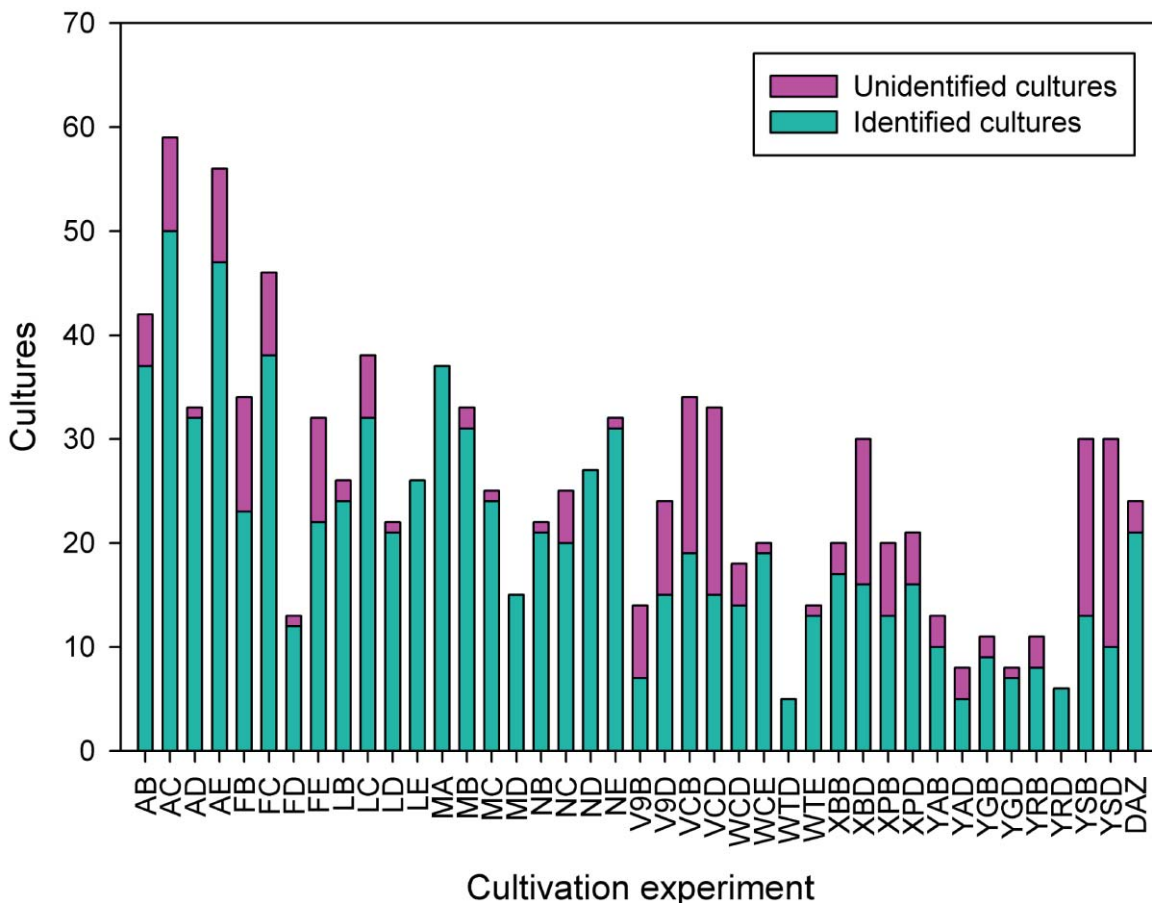


Figure 4-6. The percentage of isolates with usable 16S rRNA gene sequence reads for each sampling experiment. Identified cultures had useable 16S rRNA gene sequence reads, unidentified cultures did not have useable 16S rRNA gene sequence reads. Cultivation experiment labels are explained in section 4.3.1.

4.4.6 Phylogenetic placement of isolates

The phylogenetic positions of the new rumen isolates were assessed with three complementary methods. First, BLASTN analysis was used to give isolates a presumptive genus association by comparing their partial 16S rRNA gene sequence to their closest cultured relatives in the Ribosomal Database Project (RDP) (section 4.4.6.1). Second, the isolate sequences were analysed using the QIIME pipeline with the Greengenes database (release gg_otus_4feb2011) as a taxonomic reference (section 4.4.6.2), and, finally, evolutionary relationships between the isolates were

inferred by constructing phylogenetic trees with appropriate reference sequences (section 4.4.6.3).

4.4.6.1 BLASTN analysis

PCR, using primers 27f and 1492R, was used to amplify the 16S rRNA gene from each of the isolates. The V1-V3 region was sequenced using primers 27f and 514r. The resulting sequence was subjected to BLASTN analysis against a database created from all bacterial sequences downloaded from RDP (265493 sequences on 23/09/2011). 828 isolates produced good quality sequence and these were used for phylogenetic analyses.

The closest cultured isolate and the identity to its 16S rRNA gene for each sequence obtained from the 828 isolates is recorded in Appendix 1. Overall, 24.2% of the new isolates had partial 16S rRNA gene sequences less than 96% similar to those of previously cultured bacteria (Table 4-5). A greater percentage of novel (<96% similar to another bacteria) were isolated on the variety of 'other' media, compared to the main medium (RM02; Table 4-5). The BLASTN identity to a previously cultured bacterium for each of the cultivation experiments is shown in Figure 4-7.

There were 658 unique OTUs when clustered at 100% similarity with UCLUST within QIIME. Clusters with OTUs grouped at 100% similarity (covering 380 to 470 nt) contained 2-14 isolates. The identities of groups of isolates with identical sequences are shown in Table 4-6. All groups had >98% sequence match to a previously cultured bacterium and three OTUs were 100% identical, over the region sequenced, to *Kandleria vitulina*. These three OTUs contained sequences of differing lengths and differed from each other by one base at the termini of the sequences.

Table 4-5. Similarities of sequences from new isolates with those of previously-cultured bacteria.

Treatment	Total isolates	Total with good sequence	Similarity of isolate 16s rRNA genes (V1-V3) to previously cultured isolates		
			<92% identity	92-96% identity	>96% identity
All RM02 media	684	605	4.3%	17.6%	78.2%
All other media	353	223	12.9%	18.8%	68.2%
All samples	1037	828	6.4%	17.8%	75.8%

Table 4-6. Identity of groups of isolates with identical V1-V3 region 16S rRNA gene sequences.

Number of identical sequences in group ^a	Top BLASTN match; GenBank accession for 16S rRNA gene	Identity (%)	Lowest defined taxonomic group identified by QIIME
4	rumen bacterium NK4A237; GU324386	100%	Clostridiales
4	rumen bacterium R-7; AB239481	98%	Clostridiales
4	<i>Lachnospira pectinoschiza</i> M56; AY699283	99%	Lachnospiraceae
5	rumen bacterium R-25; AB239489	99%	Clostridiales
5	<i>Butyrivibrio hungatei</i> AR10; FJ794074	100%	Lachnospiraceae
5	rumen bacterium NK4A578; GU124465	99%	Lachnospiraceae
5	rumen bacterium NK2B42; GU324359	100%	Lachnospiraceae
5	<i>Propionibacterium acnes</i> 1570; JF277163	100%	Propionibacteriaceae
5	<i>Kandleria vitulinus</i> TB-A09; AB425916	100%	Erysipelotrichaceae
5	<i>Kandleria vitulinus</i> TB-A09; AB425916	100%	Erysipelotrichaceae
6	<i>Butyrivibrio hungatei</i> AR10; FJ794074	100%	Lachnospiraceae
6	rumen bacterium NK3A31; GU324398	98%	Clostridiales
7	rumen bacterium NK4A142; GU324402	99%	Lachnospiraceae
8	rumen bacterium NK4C38; GU324409	100%	Lachnospiraceae
9	<i>Kandleria vitulinus</i> TB-A09; AB425916	100%	Erysipelotrichaceae
9	rumen bacterium NK4A142; GU324402	100%	Lachnospiraceae
14	rumen bacterium NK4A578; GU124465	100%	Lachnospiraceae

^aOnly groups with four or more identical sequences are shown, with their top BLASTN match and the lowest defined taxonomic group defined by QIIME. Additionally, 51 groups of two identical sequences and 17 groups of three identical sequences were found.

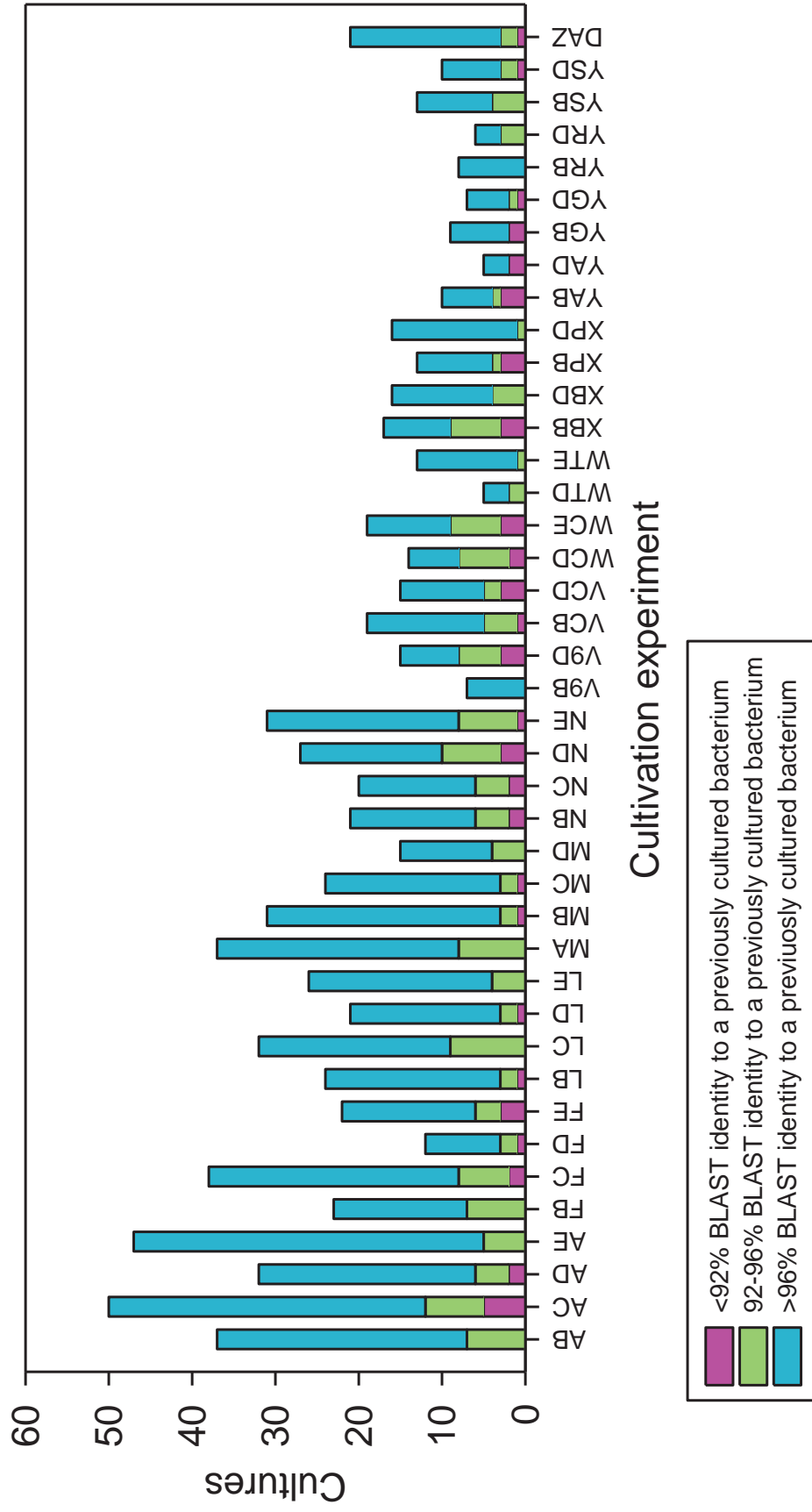


Figure 4-7. Comparison of new plant-adherent rumen isolates to previously known bacteria by level of 16S rRNA gene sequence identity. Cultivation experiment labels are explained in section 4.3.1.






























4.4.6.2 QIIME analysis

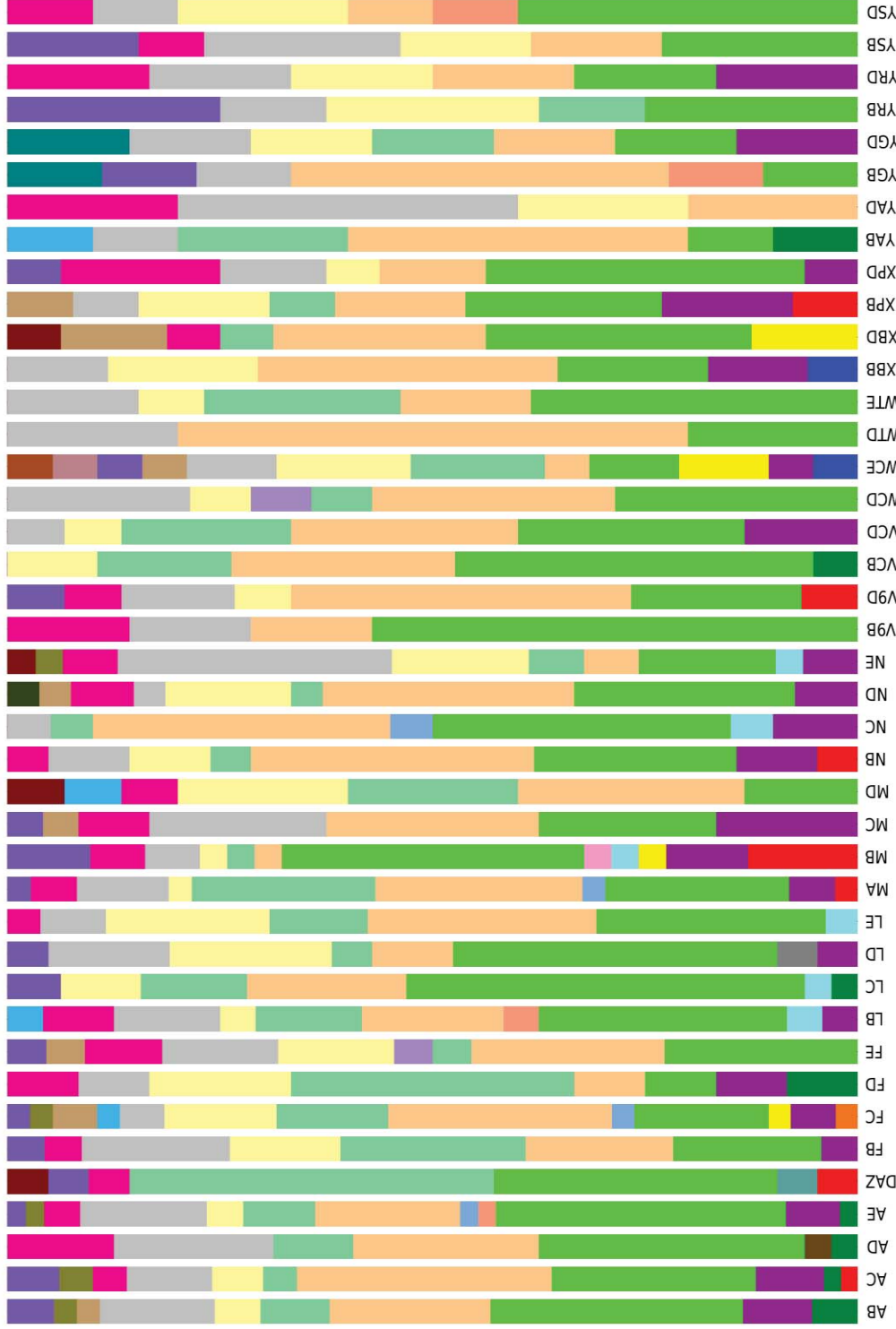
The QIIME pipeline was used to assign sequencing reads to taxonomic identities using established databases. The isolates were classified to the most detailed lineage description using the Greengenes database (release gg_otus_4feb2011) as a taxonomic reference. When analysed in QIIME, the 828 isolates grouped into 204 OTUs at 96% sequence similarity. Some could be classified to the genus level, but 0.5% could not be assigned to a phylum. Altogether five phyla were represented by the new rumen isolates. The vast majority of the isolates fell into the phylum Firmicutes (90.2%), while the others were in the phyla; Actinobacteria (1.6%), Bacteroidetes (7.0%), Fibrobacteres (0.5%) and Spirochaetes (0.3%). The taxon assignments for the isolates cultured from each cultivation experiment are shown in Figure 4-7.

Members of the genus *Butyrivibrio* were the most commonly isolated bacteria, accounting for a quarter (25.4%) of all the isolates. *Butyrivibrio* spp. were isolated from all medium and sample combinations, i.e. all cows, all seasons and on all media types, except for cow D on YA media. Undefined genera in the family Lachnospiraceae accounted for the second most abundantly isolated bacteria, with 21.55% of the total sequences, followed by undefined members of family Ruminococcaceae (12.10%), the order Clostridiales (9.65%) and the genus *Pseudobutyrvibrio* (9.62%).

The effect that the different isolation media had on the range of bacteria isolated was examined (Figure 4-9). The most diversity, determined by the number of taxa identified, was isolated in RM02 medium, but when RM02 is compared to all the 'other' medium types combined, the range of bacteria isolated is similar. Nine taxonomic groups were only isolated in RM02 medium. The smallest number of taxa was isolated in WT medium. A few taxonomic groups were isolated only in media other than RM02. A group of isolates that could only be defined as members of the phylum Firmicutes were only isolated in YG media. *Solobacterium* spp. and *Treponema* spp. were only isolated in WC medium. *Olsenella* spp. were isolated in WC medium and XB medium but not in RM02. Rarefaction analysis indicated more diversity (OTUs at 96% similarity) was being sampled by using the combination of 'other' media compared to RM02 media alone (Figure 4-10). Rarefaction from the entire dataset showed the total diversity was not greater than both subsets of isolates, showing that RM02 and the

'other' media had isolates that were broadly similar organisms at the OTU level. This confirms the finding of the high level of similarities found in Figure 4-9.

Legend	Taxonomy
	<i>Treponema</i>
	Spirochaetales
	Bacteria
	Firmicutes
	<i>Solobacterium</i>
	Erysipelotrichaceae
	<i>Selenomonas</i>
	Veillonellaceae
	<i>Anaerovibrio</i>
	<i>Ruminococcus</i>
	Ruminococcaceae
	Clostridiales
	<i>Syntrophococcus</i>
	<i>Pseudobutyrvibrio</i>
	Lachnospiraceae
	<i>Lachnobacterium</i>
	<i>Coprococcus</i>
	<i>Butyrivibrio</i>
	Incertae Sedis XIII
	<i>Anaerovorax</i>
	<i>Streptococcus</i>
	<i>Bacillus</i>
	<i>Fibrobacter</i>
	Bacteroidetes
	<i>Prevotella</i>
	Prevotellaceae
	Bacteroidales
	<i>Olsenella</i>
	<i>Propionibacterium</i>



Cultivation experiment

Figure 4-8. The effect of season, animal and medium on the proportions of different phylogenetic groups cultured from the plant-adherent fraction of rumen contents. The legend shows the lowest defined taxonomic group. Cultivation experiment labels are defined in 4.3.1



Legend on next page

Legend	Taxonomy	Total
	Bacteria; Spirochaetes; Spirochaetales; Spirochaetaceae; <i>Treponema</i>	0.10%
	Bacteria; Spirochaetes; Spirochaetales; Spirochaetaceae; Other; Other	0.10%
	Bacteria; Other; Other; Other; Other	0.50%
	Bacteria; Firmicutes; Other; Other; Other; Other	0.60%
	Bacteria; Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; <i>Solobacterium</i>	0.10%
	Bacteria; Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; Other	3.10%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; <i>Selenomonas</i>	0.40%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Other	1.10%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; <i>Anaerovibrio</i>	0.60%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; <i>Ruminococcus</i>	5.00%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Other	12.10%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Other	9.60%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; <i>Syntrophococcus</i>	0.30%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; <i>Pseudobutyrvibrio</i>	9.60%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Other	21.50%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; <i>Lachnobacterium</i>	0.30%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; <i>Coproccoccus</i>	0.70%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; <i>Butyrivibrio</i>	25.40%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Incertae Sedis XIII; Other	0.10%
	Bacteria; Firmicutes; Clostridia; Clostridiales; Incertae Sedis XIII; <i>Anaerovorax</i>	0.10%
	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; <i>Streptococcus</i>	0.10%
	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	0.10%
	Bacteria; Fibrobacteres; Fibrobacteria; Fibrobacterales; Fibrobacteraceae; <i>Fibrobacter</i>	0.60%
	Bacteria; Bacteroidetes; Other; Other; Other	0.70%
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; <i>Prevotella</i>	4.80%
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Other	1.00%
	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Other; Other	0.10%
	Bacteria; Actinobacteria; Actinobacteria; Coriobacteriales; Coriobacteriaceae; <i>Olsenella</i>	0.30%
	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae; <i>Propionibacterium</i>	1.00%

Figure 4-9. Proportions of bacteria belonging to different taxa cultured from the plant-adherent rumen fractions on different types of media. The key is shown below the graph. The media are indicated below each bar, and these codes are explained in Table 4-2. The bar charts in the blue box are comparing all isolates obtained using RM02 medium with all the isolates from all the 'other media' types. The total column in the key is the percentage of the 828 isolates assigned to each taxon.

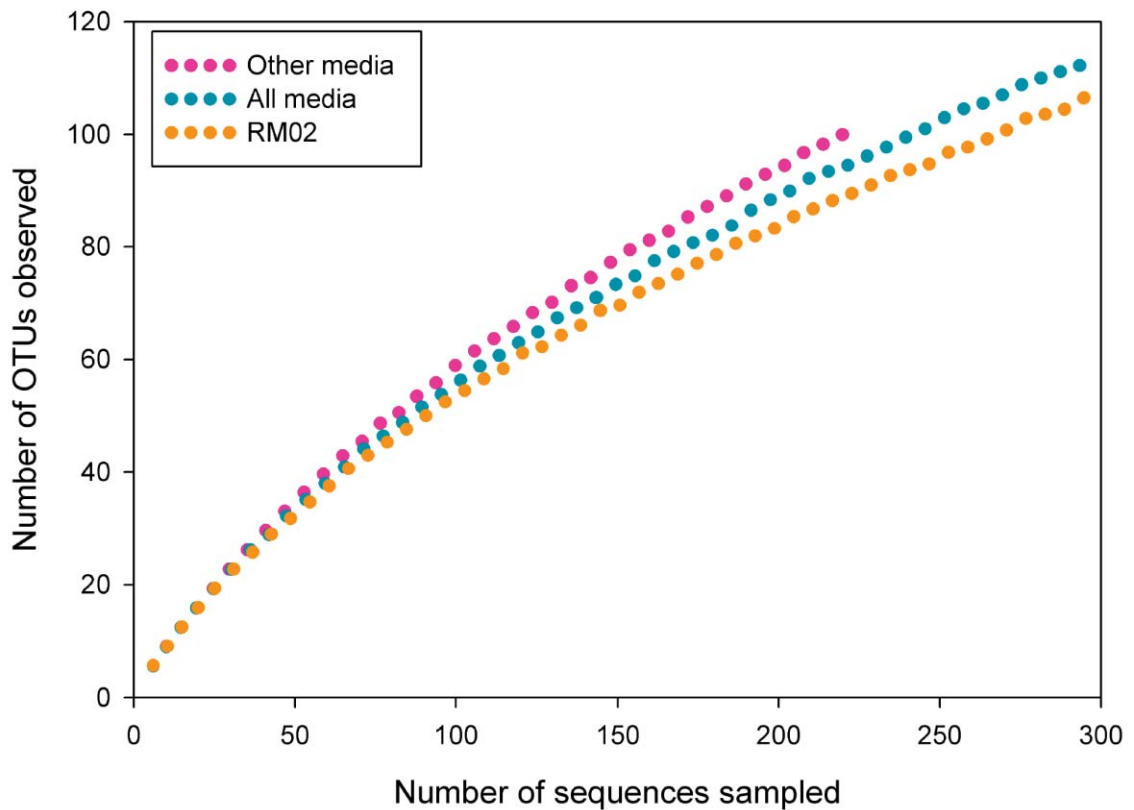


Figure 4-10. Rarefaction curve for different bacterial OTUs (grouped at 96% similarity) from isolates gained on RM02 medium, all ‘other’ media used in this study (media includes VC, WC XB YG YA WT V9 XP and YS) or all of the media types combined. Data points represent the average of 100 samplings at each step.

4.4.6.3 Phylogenetic trees

The taxonomic assignments generated in QIIME were verified by phylogenetic tree reconstruction. 828 isolate sequences were clustered into 204 OTUs at 96% similarity. A representative sequence (the most abundant in each OTU) was chosen for each OTU. The evolutionary relationships of rumen plant-adherent bacterial OTUs based on partial 16S rRNA genes sequences (V1-V3) were inferred by constructing phylogenetic dendrograms (trees) (Figure 4-11 - Figure 4-14). Sequences were manually inspected for chimeras and the alignments were manually checked. The branching order in the tree were confirmed by bootstrap analysis (1000 replicates) of neighbour joining trees using the Jukes Cantor model, p-distances, and a maximum likelihood tree with the Jukes Cantor model and the FastTree tree building method.

Nodes with low bootstrap support were not always consistent with known phylogenetic relationships and were not recovered with all three treeing methods used. In contrast clades identified with high bootstrap values were stable when tested with different treeing methods. The isolate sequences shown within the collapsed clades in Figure 4-11 reflect stable groupings. The branching order of the collapsed clades relative to each other was unstable and some phylum groups were not conserved. This is, however, expected with the short sequences used for these analyses.

The sequences belonging to the order Clostridiales fell into four clades at the family level (Lachnospiraceae, Ruminococcaceae, Veillonellaceae and Family XIII *Incertae Sedis*), and two further clades that did not have isolates assigned to named families, suggesting that these isolates are from as yet undescribed families. The clade encompassing the family Lachnospiraceae contained the majority of the OTUs (Figure 4-12), over half of which were not defined below the family level in the QIIME analysis. However, two genus groups, *Butyrivibrio* and *Pseudobutyrvibrio*, were seen. Single *Coprococcus*, *Lachnobacterium* and *Syntrophococcus* OTUs were also included in this clade. Sequences belonging to the family Ruminococcaceae were split into two separate clades. One clade contained OTUs assigned to *Ruminococcus* and the other clade contains unknown genera in the family Ruminococcaceae. The degree of separation (<79% sequence identity) between sequences in these two clades suggests this family may need to be reorganised into two family groups.

The four Fibrobacteres OTUs clustered together with 100% bootstrap support. Separate clades of Spirochaetaceae, Coriobacteriaceae and Propionibacteriaceae also had 100% bootstrap support for each clade. The sequences that could only be classified as Bacteroidetes clustered (100% bootstrap) with those of members of Prevotellaceae. Two families within the order Bacillales (Streptococcaceae and Bacillaceae) were represented by one isolate each and clustered together with 98% bootstrap support.

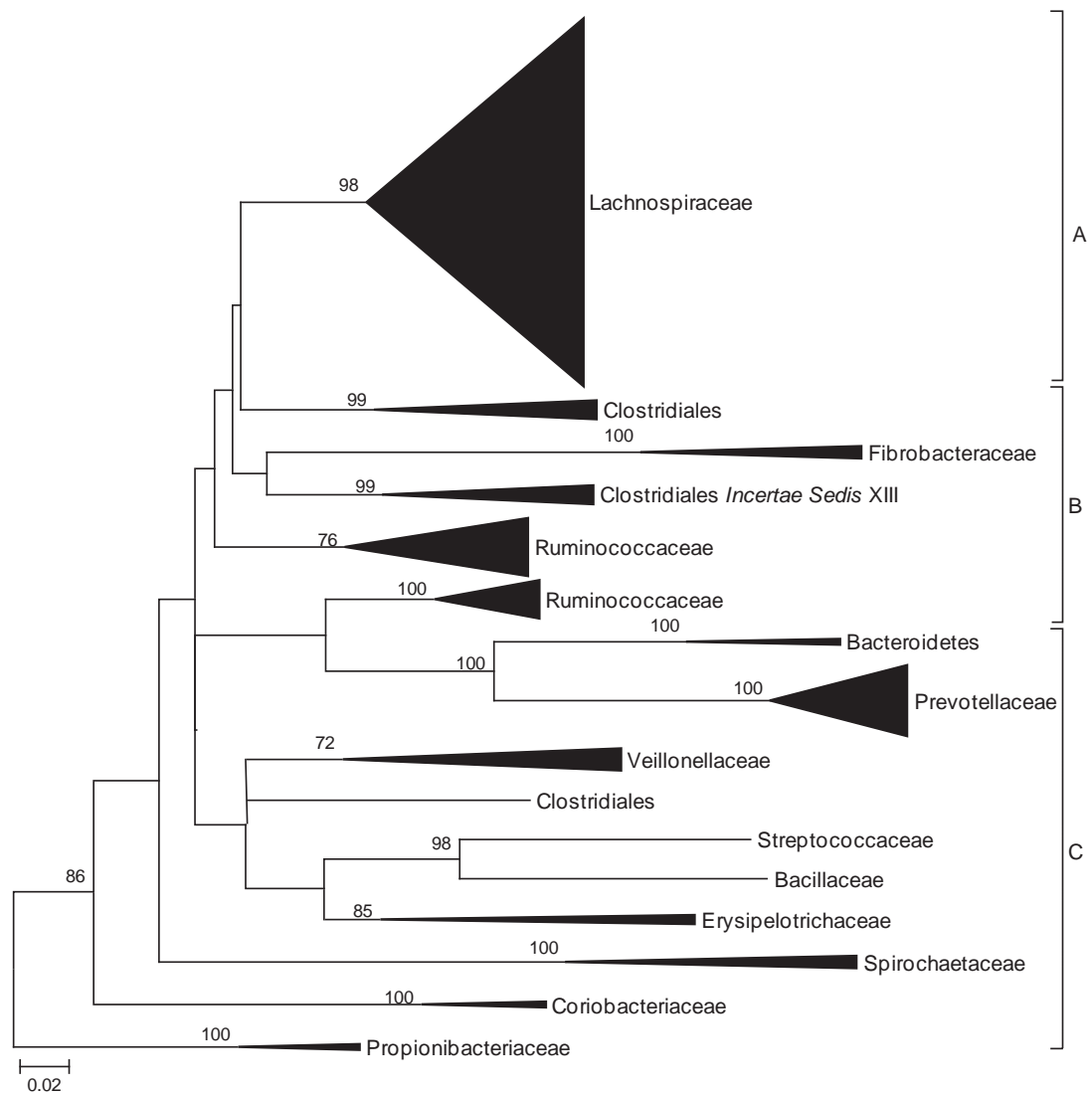


Figure 4-11. Overview of the phylogenetic relationships of rumen plant-adherent bacterial OTUs based on partial (V1-V3) 16S rRNA gene sequences. 828 isolate sequences were clustered into 204 OTUs with UCLUST (96% similarity). Representative sequences of each OTU used for the phylogenetic analysis were selected and assigned to a taxonomic group in QIIME. Clades are collapsed to show the family or higher order taxa where a family could not be assigned. Square brackets followed by the letter A, B or C indicates the part of the tree that will be shown in the expanded views following (Figure 4-12, Figure 4-13 Figure 4-14). Phylogenetic trees were constructed using the neighbour joining method, utilising the Jukes Cantor model with 1000 bootstrap replicates. Bootstrap values >70% are shown at internal nodes. The scale bar indicates nucleotide substitutions per sequence position. The tree was rooted with the sequence from *Aquifex aeolicus* VF5 (not shown).

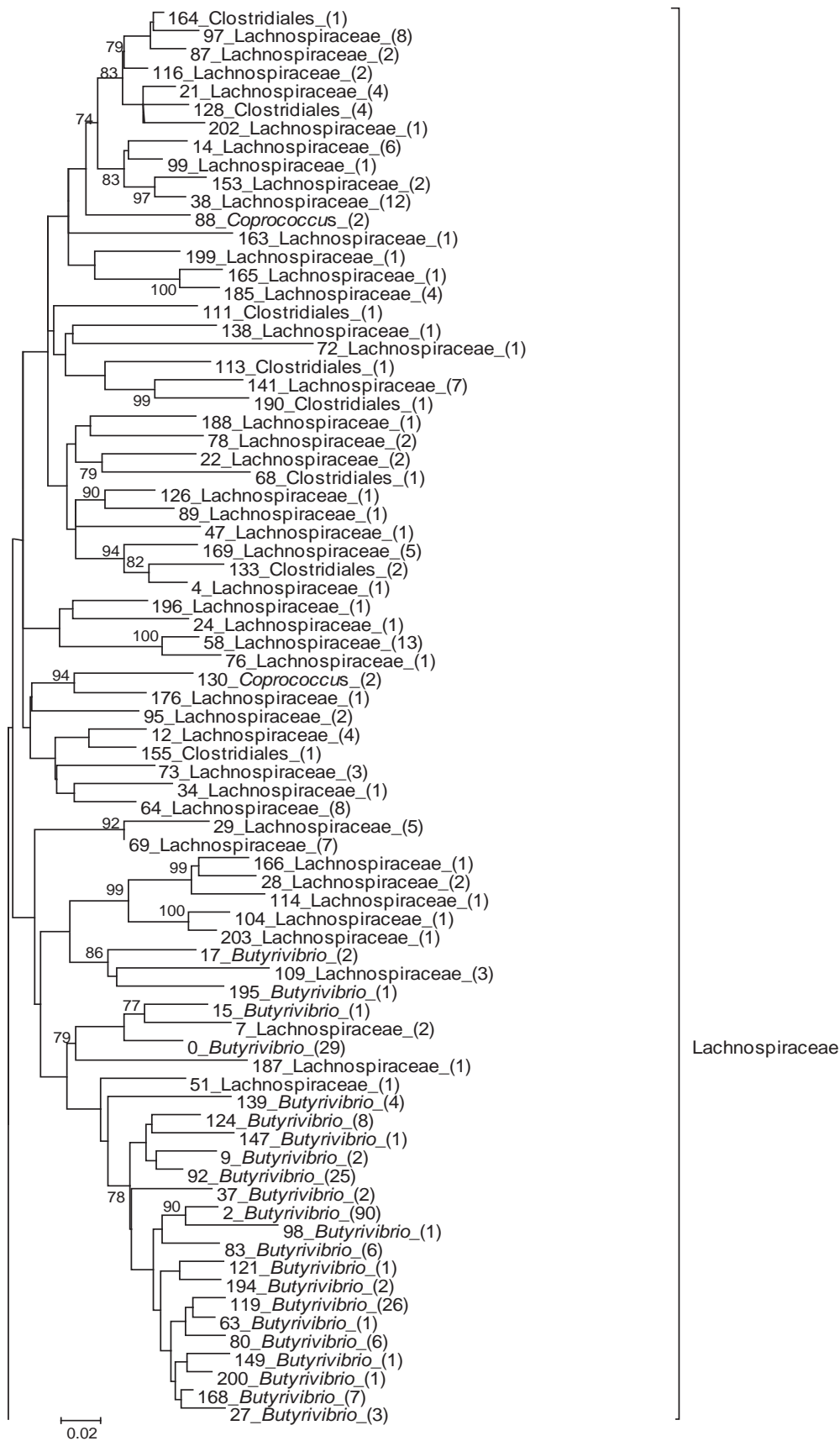


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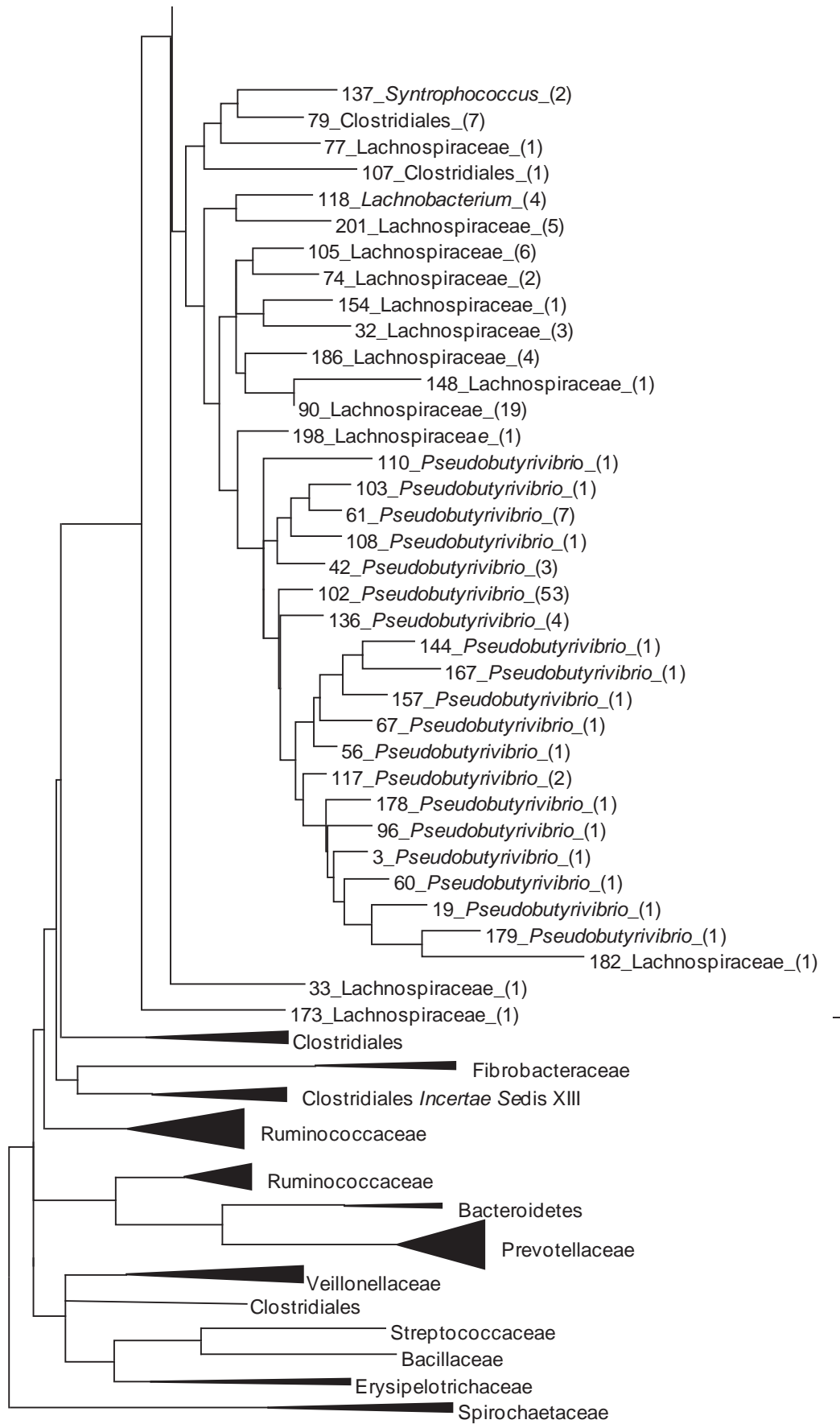


Figure 4-12. Expanded view A: The phylogenetic relationships of plant-adherent bacteria isolated from rumen contents based on partial (V1-V3) 16S rRNA gene sequences. Each leaf represents an OTU (clustered at 96% similarity). Square brackets to the right show the family or higher order taxa where a family could not be assigned. Tips are labelled with the OTU number followed by the lowest defined taxonomic group. The number in brackets represents the number of isolates found in that OTU. Phylogenetic trees were constructed using the neighbour joining method, utilising the Jukes Cantor model with 1000 bootstrap replicates. Bootstrap values >70% are shown at internal nodes. The scale bar indicates nucleotide substitutions per sequence position and is relative to each tree or part of tree it is shown with. The tree was rooted with the sequence from *Aquifex aeolicus* VF5 (not shown).

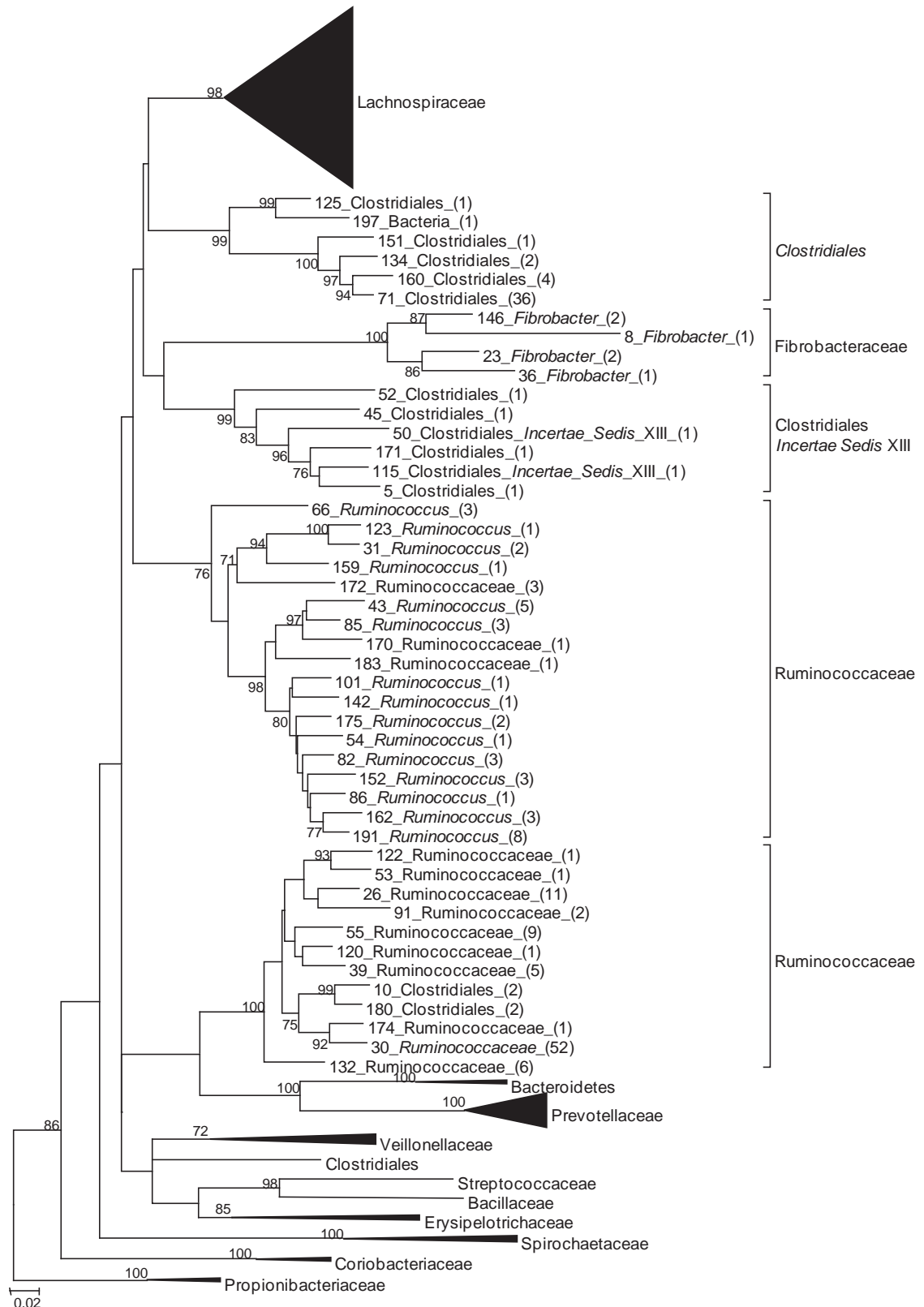


Figure 4-13. Expanded view B: The phylogenetic relationships of plant-adherent bacteria isolated from rumen contents based on partial (V1-V3) 16S rRNA gene sequences. Details are as described in the legend to Figure 4-12.

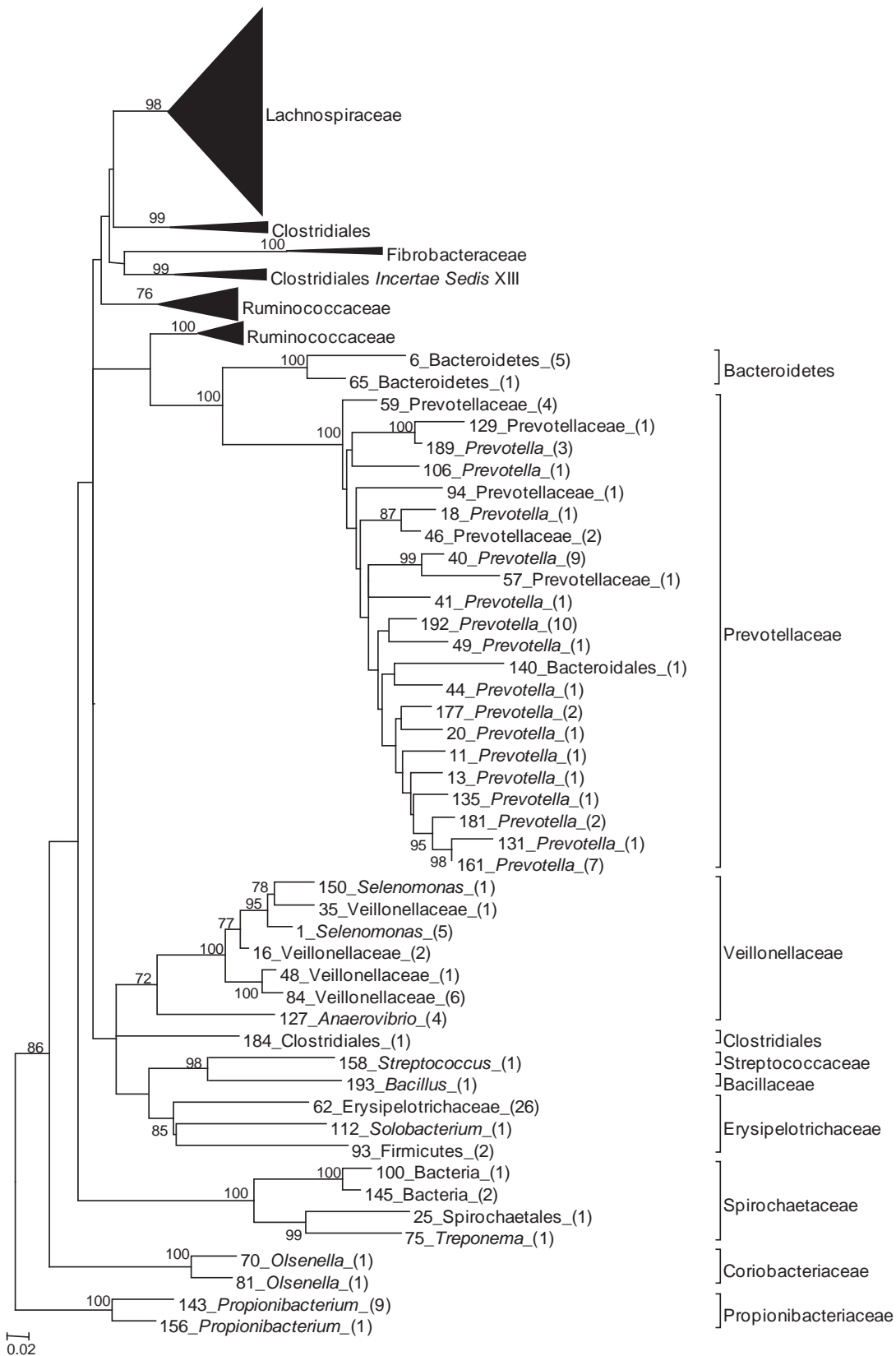


Figure 4-14. Expanded view C: The phylogenetic relationships of plant-adherent bacteria isolated from rumen contents based on partial (V1-V3) 16S rRNA gene sequences. Details are as described in the legend to Figure 4-12.

4.4.7 Selection of representative isolates for further study

Isolates that represented each 'species level' group were selected for full-length 16S rRNA gene sequencing. This was to confirm the identity and phylogenetic relationships of the new isolates. As part of the selection process, the isolates were compared with the total rumen community. To do that, the plant-adherent bacterial fractions used for isolation of new bacteria were examined by culture-independent barcoded pyrosequencing of partial 16S rRNA gene sequences. The detailed results of that study will be presented in Chapter 5. The isolate sequences were combined with the pyrosequencing reads and clustered into OTUs at 96% similarity using UCLUST, within the QIIME pipeline. The most abundant sequence type of each OTU was chosen as a representative sequence for that OTU. Representative sequences for all OTUs that contained at least one isolate (232 in total) were extracted from the dataset. The remaining OTUs without an isolate were ranked from largest to smallest in terms of number of sequences they contained. An equal number (232) of representative sequences from the largest OTUs that did not contain an isolate were extracted from the dataset. These 232 + 232 representative sequences were combined with all 828 isolate sequences. A topological tree was constructed using MEGA5. Evolutionary history was inferred using the Neighbour-Joining method and evolutionary distances calculated using the p-distance method. The resulting phylogenetic tree (not shown) was used to confirm QIIME clusters and select representative isolate sequences for full-length 16S rRNA gene sequencing.

The calculated evolutionary distances were viewed as a matrix and reordered to match the order they appeared in the tree. Clusters of sequences that were 96% similar were visualised from the distance matrix and each cluster was given a putative name (Appendix 1). This resulted in 171 species level clusters containing an isolate. Overall, the sequence clusters (96% similarity) had good agreement with the OTUs (96% similarity) with the notable exception of *Pseudobutyrvibrio* spp., which had 19 UCLUST OTUs but appeared to group into just one cluster from the phylogenetic reconstruction (not shown). Sequences classified as *Butyrvibrio* spp. also had more UCLUST OTUs identified than clusters obtained in the phylogenetic reconstruction. In both of these genera it was difficult to identify the boundaries of the clusters because they formed a continuum of related sequences. At least one representative isolate was

randomly selected from each cluster, 24 representatives of *Butyrivibrio* spp. and 10 representatives of *Pseudobutyrvibrio* spp. were selected. The isolates that were selected are recorded in Appendix 1. In large, diverse or interesting clusters more than one isolate was selected. A cluster was considered to be interesting if it contained an isolate sequence with either a representative sequence that was from a large OTU or was from part of the tree that was under-represented by isolate sequences. One example of an interesting cluster is Ruminococcaceae R-25 like cluster 6 shown in Table 6-2. In some cases, more than one isolate from a single putative species level group was included to check whether the groupings of partial (V1-V3) 16S rRNA gene sequence were maintained when nearly full-length sequence were examined. A total of 192 isolates were selected for full-length sequencing resulting in 186 good quality reads over 1300 base pairs long.

4.5 Discussion

Before the common use of the 16S rRNA gene as a phylogenetic marker to survey microbial community composition, it was postulated that most of the significant groups of rumen bacteria had already been isolated (Bryant, 1959). It was therefore assumed, at that time, that isolation studies would not yield many more new discoveries other than minor species with specialised metabolisms. However, based on studies that generated large-scale 16S rRNA gene libraries, it became evident that the full diversity of rumen bacterial communities is not well represented by cultivated micro-organisms. Only a small percentage of the rumen bacteria (perhaps as low as 10%) are available as pure cultures to study (Edwards *et al.*, 2004). In the last 10 years, more emphasis has been placed on culture-independent molecular methods to examine the rumen microbiota. The new approaches offered by molecular biology provide tools that allow populations to be examined without the need for culturing. A large number of bacteria in the rumen remain uncharacterised because there is no cultured representative and so their roles within the rumen are uncertain. Culture-independent methods in concert with the study of pure cultures are powerful tools for studying rumen ecology. In this study, the intention was to revisit the strategies of the past and examine the rumen community with a cultivation-based strategy. In tandem with molecular tools (see Chapters 5 and 6), the effectiveness of the cultivation study could be assessed.

There are many different factors influencing rumen microbial diversity. These include diet (Kocherginskaya *et al.*, 2001; Tajima *et al.*, 2001; Wanapat *et al.*, 2009), geographic area (Ramsak *et al.*, 2000), animal species (An *et al.*, 2005), growth stage (Welkie *et al.*, 2009), but not photoperiod (McEwan *et al.*, 2005) or rumen sampling technique (Lodge-Ivey *et al.*, 2009). To capture the diversity seen in New Zealand dairy cattle, four representative cows were selected. The effects of animal, feed and sampling season on the rumen bacterial communities in these four animals will be discussed in Chapter 5.

4.5.1 Culturability

Microscopic counts performed on blended digesta from the first sampling (May) were compared with the cultivation trial. Culturability was similar from all the cows,

and ranged from 3.7-5.6%. The proportion of cells adherent to the plant material was 67-84%. This is in agreement with a previous study that found ~70% of the total rumen bacteria attach to feed particles (Legay-Carmier *et al.*, 1989).

4.5.1.1 Dilution to extinction

When calculating a MPN, it is assumed that organisms are randomly distributed within the inoculum and its dilutions, that growth can be initiated by a single organism once inoculated, and that all organisms occupy a similar volume. In the blended rumen digesta, it was observed that the bacteria were not randomly distributed, but that many were in small clumps of 2- 47 cells. Including single cells, the average unit that might initiate growth contained 1.7 cells (see Chapter 3). Although the majority (77.8%) of these units were single cells, a clump of cells will act like a single cell in the MPN calculations. When comparing MPNs produced from the same inoculum, this might have an impact on the MPNs if the cells in clumps either inhibited or facilitated the initiation of growth, if the cells in clumps were in a different physiological state that affected their ability to initiate growth in artificial media, or if they were from different species. Because these were all unknowns, these effects cannot be estimated.

Although the MPN values for each cultivation experiment were different, the number of viable cells in the samples was consistent enough that the range of dilutions used for inoculation always encompassed the point where 20% of the tubes were growth positive, i.e., received close to 0.2 cells per tube. This was because the dilution steps were small (4-fold). This meant for each cultivation experiment, there were always tubes that had received suitably diluted inocula and so could be chosen for subculture. In the trial cultivation, it was observed that tubes that could have received more than one viable cell (i.e., 75% of the tubes within a dilution level grew) still largely resulted in development of just one cell type when examined microscopically (see Chapter 3). Overall, >95% of the resulting cultures from all cultivation experiments appeared to be of one cell type when examined microscopically.

4.5.1.2 Quantitative effects of medium choice on culturability

An important consideration is the medium used to isolate rumen bacteria. Hungate (1947) used media containing minerals, a carbonic acid-bicarbonate buffer, sterile rumen fluid, and cellulose to first isolate rumen anaerobes. Over the years, numerous improvements and changes have been made to the media used to isolate new rumen bacteria. RM02 was designed to simulate the rumen environment in terms of salt concentration, pH, buffering capacity, and available substrates and cofactors, and was the main medium used at all the sampling times in this study. Even though this medium has been demonstrated to allow isolation of new genera of bacteria from the rumen (Kenters *et al.*, 2011) it cannot be expected that one medium will be able to support the growth of every species present in the rumen. In parallel to the main medium, nine 'other' media were used with two of the four animals sampled. Many of these new media were based on RM02 with specific modifications. During the August sampling (the first sampling to include additional 'other' media), a control of RM02 medium (YR) was included. Because the number of tubes of 'other' media used was the same as the number of tubes of YR RM02, the MPNs are able to be directly compared. The MPN using YR (7.96×10^9 /g rumen content) was within the range of those obtained using RM02 medium with more tubes inoculated ($6.33 \times 10^9 - 1.06 \times 10^{10}$ / g of rumen content). The MPNs of all samplings on RM02 medium were not significantly different from each other. Differences in MPN were not driven by animal or season. The variability between cows was very small, and no significant differences were seen in RM02 media sampled from different seasons.

Per sample, the actual number of viable cells from the plant-adherent rumen fraction inoculated into RM02 medium and the other media types was the same, because the same inoculum was used. If more bacteria were able to grow, leading to a larger MPN, it would be because differences in the medium formulation allowed more bacteria to grow. On average, all of the 'other' media had larger MPNs than RM02, with the exception of RM02 with the addition of pectin or with titanium (III) reductant. When the MPN values for each cow were averaged for each sampling, four of the 'other' media types gave higher values than the YR control with RM02 media. These were YS, WC, V9 and VC.

The other media used in the August sampling were YA (RM02 + agar), YG (RM02 + grass), and YS (RM02 + sisal). The specific additions to these media were to add surfaces for bacterial attachment, which may be important for bacteria that prefer to grow in biofilms. In the rumen environment, many bacteria do not naturally grow in suspension, but instead grow attached to feed material (Czerkawski, 1986; Forsberg *et al.*, 1977; McAllister *et al.*, 1994), so the addition of surfaces for attachment could aid growth. It is not expected that the agar in YA medium would serve as an energy source for rumen bacteria, so in this medium only a surface for bacterial adherence was added. In contrast, adding grass (YG) and sisal (YS) to the medium provides an additional energy source as well as surfaces to allow attachment. The MPN values for YA, YS and YG media were higher than the control YR, although only YS medium was significantly higher. It appeared the addition of a surface to RM02 medium did enable more bacteria to be cultured, although this had the down side of making the visual assessment of growth more difficult in the cases of YG and YS media. YA media was still clear and growth was easily detected. YS media had the highest MPN values of all the media tested (2.66×10^{10} /g of rumen content), nearly four times that of the lowest RM02 MPN. However, many of the resulting cultures could not be sub-cultured (and some may have been false positives), so the use of this medium did not result in more isolates.

Two additional media were specific modifications of RM02 medium: XP (RM02 + pectin) and WT (RM02 + titanium (III)-NTA solution). The breakdown of pectin is one of the processes of interest in fibre degradation in the rumen and pectin is important substrate for some groups of bacteria. Plant matter is made up primarily of fibre (55-70%), which includes cellulose, hemicelluloses, lignin and pectin (Waghorn *et al.*, 2007). Red clover contains 4.7-6.7% pectin (Pressey *et al.*, 1963). A number of rumen strains including *Lachnospira multipara* have been isolated on media with pectin as the only energy source (Dehority, 1969). Further work has shown *Lachnospira multipara* is able to grow with pectin at a rate similar to growth with glucose (Dušková *et al.*, 2001). The MPN of bacteria isolated on XP media was not significantly different from YR control and was within the range seen with RM02 media.

Titanium (III)-NTA solution is a powerful chemical reducing agent shown to allow the growth of obligate anaerobes (Jones *et al.*, 1980; Moench *et al.*, 1983). RM02

contains cysteine as the reducing agent, which may also act as a sulfur source. The addition of titanium (III)-NTA should decrease the redox potential, and so may allow the growth of more fastidious anaerobes. Addition of titanium (III)-NTA solution to RM02 caused the medium to discolour over time, and a white precipitate formed. The MPN of bacteria cultured on WT media was not significantly different from the YR control and was within the range seen with RM02 media. Therefore, this modification did not increase the MPN and made it more difficult to determine growth positive tubes because of the precipitate.

Medium 98-5 (V9) is a general purpose anaerobic rumen fluid-containing medium widely used for isolation of rumen bacteria (Bryant *et al.*, 1961). At the time of its development, this medium permitted the growth of around twice the number of rumen bacteria compared to previous formulations and allowed the growth of organisms that use starch (Bryant *et al.*, 1961). As RM02 does not contain starch, this medium may allow isolation of specialist starch users. This medium was also selected to compare the diversity of isolates obtained in this study with what has been previously isolated on this or similar media. V9 medium had a significantly larger average MPN value than the control YR sampling.

The remaining three media that were tested all contain a greater proportion of rumen fluid than RM02. The addition of rumen fluid to anaerobic media has been shown to increase the number of bacteria able to be cultured from the rumen (Bryant *et al.*, 1961). BY medium (code XB for this study) is a widely-used growth medium containing 30% (v/v) rumen fluid (compared to 5% [v/v] in RM02), and was selected because it supports good growth of a large range of bacteria and methanogens from the rumen. The clarified rumen fluid used to make WC medium is the liquid component of rumen contents with much of the particulate material removed by centrifugation. VC medium was used in the last sampling and consisted of clarified rumen fluid with the addition of sisal string. It was a combination of the two most successful 'other' media used in the previous samplings, YS and WC. Both WC and VC media resulted in significantly larger average MPN values than the control YR sampling. VC media had lower although not significantly different MPN values than YS or WC, showing that combining the two media with the highest MPNs did not lead to a further improved medium. Compared to the other media, both VC and WC media were very labour

intensive to make, because they required the collection and processing of large volumes of rumen fluid. These media would not be practical for everyday use or large scale experiments unless they were shown to have clear advantages. There was also appreciable batch-to-batch variability, depending on when the rumen fluid was collected. Some batches of clarified rumen fluid formed a precipitate during the final autoclaving step, which could easily obscure growth of poorly-growing cultures.

4.5.2 Diversity of bacteria cultured

The total number of cultures obtained after all five samplings including the trial cultivation was 1037. This large number of cultures should increase the likelihood of culturing representatives of some of the ‘difficult to isolate’ or ‘unculturable’ groups of bacteria that might be isolated only on rare occasions. Greater than 95% of the initial cultures were of a single cell type. This is higher than the estimated 90% of cultures calculated to originate from a single viable cell when 0.2 cells per mL are used as the inoculum. However, the isolates have not been confirmed as pure, and it is likely that, in some cases, one of multiple cells inoculated grew so much faster than co-inoculated cells, that its descendants totally dominated the resultant cultures, masking the presence of the others.

The isolates were identified by sequencing their 16S rRNA genes. A commonly used arbitrary similarity value cut-offs for comparing 16S rRNA gene sequences is 97% for a species and 95% for a genus (Schloss *et al.*, 2004). This can be used to group isolates into species level groups based on their full-length 16S rRNA gene sequences. Here, partial 16S rRNA gene sequences encompassing the V1-V3 region were compared. Sequence divergence is not distributed evenly along the 16S rRNA gene, but the V1-V3 region can be used to estimate species richness reliably if a value of 96% similarity is used (Kim *et al.*, 2011a). By BLASTN comparisons, 24.2% (i.e., 200) of the new isolates had 16S rRNA genes sequences that were <96% similar to a previously cultured bacterium. These potentially belong to new species of rumen bacteria. Exactly how many new species or genera these may represent will be discussed in Chapter 6.

The predominance of *Butyrivibrio* spp. and unclassified members of the family Lachnospiraceae can partially account for the high proportion of Firmicutes isolated.

Over 25% of all the isolates were identified as a member of the genus *Butyrivibrio*. *Butyrivibrio* spp. were isolated from almost every cow at each sampling and the majority of the isolates had unique 16S rRNA gene sequences. Members of this genus appear to be easy to culture. Other studies have estimated the population of *Butyrivibrio* spp. at varying frequencies. In lactating cows, *Butyrivibrio* spp. contributed <0.03% of the bacterial 16S rRNA gene copies (Stevenson *et al.*, 2007) and *B. proteoclasticus* made up 2-9% of the bacterial community (Paillard *et al.*, 2007). The prevalence of this genus may vary between different animals and studies. The best way to confirm if *Butyrivibrio* spp. are over-represented in the isolate collection is by comparison to the molecularly-detected *Butyrivibrio* spp. from the same rumen samples, and this will be discussed in Chapter 6.

The majority of the isolates (79.2%) with good quality 16S rRNA gene sequences had unique sequence types. There were seventeen groups of isolates when clustered with UCLUST at 100% similarity that contained more than three sequences. Over half of these had a close BLAST match to a previously-isolated bacterium from the study of Kenters *et al.* (2011). These bacteria were isolated from a New Zealand sheep using RM02 medium and may represent the easy-to-isolate bacteria from New Zealand ruminants.

The diversity of the isolates was visualised in phylogenetic trees. Even though the deep level branching patterns in phylogenetic trees were not stable, the clades identified with high bootstrap values consistently grouped with the same members, regardless of the tree construction method. Phylogenetic tree construction revealed problems with the current classification of some groups, like the family Ruminococcaceae, which was divided into two distantly-related clades. Characterisation of the isolates within both Ruminococcaceae groups could lead to reclassifications within this family. Where it was possible to assign the isolate sequences to a genus, these were to genera of bacteria that have previously been found in the rumen. Many of the new isolates could not be classified to a genus and the study of these isolates could lead to the description of new rumen bacteria.

Perhaps the lack of competition in a single cell inoculum allowed isolation of bacteria that would otherwise have been outcompeted, or perhaps sampling from animals from a geographical area not previously sampled (NZ) resulted in a quarter of

the bacteria isolated being novel. This demonstrates that the simple isolation techniques can still be used to culture novel taxa. A greater focus on isolation studies to gain representatives of the uncultured majority seems warranted.

4.5.2.1 Taxonomic placement of new isolates

Taxonomic assignment of isolate sequences was made using several different methods. The isolates were assigned to a taxonomic group with the RDP classifier using QIIME, and by comparison to various databases of 16S rRNA gene sequences using BLASTN. Together, this gave an indication of what the newly isolated bacteria were, but many had not been cultured before and had poor matches to anything known, or appeared to be incorrectly assigned because they were similar to sequences from isolates that need to be reclassified. A more thorough inspection of their phylogenetic placement was warranted. To achieve this, near full-length 16S rRNA gene sequences were generated for 186 of the isolates and these were aligned with 618628 16S rRNA gene sequences in the SILVA database using the ARB package (see Chapter 6). Phylogenetic trees generated from these near full-length sequences should be more stable than the short length sequences showing the relationships between the taxa. While taxonomic placement has been possible, the phenotypes of the isolates remain to be characterised, especially substrate use and product formation. Any speculation into the ecology or roles of these isolates is best left until these data are available.

4.5.2.2 The effect of medium on diversity

Using the combination of ‘other’ media was better for isolating novel species than RM02 medium alone (31.7% had 16S rRNA genes <96% similar to a previously cultured bacterium compared to 21.9% from RM02 medium). This is supported by rarefaction analysis, which indicated that isolating on the ‘other’ media captured more diversity in 200 isolates than on RM02 medium alone.

The greatest diversity of identified taxa was isolated on RM02 medium, but this is likely to be the result of the larger number of isolates from this medium. Three times the number of isolates were isolated on RM02 medium resulting in nine taxonomic groups that were only isolated on RM02 medium compared to four only isolated on the ‘other’ media. Although there were fewer isolates from each of the ‘other’ media compared to

RM02, each medium type supported the growth of a range of different taxa. Using a combination of media formulations increased the variety of bacteria isolated and should be considered when designing similar isolation experiments in the future. WC medium (100% clarified rumen fluid) was very good for isolating a diverse range of bacteria. Even though there were only 33 isolates from this medium, these included 13 of the 29 taxonomic groups identified with QIIME. Two groups of bacteria (*Solobacterium* spp. and *Treponema* spp.) were isolated only on this medium. *Olsenella* spp. and a group that could only be classified as members of the phylum Bacteroidetes were isolated only on this medium and one other medium (XB), which also had a higher proportion of rumen fluid. Over 50% of the bacteria isolated on WC medium were novel (<96% 16S rRNA gene sequence similarity to a previously-cultured bacterium). VC medium, which is the same as WC medium with the addition of sisal string, had fewer novel isolates (29.4%), suggesting that the addition of sisal string was detrimental.

V9 (98-5) medium was designed by Bryant and Robinson (1961) as an improvement on RGCA medium (Bryant *et al.*, 1953), and has been widely used to isolate rumen bacteria. Here, 36.4% of the bacteria isolated on V9 media were novel (<96% 16S rRNA gene sequence similarity to a previously cultured bacterium). This shows new isolates can still be obtained using a commonly-used medium as well as newer medium formulations like RM02, using this isolation method.

If the aim is to maximise the amount of diversity recovered, more media are beneficial. However, a compromise for easier and quicker isolation using only RM02 or perhaps any suitable media captures the majority of the diversity seen in this experiment. Even with the large number of isolates it is still not clear whether the groups seen only on the 'other' media were isolated by chance as these groups were represented by only a few isolates.

4.5.3 What was missed?

Cultures were eliminated from each step of the process of identification, starting with microscopic evaluation of the cultures. If more than one cell type was observed they were discarded. The cultures that did not grow when sub-cultured eliminated themselves, and the DNA extracted from some cultures did not produce a 16S rRNA

gene sequence of acceptable quality, even after multiple attempts. From the cultures that grew from the initial inoculation and were selected for further work, 71.2% resulted in an identified putatively pure isolate. This could result in a bias in the resulting culture collection against organisms that are difficult to grow and analyse under laboratory conditions. It is not unusual for newly isolated cultures to fail to grow after the first or second passage into laboratory media (Davis *et al.*, 2011; Sait *et al.*, 2002), and the rate of failure is presumably not often reported. Of course, it is not known if the cultures that were eliminated were largely new species or contained the same degree of novelty as the isolates that were successfully identified.

Overall, the 'other' media resulted in higher MPN values compared to RM02, though only four were significantly different. However, the 'other' media also had larger percentage of cultures that could not be used in the phylogenetic analysis, 36.8% compared to 11.5% of those isolated on RM02. The apparent advantages of using a range of different media that allow growth of a larger diversity of bacteria will be lost if the resulting cultures cannot be routinely subcultured. When analysing the loss rate for individual media, WC, WT and YG were comparable with RM02 (5.3-17.6% of cultured excluded). XP, YA and XB media are within the range seen with individual RM02 samplings. The worst affected media types also had the highest MPN values, so the increased number of cultures was offset by a greater percentage of cultures that were not able to be taken through to 16S rRNA gene sequencing. YS medium produced the largest MPN and the largest proportion (61.6%) of cultures that had to be excluded from the final analysis. Most YS cultures could not be repeatedly sub-cultured and only 23 isolates were gained from 60 initial cultures. In this case it was uncertain if these cultures were real or if the very weak turbidity and observed cell-like structures that developed were a medium artefact.

Chapter 5 Plant-adherent rumen community composition

5.1 Introduction

Rumen microbes that attach to ingested plant matter within the rumen are thought to be responsible for fibre degradation (Koike *et al.*, 2003a; Weimer, 1993). The bacterial species that adhere to ingested feed are different from the planktonic bacteria in the rumen fluid and those associated with the rumen epithelium (Brulc *et al.*, 2009; Pitta *et al.*, 2009). Recently, the feed adherent community has been specifically examined because of the potential to learn more about the process of fibre degradation in the rumen (Kong *et al.*, 2010; Pitta *et al.*, 2009). A better understanding of the interactions of bacteria and the feed could lead to improvements in ruminant nutrient use and fermentation efficiency. The availability of more pure cultures would enable more detailed study of the physiology and genomes of fibre-adherent bacteria, but to date much of the diversity in the rumen remains uncultured. Furthermore, cultivation-based methods are very poor for studying and comparing complex communities, and molecular techniques using the 16S rRNA gene as a marker for phylogeny are better suited for studying rumen populations.

Denaturing gradient gel electrophoresis (DGGE) can fingerprint a microbial community to look quickly at community structure. In theory, this technique can visualise separate species, distinguishing between amplicons of regions of the 16S rRNA gene that differ in composition by as little as one base pair (Muyzer *et al.*, 1993). However, low abundance members may not be detected, and so this technique is suited to display differences or similarities between the most abundant taxa. Importantly, the communities are viewed as a fingerprint of bands where the identity and relatedness of the species that comprise those bands is unknown. Similar species will be seen as individual bands and cannot be judged as being more similar than other bands arising from quite unrelated species. In contrast, next generation sequencing methods like barcoded pyrosequencing are able to generate millions of sequence reads and can be used to detect minor taxa as well as the major taxa. Because the primary sequences are investigated, sequences can be grouped into taxa at different levels.

In this study, both DGGE and pyrosequencing were used to examine the rumen community of farmed New Zealand dairy cows. The rumen contents of four cows were sampled in each season over the course of a year. The quality of pasture feed available in each season was expected to differ, potentially affecting the rumen community composition. Therefore, examining the same animals over different seasons could capture greater ruminal bacterial diversity. DGGE was used to assess if there were differences between animals, sampling times and the bacteria in different rumen fractions, and pyrosequencing was used to focus on the variation of the plant-adherent bacterial community in individual animals and at different sampling times.

5.2 Results

5.3 Rumen and forage nutritive properties

The rumen contents of four lactating dairy cows were each sampled five times over a year. Each sampling experiment was designated by a sample code comprising of the medium code shown in Table 2-13 followed by a letter that is the cow identifier shown in Table 2-12. The sampled cows formed part of a working dairy farm grazed year round on pasture. In May 2009, August 2009, and again in May 2010, the cows were supplemented with pasture silage feed as well as pasture. The pasture silage feed was offered to the animals at about 5-10% of their recommended daily intake. The compositions of the pasture and pasture silage feeds at the time of sampling are summarised in Table 5-1. The volatile fatty acid (VFA) concentrations, pH and description of the rumen contents from each cow at each sampling are summarised in Table 5-2. May is the cows' dry period and is characterised by lower feed intake. This is reflected in lower total rumen VFA concentrations. August pasture was the best quality of the months sampled and the rumen contents at that time had the greatest concentrations of VFAs. The VFA concentrations then dropped in November when the feed quality also dropped, characterised by an increase in the fibre fractions (ADF and NDF) and lower organic matter digestibility (OMD). The pasture quality improved in February and the VFA concentrations returned to almost the same values as measured in August. One cow in February (cow E) was lame at the time of sampling, had not eaten as much as the other cows, and had lower total VFAs compared to cows B, C and D. Cow C at the time of the November sampling had an unusual fermentation product

profile, with elevated levels of lactic acid and formic acid, but the pH indicated that this animal was not suffering from acidosis. Subacute rumen acidosis is defined as periods of moderately depressed ruminal pH, from about 5.5 to 5.0 (Krause *et al.*, 2006), but the measured pH was 6.11. The animal appeared healthy and its rumen contents appeared normal, but it cannot be ruled out that the animal was recovering from a case of subacute rumen acidosis where the pH had returned to normal but elevated levels of lactic acid still remained.

Table 5-1. Nutritive value and components of pasture silage (silage) and fresh pasture (pasture) used as feed for the animals in this study.

Date	Feed	Metabolisable energy (MJ/kg of dry matter)	Organic matter ^a	Acid detergent fibre ^a	Neutral detergent fibre ^a	Crude protein ^a	Crude fat ^a	<i>in vitro</i> digestibility ^a	NH ₄ -N ^{ab}	pH ^c
May 2009	Silage	11.7	90.2	34.0	49.9	16.2	3.8	72.8	9.4	3.9
Aug 2009	Silage	11.6	89.0	35.8	47.2	18.5	3.6	72.6	7.2	4.1
May 2010	Silage	10.3	88.9	37.9	50.5	14.1	2.8	64.3	7.0	4.5
Date	Feed	Metabolisable energy (MJ/kg of dry matter)	Organic matter ^a	Acid detergent fibre ^a	Neutral detergent fibre ^a	Crude protein ^a	Crude fat ^a	Organic matter digestibility ^a		
May 2009	Pasture	11.9	90.9	22.8	38.8	22.9	2.9	82.5		
Aug 2009	Pasture	13.2	88.2	21.3	39.0	29.6	3.7	91.2		
Nov 2009	Pasture	11.7	92.6	27.4	50.8	15.6	2.2	78.2		
Feb 2010	Pasture	11.8	90.0	23.5	43.6	24.2	2.5	80.4		
May 2010	Pasture	12.6	89.4	21.4	36.4	26.9	3.4	85.2		

^aComponents estimated by NIRS analysis are reported as percent of dry matter.

^bNH₄-N, ammonia nitrogen as a fraction of total nitrogen.

^cpH estimated by NIRS.

Table 5-2. Characteristics of rumen contents of cows sampled over the course of a year.

Sample date	Cow	Sample code	Fermentation end products mmol/L					Total VFA ^a	A/P ratio ^b	Rumen pH	Description of rumen contents	
			Succinic acid	Lactic acid	Formic acid	Acetic acid	Propionic acid					Butyric acid
May 2009	A	MA	0.5	b.d. ^c	b.d.	61.6	14.0	5.2	80.8	4.4	6.68	Normal ^d
May 2009	B	MB	0.4	0.2	b.d.	64.7	12.7	2.3	79.6	5.1	6.54	Normal ^d
May 2009	C	MC	0.4	0.2	b.d.	70.1	17.5	6.0	93.6	4.0	6.76	Normal ^d
May 2009	D	MD	0.4	0.3	b.d.	42.7	10.4	5.2	58.2	4.1	6.85	Normal ^d
Aug 2009	B	AB	0.9	0.3	b.d.	92.6	23.2	12.8	128.6	4.0	6.09	Normal
Aug 2009	C	AC	0.6	0.6	b.d.	92.6	24.0	13.5	130.1	3.9	5.88	Normal
Aug 2009	D	AD	0.8	0.4	b.d.	97.9	24.3	13.5	135.8	4.0	6.12	Normal
Aug 2009	E	AE	0.6	0.3	b.d.	79.7	19.5	11.8	111.0	4.1	6.30	Slimy
Nov 2009	B	NB	0.2	0.2	b.d.	73.5	17.1	13.1	103.7	4.3	6.37	Normal
Nov 2009	C	NC	0.2	11.2	6.1	64.5	14.8	13.0	92.3	4.4	6.11	Normal
Nov 2009	D	ND	0.2	0.2	b.d.	73.3	14.9	13.3	101.5	4.9	6.33	Normal
Nov 2009	E	NE	b.d.	0.3	b.d.	57.0	10.9	10.2	78.2	5.2	6.65	Normal
Feb 2010	B	FB	0.2	b.d.	b.d.	99.4	23.6	17.0	140.1	4.2	6.03	Full rumen
Feb 2010	C	FC	0.2	b.d.	b.d.	93.8	23.0	16.1	132.9	4.1	6.02	Normal
Feb 2010	D	FD	b.d.	b.d.	b.d.	69.0	14.7	9.7	93.4	4.7	6.82	Very full rumen
Feb 2010	E ^e	FE	b.d.	b.d.	b.d.	59.7	14.9	7.1	81.7	4.0	6.70	Half full rumen
May 2010	B	LB	b.d.	b.d.	b.d.	63.7	15.3	6.7	85.7	4.1	6.66	Normal ^d
May 2010	C	LC	0.2	b.d.	b.d.	71.0	17.3	9.0	97.3	4.1	6.52	Normal ^d
May 2010	D	LD	b.d.	b.d.	b.d.	54.4	12.3	6.2	72.9	4.4	6.78	Normal ^d
May 2010	E	LE	0.4	0.3	b.d.	60.7	14.5	7.1	82.4	4.2	6.66	Normal ^d

^aTotal VFA is the sum of acetic, propionic and butyric acids. ^bThe A/P is the ratio of acetic acid to propionic acid. ^cb.d., below detection limit.

^dRumen was half full but this is the normal level for the cows dry period. ^eCow lame.

5.4 Analysis of plant-adherent bacterial communities

The composition of the bacterial communities firmly attached to ingested plant material, free within the rumen fluid, or loosely attached to ingested plant material (and removed by washing) was examined using DGGE. DNA for DGGE analysis was obtained from the rumen samples fractionated for the cultivation experiment (Chapter 4). The fractionation procedure, explained in Figure 2-2, resulted in four fractions from rumen contents from each animal at each sampling date. The fractions were total rumen contents with no blending treatment (UT), total rumen contents after blending treatment (BT), the adherent fraction which was the blended digesta (solids) after washing (BD), and the combined wash fraction (TW) which comprised the liquid portion of total rumen contents combined with all the material that was washed off the digesta.

The V3 region of the 16S rRNA gene was amplified and community fingerprints visualised by DGGE. Banding patterns were assessed and clustered using the BioNumerics software package. Due to gel-to-gel variations and the complex banding profiles, each gel was analysed separately because between-gel comparisons were not feasible. The restriction on the number of samples that can be analysed on a single gel meant eight gels were used to compare different combinations of samples. Densitometric values for each band were captured and exported using the BioNumerics package and statistically analysed with the R software package using with Adonis (Anderson, 2001), a nonparametric multivariate statistical test.

The combined wash fractions (TW) (Figure 5-1) and the blended digesta fractions (BD) (Figure 5-2) from each animal and season were compared by DGGE. The TW fraction samples were loaded onto the gel in month order, i.e., samples from the May 2009 collection were loaded into lanes 1-4, then all the samples from the August 2009 collection, and so on. The banding patterns were similar for all animals and months sampled (Figures 5–1B). Clustering analysis (dendrograms) did not reveal any strong patterns with regard to month or animals. However, there was a statistical difference in bacterial communities from different months ($p=0.001$) but not from different animals ($p=0.655$), which supports the grouping of the lanes determined using the BioNumerics package (Figure 5–1A). In contrast, the BD fraction samples were loaded onto the gel in cow order, i.e., the sample from cow A was loaded into lane 1, then all sample months from Cow B were loaded in lanes 2-6, and so on. The banding patterns were similar for

all animals and months sampled (Figure 5–2B). DGGE fingerprints from the same animal tended to cluster together (Figure 5–1A). There was a statistical difference in the bacterial communities in different animals ($p=0.002$) but not from different months ($p=0.1399$).

Overall the differences between the samples were small, and the order in which samples were run on the DGGE gels seemed to have had a major effect on the differences detected by statistical analysis. Samples typically do not migrate evenly in all lanes across the width of a DGGE gel. Lanes on the edge of the gel tend to run faster than those in the middle. This is corrected for by band normalisation, but lanes that are next to each other tend to appear more similar compared to distant wells if the banding pattern is very similar across all samples (Figures 5–1B and 5–2B). Certainly, the band profiles from the different samples all appear visually very similar (Figures 5–1A and 5–2A), and it seems that lane-to-lane artefacts may be increasing the variation between samples. This highlights the difficulties of using DGGE for this type of analysis. Visual examination of the unprocessed gel pictures (Figure 5-2B and Figure 5-2B) shows there are very few differences between the fractions, animals and months. Statistical analysis showed significant differences that were probably due to noise of the technique (i.e., lane position) rather than true variation. Because of this, visual inspection and clustering analysis were both used to assess DGGE gels.

The washed rumen digesta were blended to separate tightly-attached bacteria from the plant material and to reduce the particle size of material in the digesta, so they could be used as inocula for cultivation. However, the effects that blending had on the bacteria was unknown. To assess the effects of the mechanical blending process on the apparent community of the rumen contents, unblended total rumen contents were compared with blended total rumen contents. The DGGE fingerprints are shown in Figure 5-3 and showed similar banding profiles across all fractions, animals and months. Both the blended and unblended rumen samples for each animal tended to cluster together, indicating that blending is not affecting the observed community.

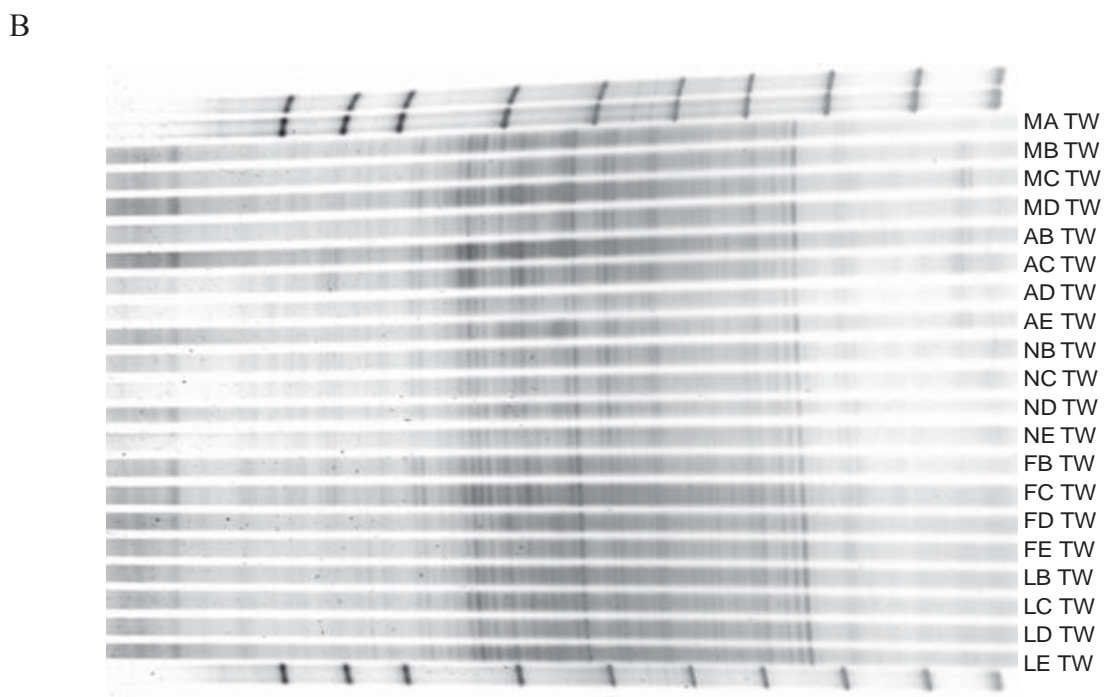
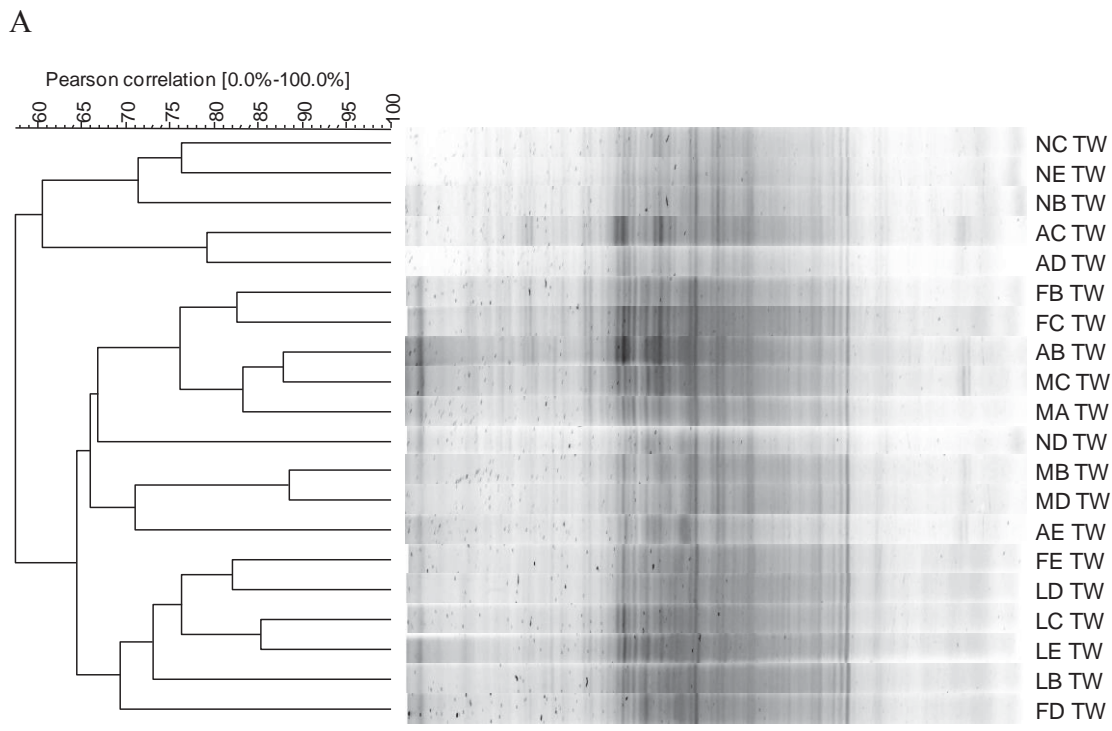
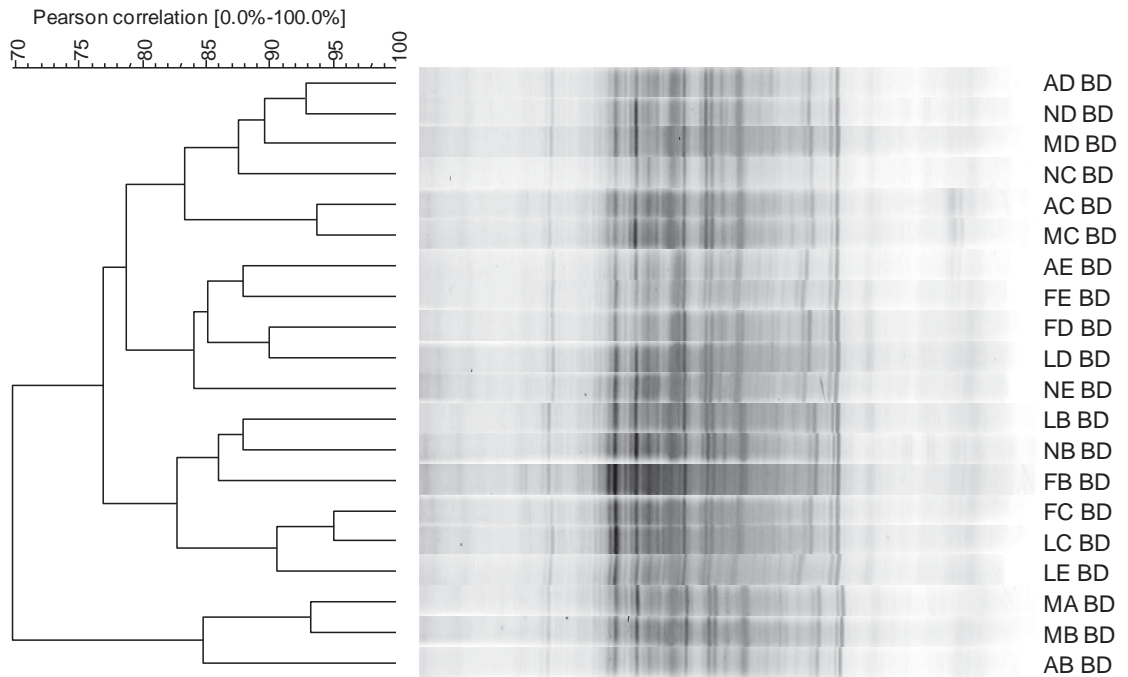


Figure 5-1. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) from combined wash fraction (TW) from five animals sampled in five seasons.(A) Differences between the patterns were analysed using the Pearson correlation and the pattern relatedness is shown by branch length using the UPGMA method, in the dendrograms to the left of the gels. (B) DGGE samples in the order they were loaded onto the gel. This image was processed by the BioNumerics package to generate the image shown in panel A. The marker in the first two lanes and the final lane is DGGE MII (Invitrogen). The lanes are labelled to the right (A) or left (B) with their sample code followed by the fraction type.

A



B

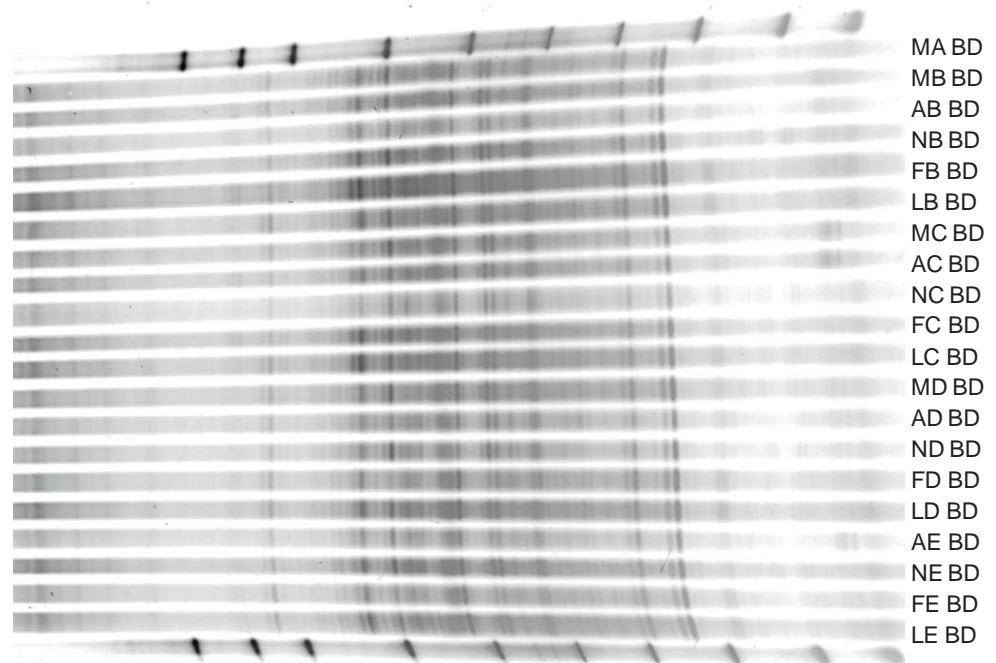
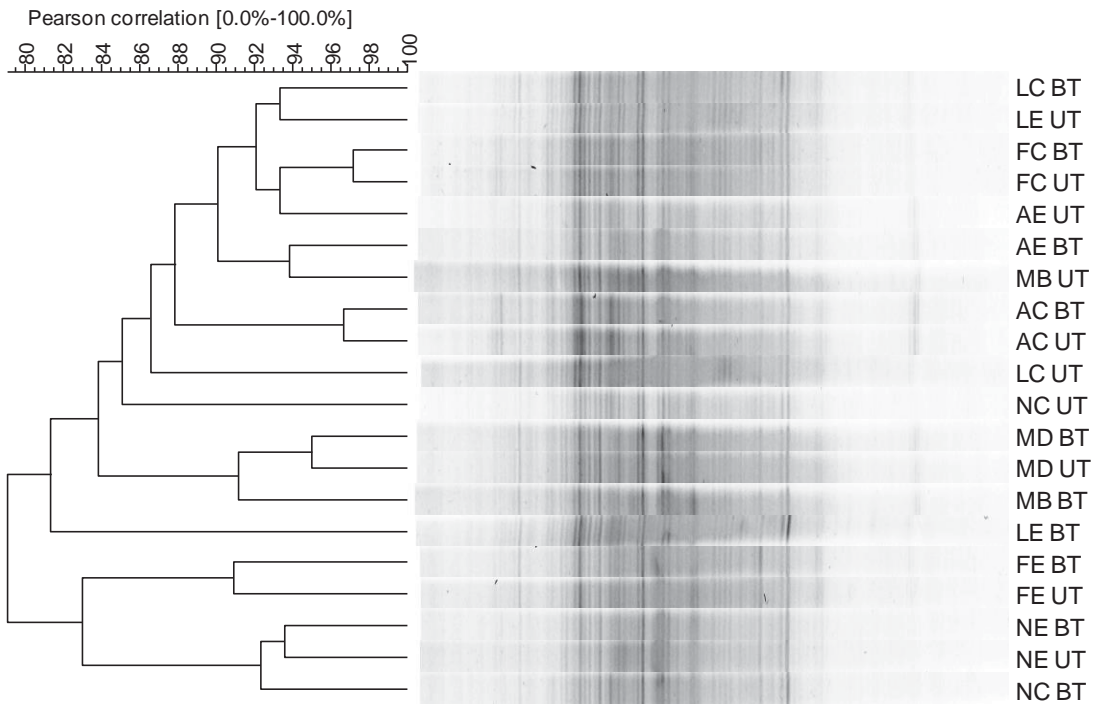


Figure 5-2. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) from blended digesta fraction (BD) from five animals sampled in five seasons.(A) Differences between the patterns were analysed using the Pearson correlation and the pattern relatedness is shown by branch length using the UPGMA method, in the dendrograms to the left of the gels. (B) DGGE samples in the order they were loaded onto the gel. This image was processed by the BioNumerics package to generate the image shown in panel A. The marker in the first lane is DGGE MIV and the second and final lanes are DGGE MII (Invitrogen). The lanes are labelled to the right (A) or left (B) with their sample code followed by the fraction type.

A



B

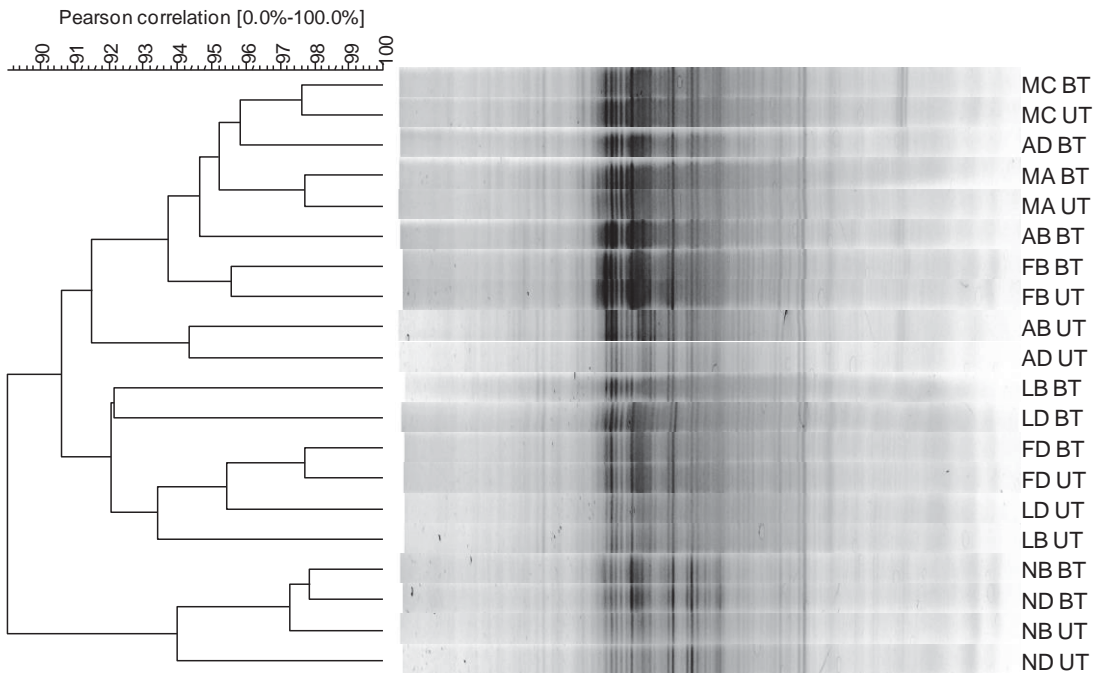
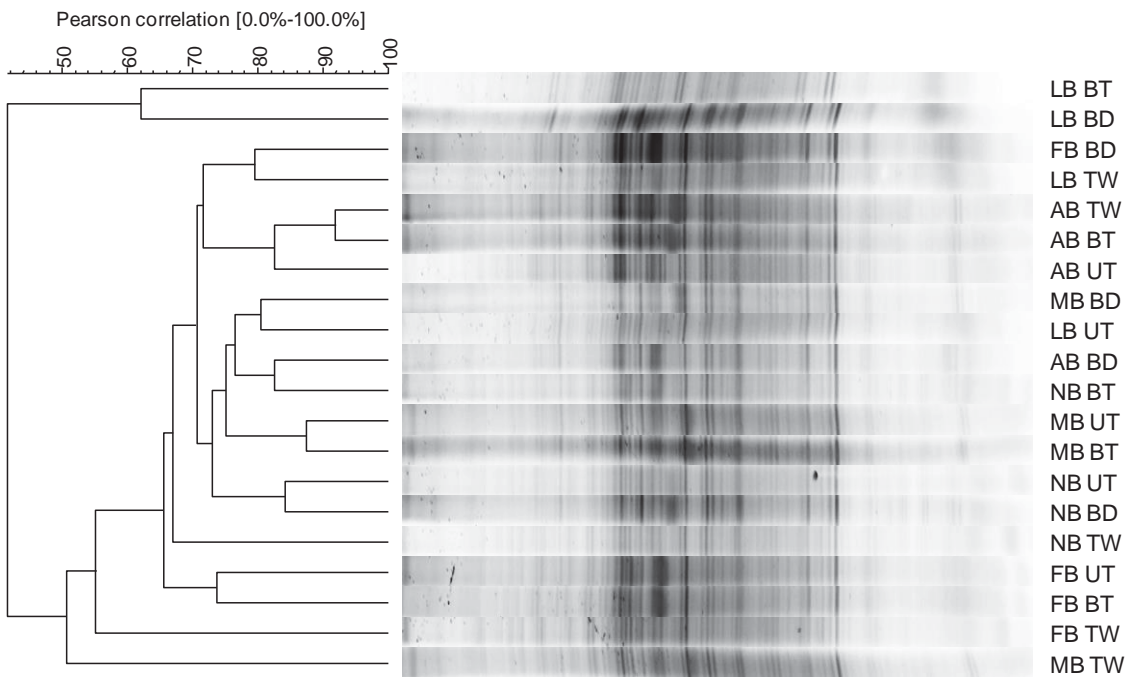


Figure 5-3. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) from blended total (BT) and unblended total (UT) rumen contents from five animals sampled in five seasons. The sample separated in each lane is named on the right of the gels. Differences between the patterns were analysed using the Pearson correlation and pattern relatedness is shown by branch length using the UPGMA method, in the dendrograms to the left of the gels. Half of the samples (sample code shown to the right of the gel) are shown on one gel (A) and half on a second gel (B).

All samples generated from a single cow were analysed together on a gel (one for each animal, four in total) shown in Figure 5-4 and Figure 5-5. Samples from Cow A were loaded on the same gel as those from Cow E (Figure 5-5B). These gels show each fraction type and sampling month combination for each animal, to assess changes in the community due to fraction type or month sampled excluding animal variation as a factor. The samples were loaded onto the gels in order of month, i.e., the four fractions for May 2009 were loaded into wells 1-4, then all the samples from the August 2009 collection, and so on. Overall, the banding patterns for each sample were very similar for each of the animals. Clustering analysis (dendrograms) of banding patterns from each of the cows; B (Figure 5-4A), C (Figure 5-4B), D (Figure 5-5A), and A combined with E (Figure 5-5B) tended to show no clustering of bacterial community banding patterns due to fraction type, but a weak association due to month. The clustering analysis of the DGGE fingerprints revealed each gel was on average ~70% similar (Pearson correlation~40-88%), therefore the differences detected in bacterial communities are only small. Because there was difficulty in recognising and matching each individual band (99 – 105 bands per gel), some of the variation may be due to noise of the technique. This is probably the case for the gel containing samples from cow B (Figure 5-4A), which showed the most variation in the clustering analysis. Here, samples LB BT and LB BD clustered separately from the other samples from Cow B, but this appears to be a gel artefact due to gel smiling effects where bands in these two lanes migrated faster on the right edge of the gel.

A



B

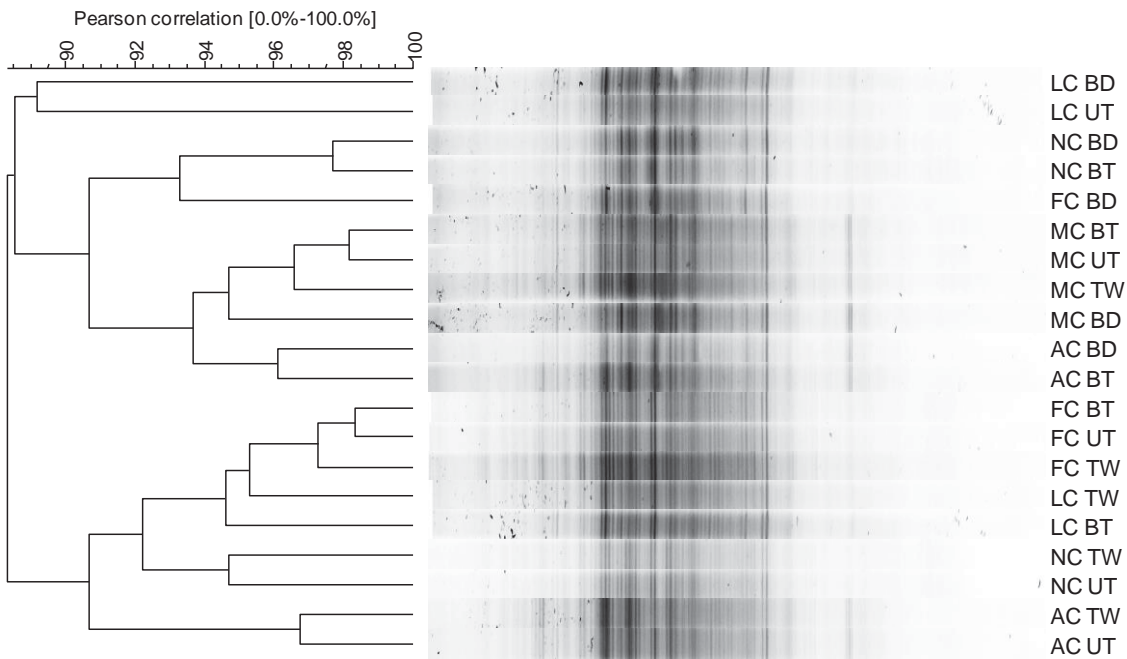


Figure 5-4. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) from (A) cow B and (B) Cow C from five animals sampled in five seasons. Differences between the patterns were analysed using the Pearson correlation and the pattern relatedness is shown by branch length using the UPGMA method, in the dendrograms to the left of the gels. The lanes are labelled to the right with their sample code followed by the fraction type.

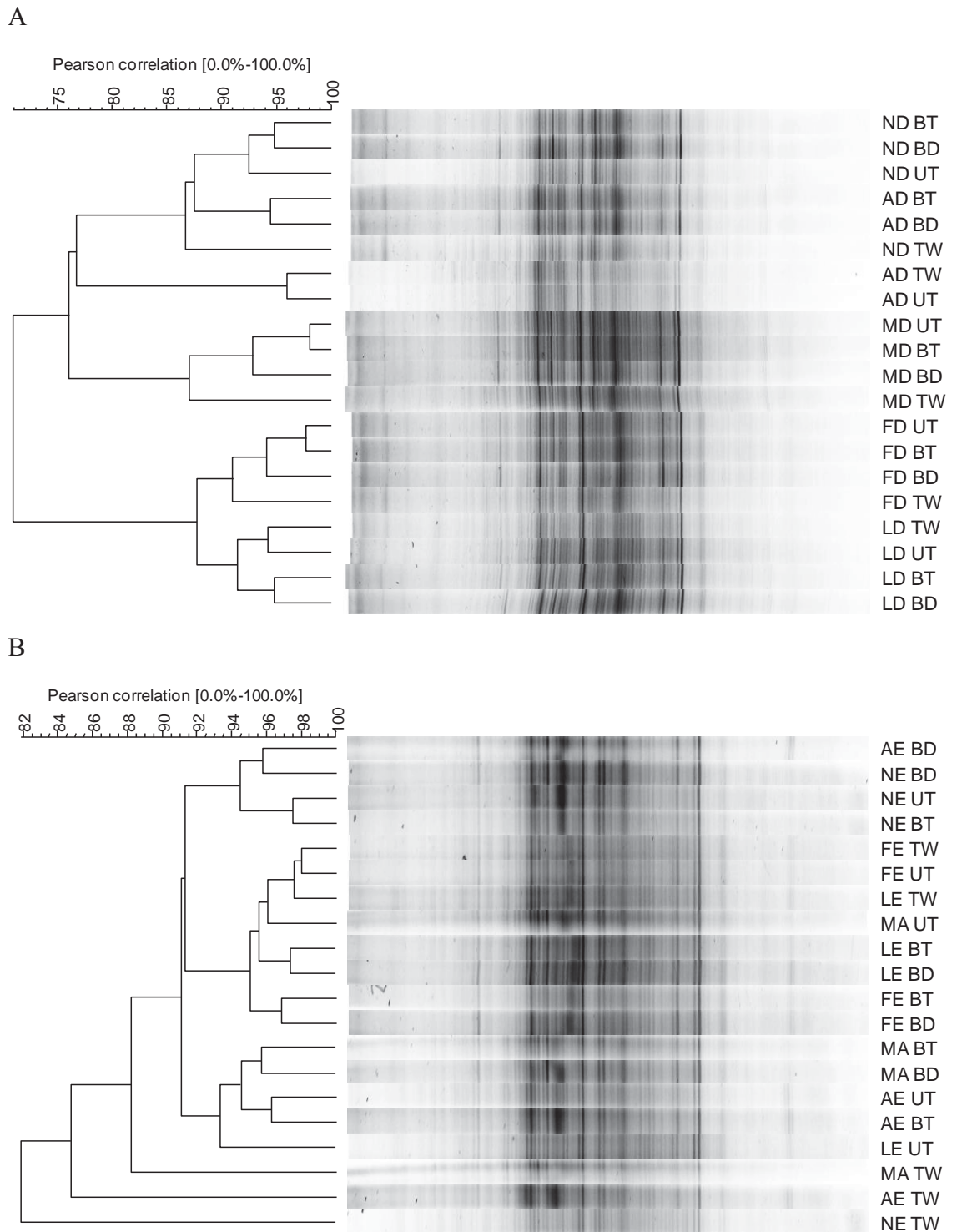


Figure 5-5. Comparison of DGGE profiles of bacterial 16S rRNA genes (V3 region) from (A) cow D from five animals sampled in five seasons, and (B) Cow E (sampled in four seasons) and Cow A (sampled in one season). Differences between the patterns were analysed using the Pearson correlation and the pattern relatedness is shown by branch length using the UPGMA method, in the dendrograms to the left of the gels. The lanes are labelled to the right with their sample code followed by the fraction type.

5.4.1 Culture independent assessment of community structure

A small scale clone library was constructed to check one of the samples to be analysed by pyrosequencing. The plant-adherent rumen fraction from the May sampling of cow A was used to prepare a library of 16S rRNA genes. DNA extracted from the blended digesta fraction was used as the template and the V1-V3 region of the 16S rRNA gene amplified. The amplicons were ligated into the pCR 2.1 TOPO cloning vector and the plasmids transformed into TOP10 competent *Escherichia coli* cells. A selection (54) of the resulting clones was checked for inserts of the correct size, by PCR amplification using vector-targeted primers and using a small part of the colony as the template source. These inserts were sequenced and quality checked sequences were compared to their closest cultured relative in Greengenes and assigned a phylogeny with RDP (Figure 5-6). Greengenes calculates the sequence similarity values as the identity between the user sequence and the reference considering only conserved bases. The majority of sequences (42, 78%) had $\leq 96\%$ similarity match to anything previously cultured. The clones were identified as belonging to five phyla: Firmicutes (27 clones), Bacteroidetes (19 clones), Fibrobacteres (2 clones), Actinobacteria (1 clone) and Proteobacteria (1 clone). Four clones were not able to be assigned to a phylum.

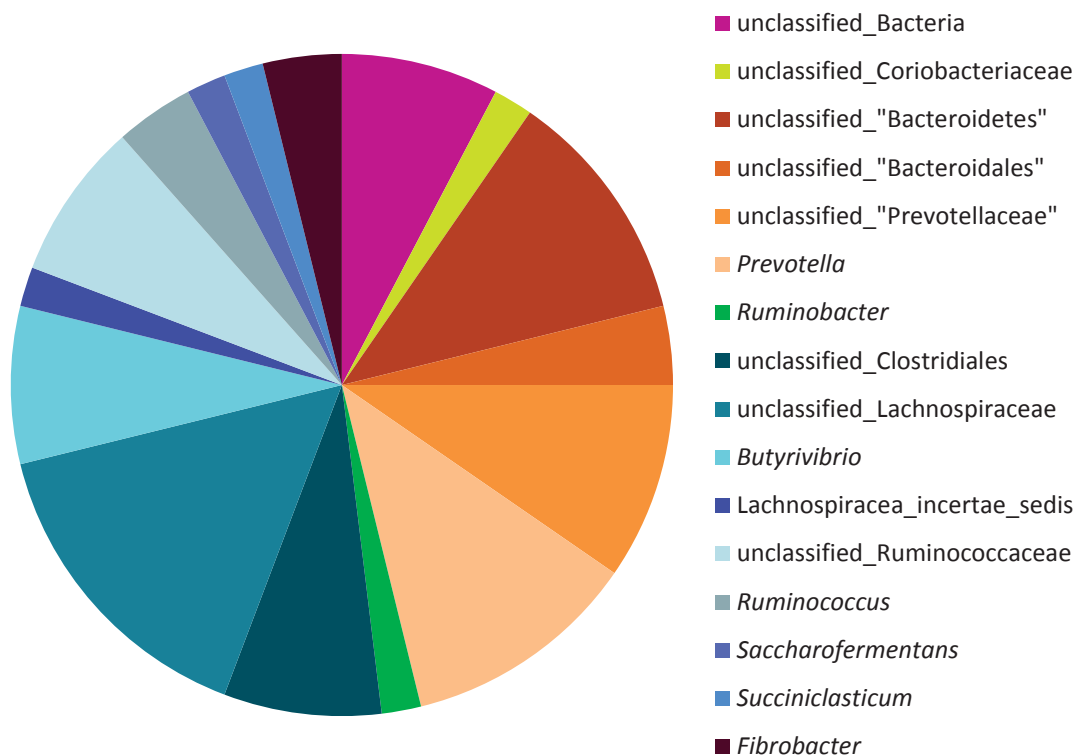


Figure 5-6. Identity of 16S rRNA genes amplified from plant-adherent fraction of rumen contents from cow A in May 2009. Sectors in brown/orange tones are taxa within the phylum Bacteroidetes and sectors in blue tones are taxa within the phylum Firmicutes.

5.5 Pyrosequencing

The plant-adherent rumen bacterial communities from four lactating dairy cows, each sampled five times over a year, were examined using barcoded 454 titanium pyrosequencing. DNA for the pyrosequencing was extracted from washed and blended rumen digesta that had been fractionated as described in Figure 2-2. These same samples were also used to isolate new cultures as described in chapter 4. The V1-V3 region of the 16S rRNA gene was amplified from each DNA sample using a conserved primer pair with a unique 10-nucleotide barcode at the 5'-end of the forward primer (see Table 2-19). Equal amounts of each barcoded PCR product were mixed together and pyrosequenced, resulting in 594,271 pyrotag reads. After pre-processing and quality control of the reads in QIIME (section 2.7.5), 327,300 high quality sequences ranging in length from 370-409 bases were obtained. The pyrosequence reads underwent further quality control and clustering analysis using CD-HIT-OTU, which removed noise and chimeric reads and then clustered the denoised reads. Demultiplexed and quality trimmed sequences were parsed into the CD-HIT-OTU pipeline where they underwent the following steps. (1) Low quality reads were filtered out and reads with extra long tails were trimmed. No reads were removed in this step because they had already been quality checked. (2) Filtered reads were clustered at 100% identity using CD-HIT-DUP, resulting in 209,433 clusters. (3) 8,228 chimeric clusters were identified and removed. (4) Secondary clusters were identified and recruited into primary clusters. (5) Noise sequences were designated as those in clusters of one or two reads. Consequently, 187,300 clusters of $n \leq 2$ were removed. (6) The remaining processed dataset of 110,585 reads from non-chimeric clusters was reclustered into 1539 OTUs at 96% similarity level. The average number of reads, OTUs and taxa for each animal are summarised in Table 5-3. Comparable numbers of trimmed reads, clustered reads and OTUs were obtained for each sample.

Table 5-3. Number of sequences^a and taxa obtained from all animals and sampling times^b.

Parameters	Total	Average per animal	s.d.	Min	Max
Trimmed reads ^c	327300	15585.71	1324.42	12299	17341
CD-HIT-OTU quality reads ^d	110585	5265.95	360.44	4607	5986
Number of OTUs ^e	1539	820.95	70.31	653	926
Bacterial taxa groups ^f	58	45.9	1.87	42	50

^a Average length of sequence (nt) = 519 and covers the V1-V3 region.

^b Number of samples = 21.

^c Sequence reads after quality trimming in QIIME.

^d Sequence reads after clustering with CD-HIT-OTU.

^e Number of OTUs clustered at 96% similarity.

^f Number of taxonomic groups, identified to the genus level if possible. The higher level taxa may contain several genera.

5.5.1 Sequencing controls

Two control samples, each with their own unique identifying barcode, were included in the pyrosequencing. The first control sample was a negative control (no sample DNA, just water added) from the initial PCR run that generated amplicons for the pyrosequencing runs. This had its own barcoded forward primer (Adf*MID54 on Table 2-20). No bands were visible in electrophoresis gels and no pyrosequences were able to be assigned to the negative control barcode. The second control sample was included to test the effects of the barcode on primers pairs with different amplification efficiencies. The barcode was only incorporated into the forward primer and the reverse primer was the same for each reaction. A few primer pairs consistently produced less PCR product (example shown in Figure 5-7), even though the amount of template DNA for each PCR was normalised. The same DNA preparation from the worst affected sample (sample FD with barcode MID65) was amplified with a second primer pair containing a different barcode (MID51). This resulted in a greater PCR product yield, and indicated that the differences were due to the barcode and not the sample DNA. The amplicons from both PCRs, with the different barcodes, were pyrosequenced to assess the influence that barcode choice had on the observed sequenced community. Thus sample FD was included twice in the pyrosequencing analysis. The read sets are labelled FD.51 and FD.65 (corresponding to barcoded primer MID51 and MID65 on Table 2.19).

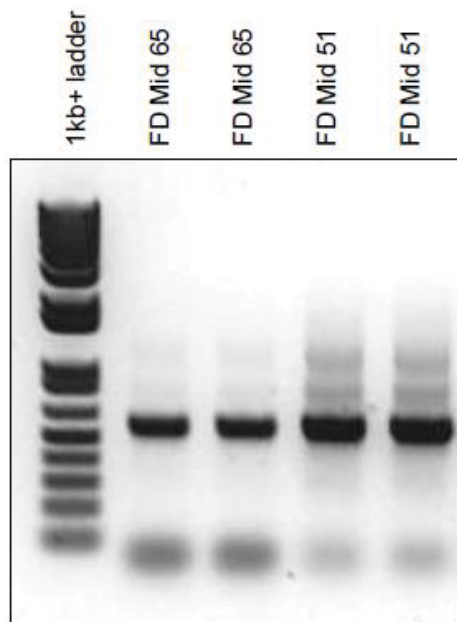


Figure 5-7. Amplification of V1-V3 region of the 16S rRNA gene with two different barcoded forward primers paired with the same reverse primer from the same DNA aliquot extracted from sample FD. The left lane contains a DNA standard. Each PCR was performed twice and the products from each were run on separate lanes.

5.5.2 OTU analysis

Clusters of sequences, designated as OTUs, were produced in the QIIME pipeline at different similarity values or thresholds. Sequences with similarities greater than the threshold were grouped into the same OTU. The numbers of OTUs increased when the threshold for clustering was increased (Figure 5-8). Clusters of sequences at similarities of 96% were chosen to represent ‘species-like groups’. The proportion of OTUs unique to each sample was determined and the results are summarised in Table 5-4. Twelve of the 21 samples had no unique OTUs and the OTUs that were unique to the remaining samples represented less than 0.3% of all the OTUs and less than 0.01% of all the reads in the processed dataset. This demonstrated that the differences observed between the individual communities were very small. There were a greater proportion of OTUs unique to any sample month than the total of unique OTUs for each animal sampled in the same month. Similarly, there were a greater proportion of OTUs unique to an animal than the total of unique OTUs in each month that the same animal was sampled in. This shows that there were some OTUs specific to either the animal or month sampled. However, these unique OTUs still represented only a small fraction of the total OTUs. The largest percentage of unique OTUs (0.9%) was found in the February samples. Of the all the OTUs, 11.9% were found in all 21 of the samples, 21.1% were found in more than 90% of the samples, and 50.3% were found in more than 50% of the samples.

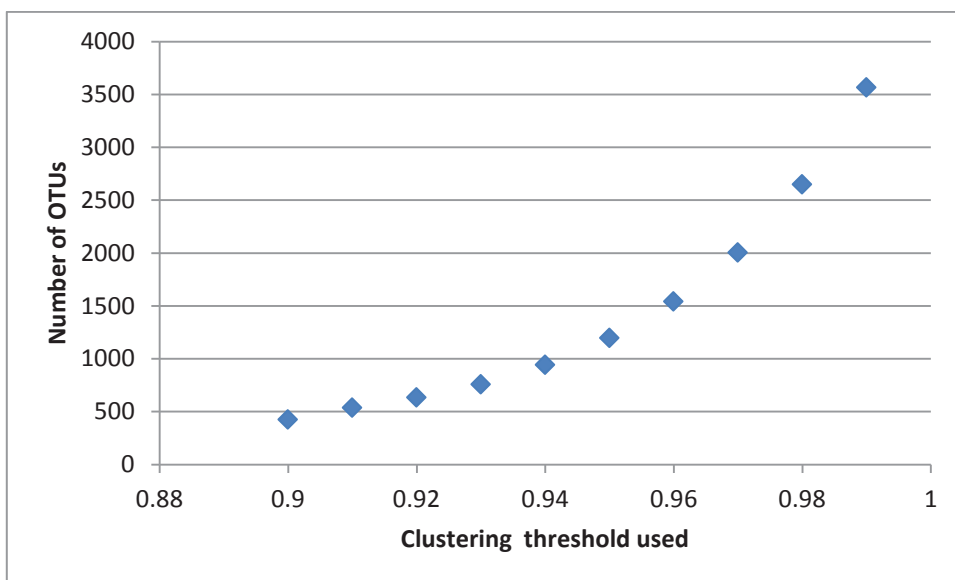


Figure 5-8. The number of OTUs generated when different clustering thresholds were applied to the processed dataset of 110,585 sequence reads.

Table 5-4. Percentage of unique OTUs and percentage of sequences in the processed dataset represented by those OTUs in each animal at each sampling^a.

Months	Animal					Total ^b	All animals in month ^c
	A	B	C	D	E		
May - M	0 (0)	0.195 (0.014)	0 (0)	0 (0)	0 (0)	0.195 (0.014)	0.585 (0.038)
August - A		0.065 (0.008)	0 (0)	0.195 (0.011)	0 (0)	0.260 (0.019)	0.520 (0.059)
November - N		0.065 (0.005)	0.065 (0.008)	0 (0)	0 (0)	0.130(0.013)	0.585 (0.044)
February - F		0 (0)	0 (0)	0.130 (0.007)	0.130 (0.007)	0.260 (0.014)	0.910 (0.080)
May - L		0 (0)	0 (0)	0.065 (0.006)	0.065 (0.004)	0.130 (0.010)	0.455 (0.036)
Total	0 (0)	0.325 (0.027)	0.065 (0.008)	0.390 (0.024)	0.195 (0.011)		
All months for each animal^d	0 (0)	0.455 (0.034)	0.130 (0.018)	0.585 (0.035)	0.260 (0.014)		

^a The percentage of OTUs that were unique to only that fraction are shown; the percentage of sequences this represents is shown in brackets.

^b The total of unique OTUs and sequences found in fractions for each month or animal.

^c OTUs and sequences unique to that month of sampling.

^d OTUs and sequences unique to each animal.

5.5.3 Plant-adherent rumen community composition

The pyrosequence reads in the processed dataset were classified into 14 phyla (Figure 5-9). The majority of the sequences fell into the phylum Firmicutes (79.5%), followed Bacteroidetes (12.1%). Nearly 5% of the sequences were only identified to the bacterial domain level. For a more detailed examination of the phylogenetic composition of the plant-adherent community, the pyrosequence reads were classified using QIIME, to the most detailed taxonomic description possible. In some cases this was to the genus level, but in others to the family, order, class, phylum or domain level. A small percentage (0.05%) of the sequences could not be classified at all. The taxa identified in each sample are shown in Figure 5-10. Altogether, 58 taxonomic groups were identified. Taxonomic groups classified at levels higher than the genus level could represent several genera each. Therefore 58 is the lowest possible number of genera found in these samples. The community composition differences between samples FD.51 and FD.65 (Figure 5-10) show the amount of variation from the same sample amplified by primers with different barcodes. Interestingly, by visual inspection, these differences appear around as great as between any two different samples. This indicates the choice of primer may have an effect on the observed community composition.

Overall, the bacterial communities identified in each sample were similar. The largest taxonomic groups of pyrosequences identified in plant-adherent rumen fractions were those of undefined genera in the family Lachnospiraceae, followed by those that could only be assigned to the order Clostridiales. Details of all the major taxa (those that comprise of over 0.5% of the total sequences) are shown in Table 5-5. Nearly half of the major taxa groups could not be classified to the genus level. Of those that could, there were 10 described genera with >0.5% of the total sequences, including (from the largest to smallest) *Ruminococcus*, *Prevotella*, *Pseudobutyrvibrio*, *Fibrobacter*, *Butyrvibrio*, *Mogibacterium*, *Coprococcus*, *Anaerovorax*, *Succinivlasticum* and *Oscillibacter*. Minor taxa, each with less than 0.5% of the total sequences, contributed to most of the diversity seen within sequences (Table 5-6). Forty out of the 58 taxa groups each contained less than 0.5% of the total sequences.

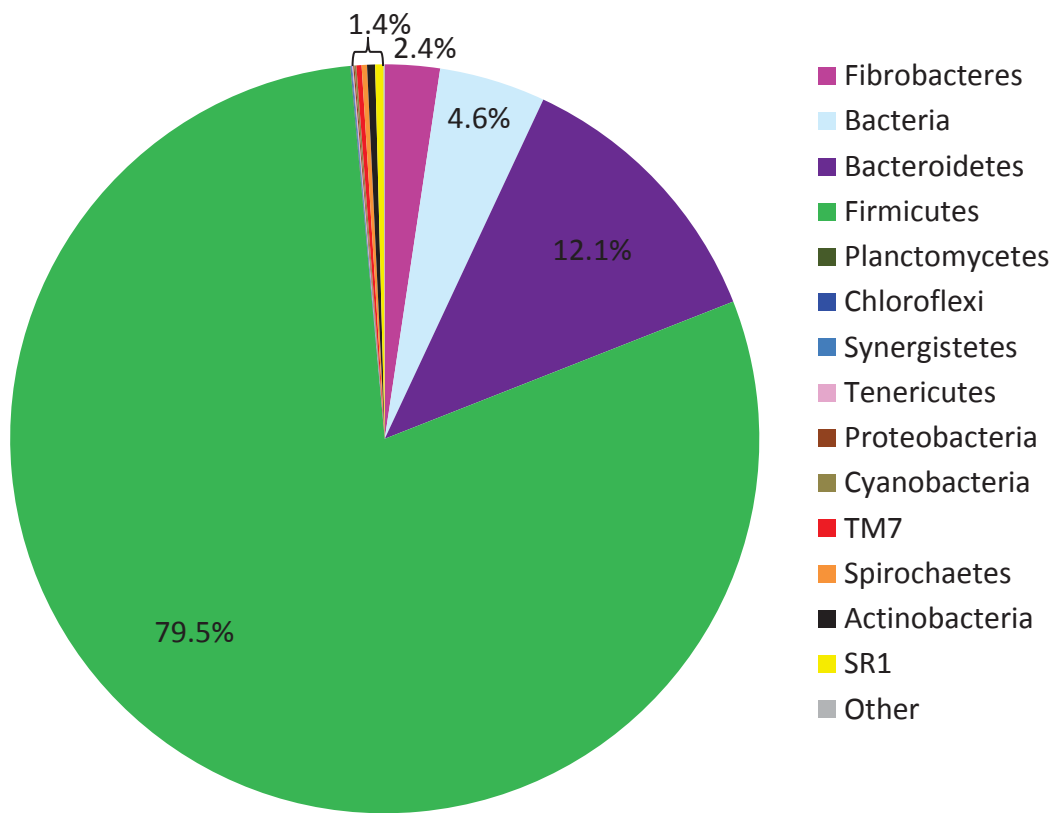






























Figure 5-9. Phylum-level assignment of sequences obtained from the plant-adherent fraction of rumen contents, summarised from all 21 samples.

Key	Taxa > 0.1%
	<i>TM7_genera_incertainae_sedis</i>
	<i>SR1_genera_incertainae_sedis</i>
	<i>Treponema</i>
	Bacteria
	Firmicutes
	Erysipelotrichaceae
	Clostridia
	<i>Succiniclasticum</i>
	<i>Selenomonas</i>
	<i>Ruminococcus</i>
	Ruminococcaceae
	<i>Oscillibacter</i>
	Clostridiales
	<i>Roseburia</i>
	<i>Pseudobutyrvibrio</i>
	Lachnospiraceae
	Lachnobacterium
	<i>Coprococcus</i>
	<i>Butyrivibrio</i>
	Clostridiales;incertae Sedis XIII
	<i>Magibacterium</i>
	<i>Anaerovarax</i>
	<i>Fibrabacter</i>
	Bacteroidetes
	<i>Prevotella</i>
	Prevotellaceae
	Bacteroidales
	<i>Olsenella</i>

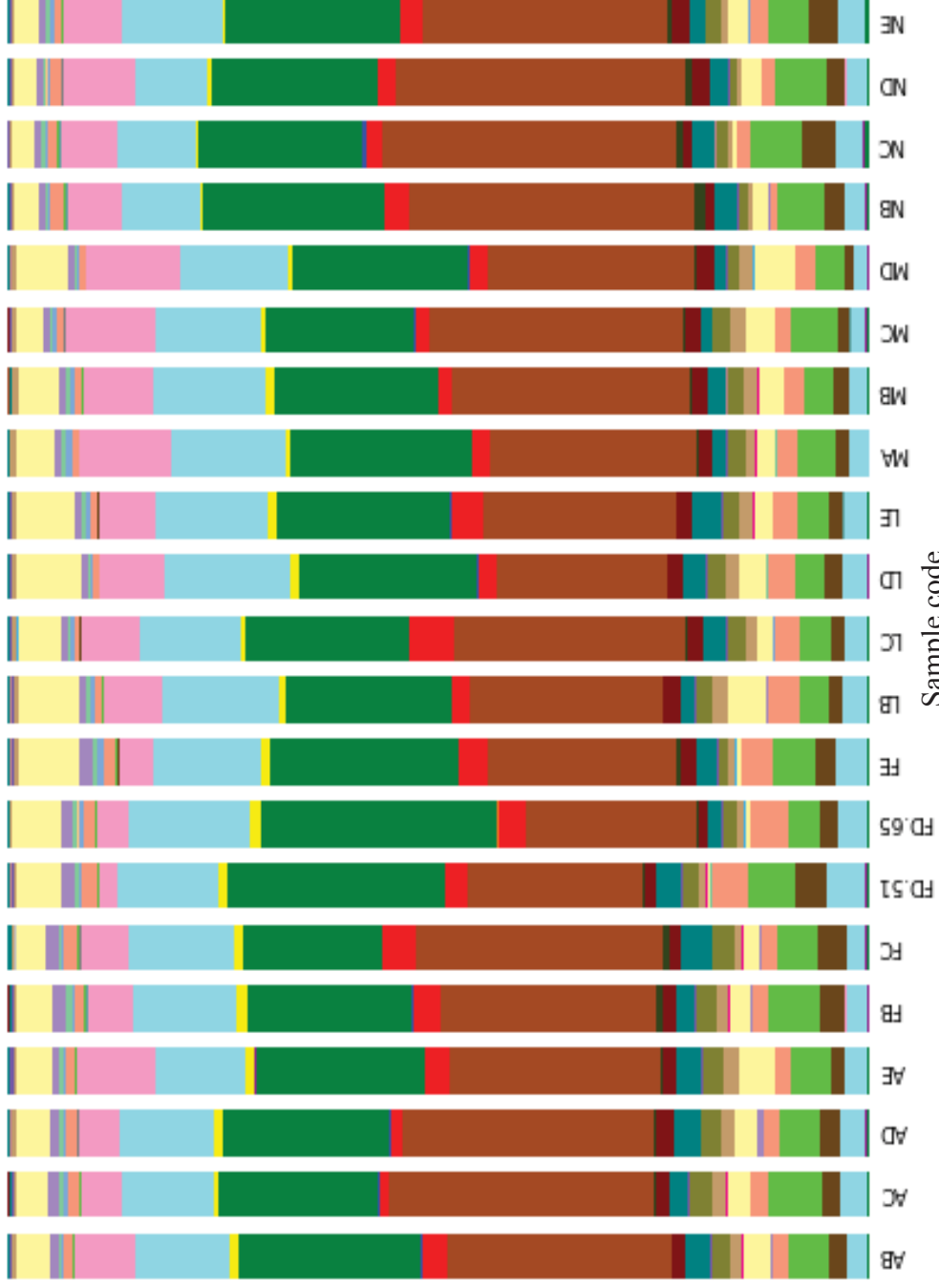


Figure 5-10. Bacterial taxa from the plant-adherent fraction of rumen contents. The sample code is explained in Table 5-2. The legend shows the lowest defined taxonomic group for all taxa that contained at least 0.1% of the total reads.

Table 5-5. The percentage of major bacterial taxa found plant-adherent rumen samples.

Major taxa (>0.5% of the total)	% of total
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Other;Other	2.688
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Other	2.235
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae; <i>Prevotella</i>	4.626
Bacteria;Bacteroidetes;Other;Other;Other;Other	2.423
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae; <i>Fibrobacter</i>	2.368
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIII; <i>Anaerovorax</i>	1.204
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIII; <i>Mogibacterium</i>	1.858
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Butyrivibrio</i>	2.309
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Coprococcus</i>	1.713
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Other	26.276
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Pseudobutyrvibrio</i>	2.549
Bacteria;Firmicutes;Clostridia;Clostridiales;Other;Other	20.170
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae; <i>Oscillibacter</i>	0.787
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Other	11.779
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae; <i>Ruminococcus</i>	6.765
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae; <i>Succiniclaticum</i>	1.065
Bacteria;Firmicutes;Other;Other;Other;Other	1.013
Bacteria;Other;Other;Other;Other;Other	4.593

Table 5-6. Minor bacterial taxa found in plant-adherent rumen samples.

Minor taxa (<0.5% of the total)	% of total
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae; <i>Atopobium</i>	0.015
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae; <i>Denitrobacterium</i>	0.006
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae; <i>Eggerthella</i>	0.006
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae; <i>Olsenella</i>	0.205
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae;Other	0.108
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae; <i>Slackia</i>	0.004
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Other	0.038
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae; <i>Hallella</i>	0.008
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae; <i>Paraprevotella</i>	0.012
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Other;Other	0.035
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Sphingobacteriaceae;Other	0.004
Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;Other	0.032
Bacteria;Cyanobacteria;Cyanobacteria;Chloroplast;Streptophyta;Other	0.082
Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae; <i>Lactobacillus</i>	0.005
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae; <i>Streptococcus</i>	0.069
Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae; <i>Anaerofustis</i>	0.053
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIII;Other	0.186
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Anaerosporobacter</i>	0.034
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Lachnobacterium</i>	0.340
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Roseburia</i>	0.165
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae; <i>Syntrophococcus</i>	0.005
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae; <i>Anaerotruncus</i>	0.019
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae; <i>Anaerovibrio</i>	0.045
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Other	0.042
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae; <i>Schwartzia</i>	0.040
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae; <i>Selenomonas</i>	0.132
Bacteria;Firmicutes;Clostridia;Other;Other;Other	0.423
Bacteria;Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae; <i>Bulleidia</i>	0.035
Bacteria;Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae;Other	0.418
Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales;Planctomycetaceae;Other	0.017
Bacteria;Proteobacteria;Alphaproteobacteria;Other;Other;Other	0.004
Bacteria;Proteobacteria;Other;Other;Other;Other	0.045
Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Other;Other	0.028
Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae; <i>Treponema</i>	0.203
Bacteria;SR1;SR1_genera_incertae_sedis;Other;Other;Other	0.361
Bacteria;Synergistetes;Synergistia;Synergistales;Synergistaceae;Other	0.038
Bacteria;Tenericutes;Mollicutes;Anaeroplasmatales;Anaeroplasmataceae; <i>Anaeroplasma</i>	0.041
Bacteria;Tenericutes;Mollicutes;Other;Other;Other	0.005
Bacteria;TM7;TM7_genera_incertae_sedis;Other;Other;Other	0.224
None;Other;Other;Other;Other;Other	0.049

5.5.4 Comparing sequences from different animals or sample months

The proportions of bacterial families identified from the pyrosequences when the samples are grouped by animal or sample month are shown in Figure 5-11. The family level groups appear similar for each animal and each month, with only small differences in the abundance of the major groups (>0.5% of the total reads) and presence or absence of minor groups (<0.5% of the total reads). Differences in the overall community composition were visualised with principal coordinates analysis (PCoA). Communities were clustered using PCoA of weighted and unweighted UniFrac distance matrices implemented in QIIME, and the resulting plots were colour-coded by animal (Figure 5-12) and month (Figure 5-13). The percentage of community variation at the family-level explained by the plots was around 7-36%. These small UniFrac distances indicate the communities were similar. PCoA revealed no clustering of samples by animal and a weak relationship indicated by overlapping clusters by month. No single month clustered completely separately from the other months on all plots. Interestingly, communities in the samples taken in May 2009 and May 2010 clustered together even though these were taken one year apart (not shown). To determine whether the grouping of samples by month or animal was statistically significant, two nonparametric multivariate statistical tests, ANOSIM and Adonis, were implemented within QIIME on the weighted and unweighted UniFrac distance matrices. ANOSIM and Adonis analysis showed significant differences in the bacterial communities from different months with the weighted ($p=0.001$ for both analyses) and the unweighted ($p=0.001$ for both analyses) UniFrac distance matrices. Differences in bacterial communities between different animals were not significant for the weighted ($p=0.78$, $p=0.745$ for ANOSIM and Adonis, respectively) and the unweighted ($p=0.905$, $p=0.53$) UniFrac distance matrices. These results support the observations in the PCoA plots.

Legend ^a	Taxonomy ^b
■	TM7_genera_incertain_sedis
■	Anaeroplasmataceae
■	SR1_genera_incertain_sedis
■	Spirochaetaceae
■	Bacteria
■	Firmicutes
■	Erysipelotrichaceae
■	Clostridia
■	Veillonellaceae
■	Ruminococcaceae
■	Clostridiales
■	Lachnospiraceae
■	Clostridiales
■	Incertain Sedis XIII
■	Eubacteriaceae
■	Streptococcaceae
■	Fibrobacteraceae
■	Streptophyta
■	Anaerolineaceae
■	Sphingobacteriales
■	Bacteroidetes
■	Prevotellaceae
■	Bacteroidales
■	Coriobacteriaceae

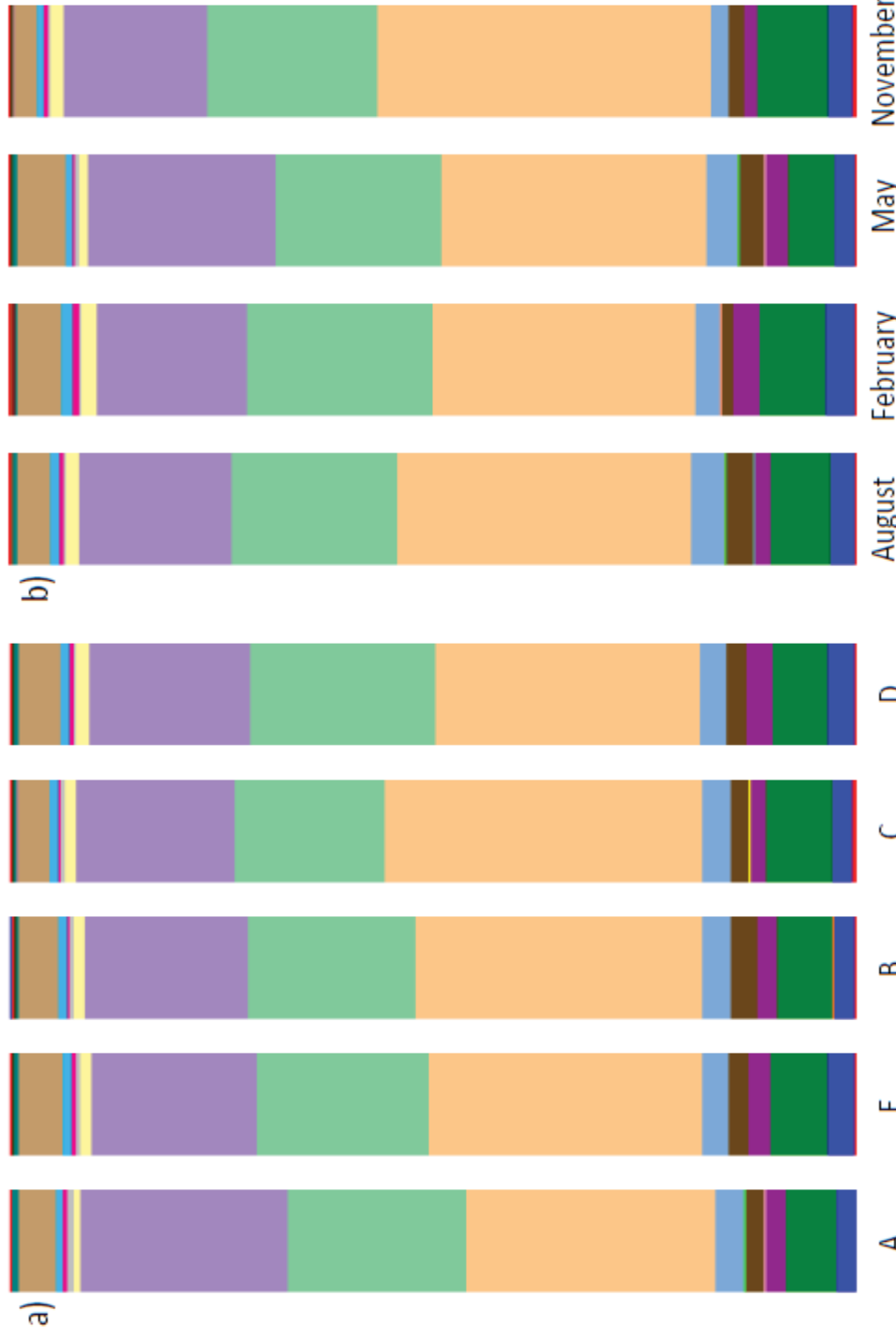


Figure 5-11. The effect of (a) animal and (b) sample month on the bacterial families identified from the plant-adherent fraction of rumen contents. (a) Samples from each animal (A-E) over the course of a year. Animal A was sampled in 1 season and animal E was sampled in 4 seasons. Animals B, C and D were sampled in five seasons. (b) Four animals were sampled once in each season (month) except for May which was sampled twice, a year apart.

^a Taxa with $\geq 0.1\%$ of the sequence reads.

^b The taxonomy is shown to the family level where possible or to the lowest defined rank it could be assigned.

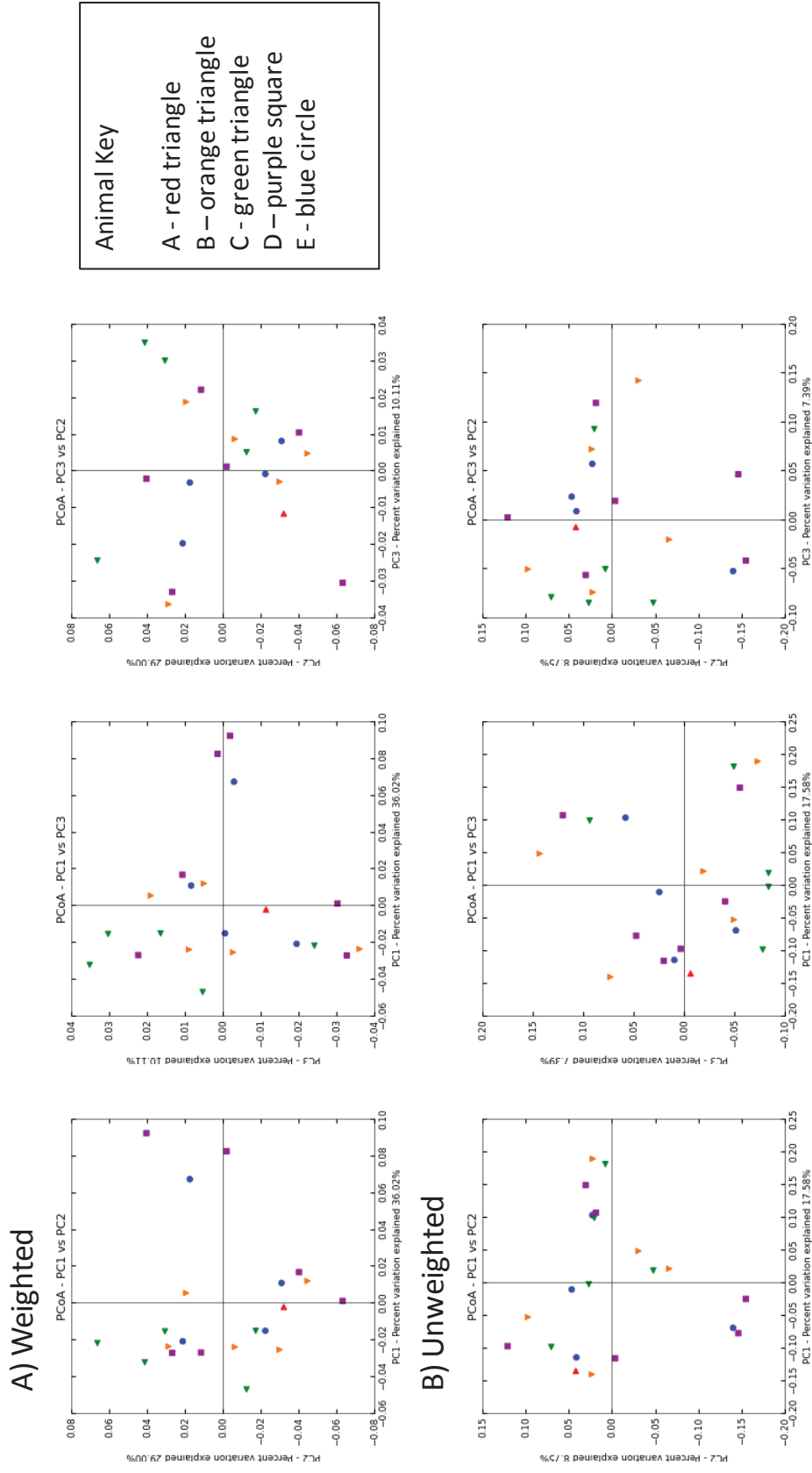


Figure 5-12. Principal coordinate analysis (PCoA) showing the similarity of sequenced plant-adherent rumen bacterial communities. Plots were generated using the weighted (A) and unweighted (B) versions of the Unifrac distance matrix. Each point corresponds to a sample coloured by animal. The percentage variation explained by the plotted coordinates is indicated on each axis.

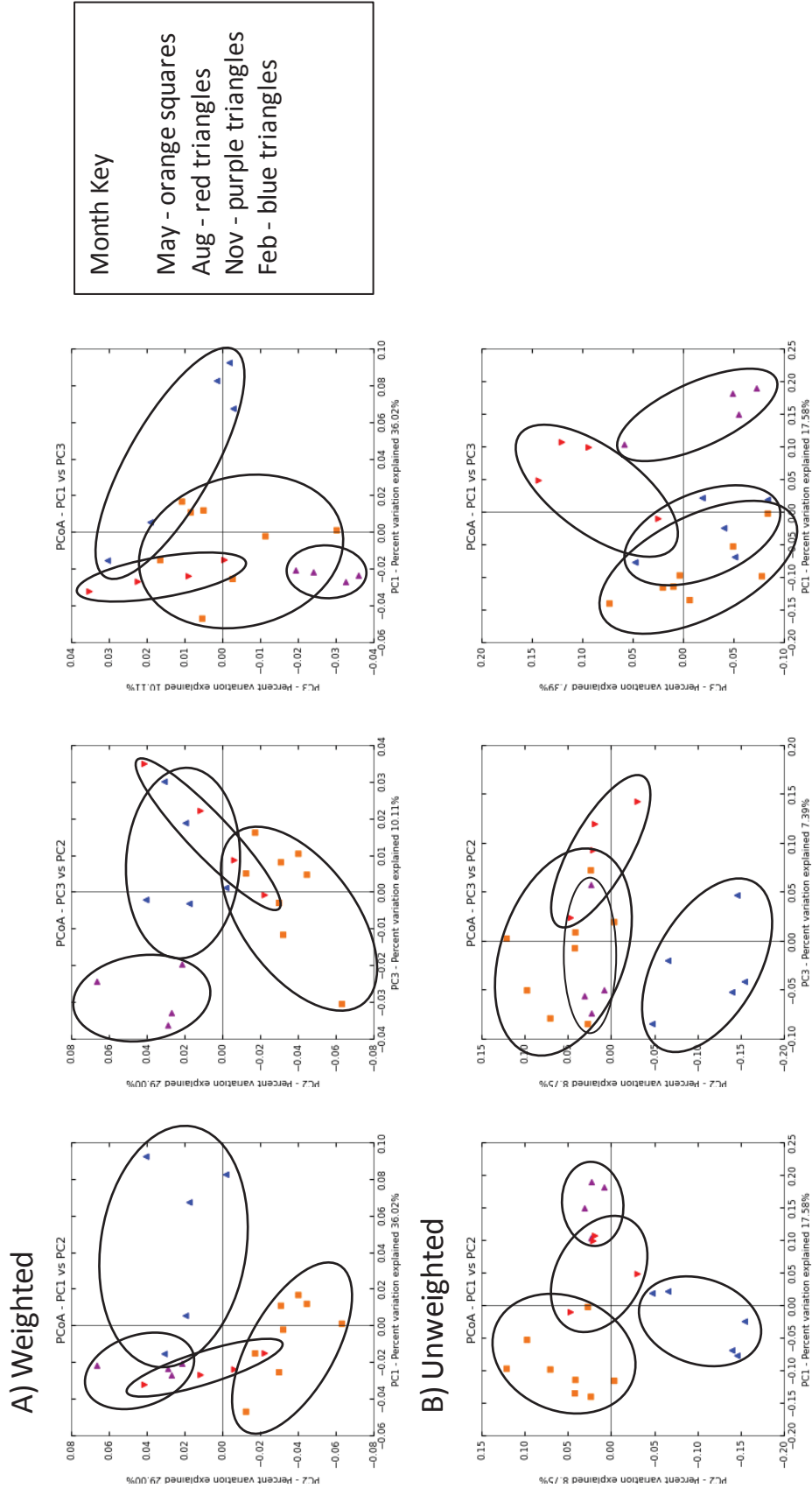


Figure 5-13. Principal coordinate analysis (PCoA) showing the similarity of sequenced plant-adherent rumen bacterial communities. Plots were generated using the weighted (A) and unweighted (B) versions of the Unifrac distance matrix. Each point corresponds to a sample coloured by month. The percentage variation explained by the plotted coordinates is indicated on each axis.

5.6 Discussion

The rumen contains a vast number of microorganisms, most of which have not been cultured and defined *in vitro* (Edwards *et al.*, 2004). Molecular techniques based on sampling the 16S rRNA gene yield information about community structure without the need for culturing. Rumen samples (n=20) were taken from four animals, once in each season over the course of a year and again in the first season of the next year. The rumen pH for each animal was within the normal range at each sampling. Sample NC had an unusual fermentation end product profile compared to all the other rumen samples, with elevated levels of lactic acid and formic acid and reduced levels of acetic acid. However, this sample did not have a noticeably different bacterial community detected by either DGGE or pyrosequencing. There was only one OTU unique to this sample and it was assigned to Coriobacteriaceae. The cause of unusual fermentation profile could not be determined but may have been caused by the other rumen microbiota (not bacteria) or be from a disturbance in the bacterial microbial community that was in the process of returning to normal. Because only bacteria were examined and only one sample was taken at each sample month, this dataset did not allow us to verify either of these theories.

5.6.1 Discussion of methods

Once pyrosequencing data has been obtained there are many ways to analyse the data. Diversity estimates are affected by quality control and pre-processing steps as well as the different clustering methods and taxonomic assignment methods (Kunin *et al.*, 2010a; Schloss, 2010). Simply removing the reads with ambiguous base calls and abnormal length is not enough to ensure accurate diversity measures (Kunin *et al.*, 2010a). The errors in PCR amplification cause sequencing noise leading to estimations of OTUs that can be orders of magnitude larger than the correct number. Flowgram clustering denoising methods such as PyroNoise, Denoiser and AmpliconNoise have been developed to reduce the noise in pyrosequence data. CD-HIT-OTU has also been developed to denoise large pyrosequencing dataset with comparable accuracy and has the advantage of being less computationally intensive than the flowgram clustering

approaches. CD-HIT-OTU removes chimeric OTUs and denoises the pyrosequencing data before clustering into OTUs. It achieves this by clustering the pyrosequencing reads at 100% and identifying error free clusters from sequences with errors. Due to the depth of sequencing, each individual species should have been sequenced multiple times, and therefore the error-free reads will cluster together at 100% sequence identity. Secondary clusters can be formed that contain one error at exactly the same position, but are two orders of magnitude smaller than the primary clusters. Tertiary clusters are also possible with two errors at exactly the same position but these are smaller than the secondary clusters. Singleton OTUs are likely to be chimeras or artefacts. Only the primary clusters are needed for analysis and the other reads are discarded. An OTU clustering threshold of 96% was chosen, as this has been shown to most accurately represent OTU clusters gained from the full-length 16S rRNA gene sequence (clustered at 97% similarity), when only the V1-V3 region of the 16S rRNA gene is used (Kim *et al.*, 2011a).

The pyrosequenced bacterial communities were very similar between samples from different animals and months. Examining one sample twice, with two different barcoded primers, shows the biases introduced by the choice of barcode. The differences in community profiles between samples FD.51 and FD.65 appeared nearly as great as the differences between any two samples. Therefore, the variation between samples could be interpreted as process-inherent noise rather than true rumen-to-rumen variation.

5.6.1.1 Bacterial communities associated with rumen fractions

DGGE analysis was used to examine bacterial communities associated with the different rumen fractions as well as those associated with the cows and sampling months. Previous studies have shown clear differences between fluid-associated and plant-adherent bacterial communities (de Menezes *et al.*, 2011; Fouts *et al.*, 2012; Larue *et al.*, 2005), but the differences were not clear with this approach. DGGE comparison of all the samples from different fractions revealed the bacterial communities were remarkably similar. None of the fraction types from the four rumen fractions tested (UT, BT, BD and TW) clustered together, when all samples from an individual animal were

compared. Fractions UT and BT were essentially the same except BT had undergone mechanical blending. DGGE fingerprint clustering analysis showed blending had no effect on the observed community. This implies the blending process used to prepare the plant-adherent fractions for cultivation does not bias the sample by destroying whole groups of fragile cells.

In the DGGE analysis performed on samples washed nine times with basal RM02 for the method development described in chapter 3 (Figure 3-6), the DGGE profile for the plant-adherent fraction could be distinguished from the liquid fractions. A possible explanation for the results seen here is the TW fraction was a combination of the liquid rumen fraction and the four wash fractions compared to the DGGE analysis presented in method development (Figure 3-6) where each of the liquid rumen and wash samples were examined separately. It was noted in the method development (Figure 3-6) experiment that as the digesta were washed, the cells that were washed off increasingly resembled the total community sample. Small particles with attached cells are able to pass through the cheesecloth and end up in the wash fraction. Considering that the attached cells greatly outnumber the unattached cells, this may end up masking the differences of the liquid community in the combined wash (TW). Therefore the TW, BT and UT communities could be expected to be similar to each other. It also should be noted there may also have been subtle community differences that were beyond the detection limit of DGGE. The analysis of rumen fractions was only examined using DGGE so no comparison can be made to the pyrosequencing results.

5.6.1.2 Bacterial communities associated with different animals

The bacterial communities observed with DGGE were very similar between the five animals sampled. Analysis of the pyrosequences revealed the differences between the animal bacterial communities were small, and that only between 0.13-0.59 percent of the OTUs were unique to each animal. The differences were not statistically significant and principal coordinate analysis showed no clustering due to animal variation. Taken together, these data indicate there is no difference in the bacterial communities from the different animals.

5.6.1.3 Bacterial communities associated with different sample months

One of the aspects examined in this study was the change in rumen bacterial communities over time with the naturally changing seasonal forage diet. Previous work has examined changes in rumen bacterial communities when cows were transitioned from one diet to another (Pitta *et al.*, 2009) or fed different diets (Kong *et al.*, 2010). To the best of my knowledge, this is the first study to examine rumen bacterial communities in animals grazed year round on pasture, under normal dairy farming conditions. The nutritive value of the pasture feed changed throughout the year and this is reflected by statistically different bacterial communities detected by pyrosequencing in the months sampled. This indicates the rumen bacterial community is not static and adapts to the more modest changes in pasture diet of different qualities and not just too major dietary changes i.e. between forage and grain.

Clustering analysis of DGGE fingerprints did not show strong patterns of month or animal groupings. However principal coordinate analysis of the pyrosequences showed overlapping clusters of samples related to the month they were sampled. The differences seen in the statistical analysis though significant are small.

5.6.2 Bacterial diversity

DGGE analysis and pyrosequencing revealed a high diversity in the rumen of animals grazing pasture under normal dairy farming practices. In total, 1539 OTUs (96% similarity) were found in the plant-adherent fraction of all the rumen contents, ranging from 653 to 926 OTUs per sample. These OTU estimates are similar to those reported in other studies using 16S rRNA gene sequence analysis of rumen communities, with 613 OTUs (97% similarity) (Kong *et al.*, 2010) and 739-946 OTUs (estimates based on rarefaction curve at 97% similarity) (Pitta *et al.*, 2009). Most OTUs found in this study were present in more than one sample. Around 50% of the OTUs were found in more than half the samples and 21% of the OTUs were found in more than 90% of the samples. These OTUs could represent a core microbiome for plant-adherent bacteria in New Zealand pasture fed dairy cows.

A small scale clone library was constructed using DNA from one sample (cow A, May 2009). Even with the limited number of clones (54), five phyla were identified,

with Firmicutes and Bacteroidetes being predominant. These results are in agreement with those found using pyrosequencing analysis on 20 samples, where the plant-adherent bacterial community was represented by 14 phyla but was dominated by Firmicutes followed by Bacteroidetes. The prevalence of Firmicutes has been found in many other studies on rumen bacteria (Edwards *et al.*, 2004; Kim *et al.*, 2011c; Kong *et al.*, 2010; Tajima *et al.*, 2000), although a few studies show Bacteroidetes as the most prevalent phylum (An *et al.*, 2005; Jami *et al.*, 2012a; Li *et al.*, 2012a). Together, Firmicutes and Bacteroidetes are always the most prevalent phyla detected in the rumen by molecular methods. The prevalence of Firmicutes found in this study (79.5%) is one of the highest values reported for the rumen. Higher values of 90.2% and 95% of sequences assigned to Firmicutes were reported for Holstein cows on a high roughage diet and grain diet respectively (Tajima *et al.*, 2000), but most reported values are less than 70% (shown in Table 1-2).

The 1539 OTUs were classified into at least 58 genera. This is more than the 23-27 genera reported by Kong *et al.* (2010) from four cows on alfalfa or triticale diets but less than the 122-149 genera reported by Pitta *et al.* (2009) from 14 steers fed bermudagrass or wheat and the 180 known genera represented in the meta analysis of all 16S rRNA gene sequences of rumen origin from the RDP database by Kim *et al.* (2011c). Some of the differences in reported genera are likely to be due to the use of different taxonomic assignment methods (Liu *et al.*, 2008b).

Together, the genera *Ruminococcus*, *Prevotella*, *Pseudobutyrvibrio*, *Fibrobacter*, *Butyrvibrio*, *Mogibacterium*, *Coprococcus*, *Anaerovorax*, *Succiniclasticum* and *Oscillibacter* contain ~ 25% of the pyrosequences. Many studies have found *Prevotella* the most prevalent genus in the rumen (Callaway *et al.*, 2010; Jami *et al.*, 2012a; Wu *et al.*, 2012). In this study *Ruminococcus* was the largest of the known genera and contained 6.7% of the sequences, followed by *Prevotella* with 4.6% of the sequences. This is perhaps due to the prevalence of Firmicutes in this study. However, undefined genera in Lachnospiraceae, Clostridiales and Ruminococcaceae account for 58% of the sequences, illustrating the need for more pure cultures of these groups to be obtained and classified in order to accurately classify the molecularly-detected members of the rumen bacterial community. The most prevalent genera here were similar to those found in other studies utilising pyrosequencing. Fouts *et al.* (2012) found *Prevotella*,

Oscillibacter, *Coprococcus*, unclassified Ruminococcaceae and *Butyrivibrio* as the most prevalent. Pitta *et al.* (2009) found *Prevotella*, *Rikenella*, *Butyrivibrio*, *Sporobacterium*, *Roseburia*, *Coprococcus* and *Pseudobutyrvibrio*, while Kong *et al* (2010) found *Prevotella*, *Butyrivibrio*, unclassified Lachnospiraceae, *Pseudobutyrvibrio*, and *Ruminococcus* to be the most prevalent genera.

Chapter 6 Comparison of isolates with the plant-adherent bacterial community

6.1 Introduction

Some estimates put the number of cultured species from the rumen at around 7-10% of what can be detected in the rumen (Edwards *et al.*, 2004; Kim *et al.*, 2011c). It is difficult to gauge what proportion of bacterial species in the rumen have been isolated if the total number of species is not known. Also, comparing all bacterial isolates from the rumen with all sequences detected in molecular studies from different rumen samples doesn't show the coverage of the species diversity within one system and may be making unfair comparisons of unlike environments. One way to get a true estimate of cultivation success is to directly compare sequences of a marker gene like the 16S rRNA gene from cultured bacteria with that of the total bacteria from the same rumen environment. In the current study, a large number (~1000) of isolates were cultured (see Chapter 4) from the same samples that were analysed by pyrosequencing (see Chapter 5).

In this chapter, results and discussion are presented together. The phylogenetic placement of the isolates for which near full-length 16S rRNA gene sequences were obtained will be discussed and then the success of the isolation strategy estimated by comparison with the plant-adherent bacterial community. Bacterial 16S rRNA gene sequences were grouped into operational taxonomic units (OTUs) based on their sequence relatedness. The number of OTUs with a cultured representative gives an estimate of how much of the bacterial diversity was able to be cultured.

16S rRNA gene sequences can be grouped into species and genus groups using 97% and 95% sequence similarity respectively (Schloss *et al.*, 2004). However, these sequence similarities do not always apply to established phylogenetic groups. It appears the degree of sequence similarity that defines a species, genus or even family is specific to the bacterial group in question. For example, the members of the genus *Treponema* have only 86-89% sequence similarity (Paster *et al.*, 1991). In this study, different levels of similarity were used to define species and genera after first establishing the sequence similarities that defined already established taxonomic groups within each phylum. It

also became obvious in this analysis that some recognised taxa are polyphyletic and that some members need to be reclassified. In these cases, suggestions are made but the isolates are named according to currently accepted taxonomies.

6.2 Results and discussion

6.2.1 Phylogenetic placement of isolates with near full-length 16S rRNA gene sequence

There were 828 isolates with good partial (V1-V3) 16S rRNA gene sequences, and these were analysed with QIIME (described in chapter 4). Near full-length 16S rRNA gene sequences were generated from 186 of these isolates. These 186 isolates hereafter are referred to as the “selected isolates” and the 828 isolates with good partial (V1-V3) 16S rRNA gene sequences as the “total isolates” sequenced. The selection of the isolates for near-full-length sequencing was described in section 4.3.12. Each selected isolate for which near-full-length 16S rRNA gene sequence data was obtained, represents a species-level group (96% sequence similarity) in the analysis of the total isolates. A few species-level groups had more than one representative isolate (details of the selected isolates are in Appendix 1). Altogether, the selected isolates represented 801 of the total isolates sequenced. The evolutionary relationships of each of the 186 selected isolate were inferred by comparing the aligned 16S rRNA gene sequences with the sequences in the SILVA database (Pruesse *et al.*, 2007). An overview tree of bacterial classes containing an isolate is shown in Figure 6-1. Names for taxonomic groups follow the scheme presented in Bergey’s Manual of Systematic Bacteriology (Krieg *et al.*, 2010; Vos *et al.*, 2009). The phylogenetic trees shown were inferred using a maximum likelihood (ML) method (algorithm general time-reversible, GTR plus gamma distribution) in RaxML with bootstrap analysis of 500 re-samplings to estimate support for the branch order. The proposed groupings of the isolates within the trees are based on the similarity matrix using cut-off values consistent with the established phylogenetic groupings within the phylum being analysed. Information from both Neighbor Joining (NJ) trees and ML trees were combined to propose the final taxonomic assignment of the new isolates.

Trees were rooted with eight deeply-branching reference sequences: *Sulfurihydrogenibium subterraneum* HGM-K1 (AB071324), *Thermotoga maritima* MSB8 (AE000512), *Aquifex aeolicus* VF5 (AE000657), *Thermodesulfator indicus*

CIR29812 (AF393376), *Thermodesulfobacterium commune* YSRA-1 (AF418169), *Fervidobacterium nodosum* Rt17-B1 (CP000771), *Desulfurobacterium thermolithotrophum* BSA (CP002543) and *Caldimicrobium rimae* DS (EF554596).

The arrangement of the higher-level taxa agrees with tree topologies found by other authors. As found for the trees generated from the short (V1-V3) length 16S rRNA gene sequences (Figure 6-1), the phylum Firmicutes contained the majority of the selected isolates. This phylum was separated into four clades, three of which are well-accepted classes of the Firmicutes: Bacilli, Clostridia and Erysipelotrichia. The fourth clade consisted of the family Veillonellaceae. This family is currently classified in the order Clostridiales of the class Clostridia (Ludwig *et al.*, 2009b), but here it formed a separate clade. It has been proposed that the family Veillonellaceae be elevated to a novel class, Negativicutes, within the phylum Firmicutes (Marchandin *et al.*, 2010). This tree supports that proposal, as the family Veillonellaceae formed a robust lineage separated from the classes Bacilli, Clostridia and Erysipelotrichia.

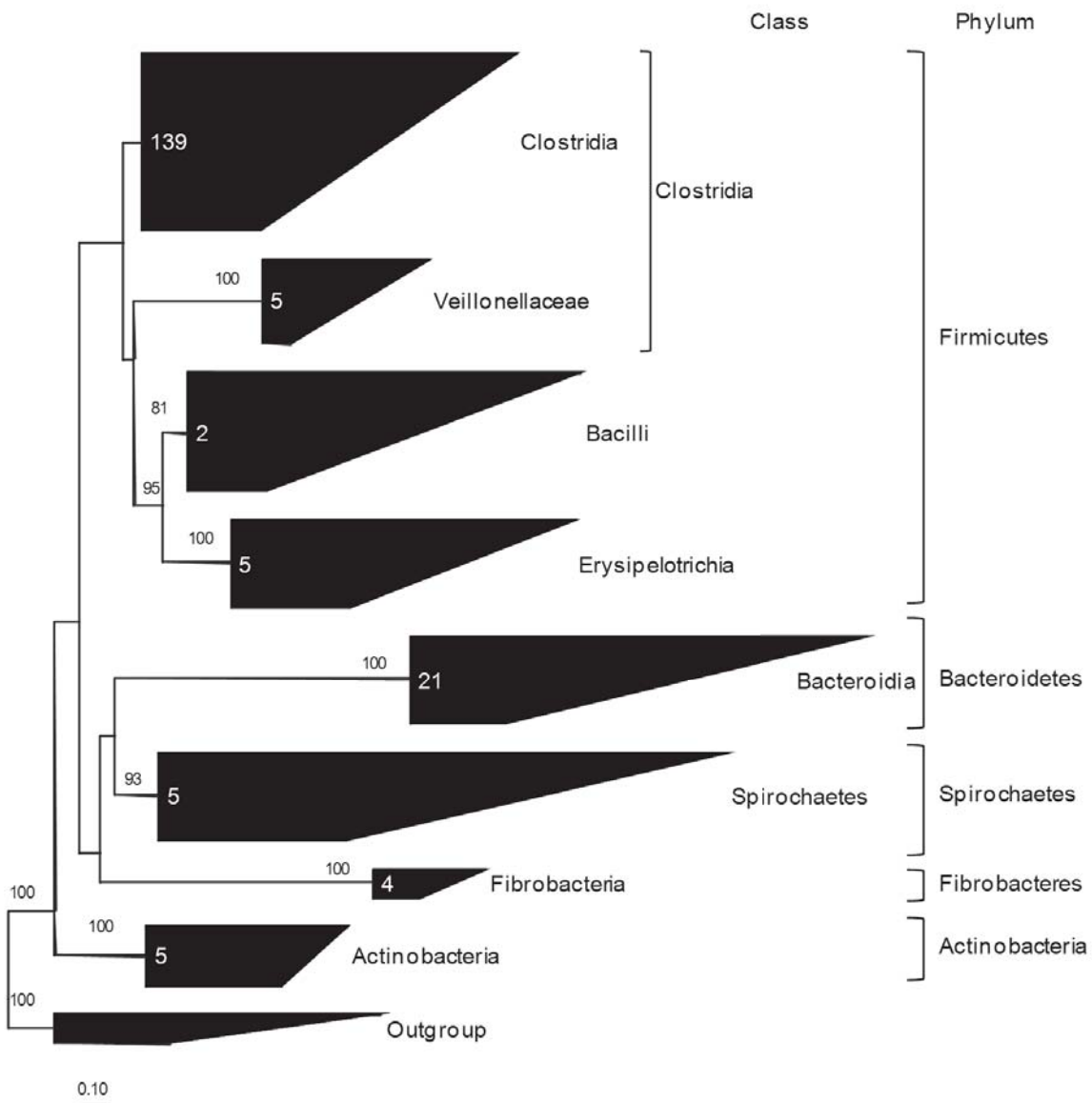


Figure 6-1. Overview of the phylogenetic relationships of new isolates cultured in this study. Each phylum demarcated by square brackets to the right will be shown in expanded views in subsequent trees. The tree was based on a ML analysis in RaxML of a dataset comprising 1075 near full-length high quality 16S rRNA gene sequences from the selected isolates, their closest relatives and representatives of the phyla depicted. The number of isolates within each group is shown in white. Bootstrap values >70% are shown at internal nodes. The scale bar indicates the mean number of nucleotide substitutions per site. The tree was rooted with eight reference sequences: *Sulfurihydrogenibium subterraneum* HGM-K1 (AB071324), *Thermotoga maritima* MSB8 (AE000512), *Aquifex aeolicus* VF5 (AE000657), *Thermodesulfator indicus* CIR29812 (AF393376), *Thermodesulfobacterium commune* YSRA-1 (AF418169), *Fervidobacterium nodosum* Rt17-B1 (CP000771), *Desulfurobacterium thermolithotrophum* BSA (CP002543) and *Caldimicrobium rimae* DS (EF554596).

6.2.2 Description of isolates within Firmicutes

Most of the isolates (723 of the 801 isolates represented by the 186 selected isolates) were members to the phylum Firmicutes, in agreement with the finding that 79.5% of the 16S rRNA gene sequences from the total plant-adherent community were also assigned to Firmicutes. An overview of families within Firmicutes that contained isolates is shown in Figure 6-2. Each collapsed clade will be shown and discussed in detail in sections (6.2.3 – 6.2.5). Several families (Enterococcaceae, Bacillaceae and Paenibacillaceae) within Bacilli did not form coherent clusters in the ML tree (Figure 6-2), but they did in the NJ tree (not shown). Three classes are defined within the phylum Firmicutes in the current taxonomic backbone defined in Bergey's Manual of Systematic Bacteriology: Bacilli, Clostridia and Erysipelotrichia. New isolates were found in each of these classes. The majority (139 of 186) of all the selected isolates clustered within Clostridia, and only two selected isolates were associated with the class Bacilli and four selected isolates were in the class Erysipelotrichia.

6.2.3 Firmicutes, class Clostridia (order Clostridiales)

The class Clostridia currently has three recognised orders: Clostridiales, Thermoanaerobacteraceae and Halanaerobiales, with the first two being paraphyletic and the last monophyletic (Ludwig *et al.*, 2009b). Close to 86% of all the isolates (688 of the 801 isolates represented by the 186 selected isolates) were within the order Clostridiales, and no isolates fell in the other two orders. The tree-based analysis of near full-length 16S rRNA gene sequences showed that the selected isolates fell into the families *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae* and Family XIII *incertae sedis*, as well as into a new group designated here as the R-7 group. The R-7 group appears to be a new family within Clostridia in ML trees but had uncertain phylogenetic placement within NJ trees, and may even represent a new order of within the class Clostridia. The R-7 group is affiliated with the families Catabacteriaceae and Christenellaceae with high bootstrap support (84%), but its members share only 84.3% and 84.6% average sequence similarity (respectively) with members of these two families.

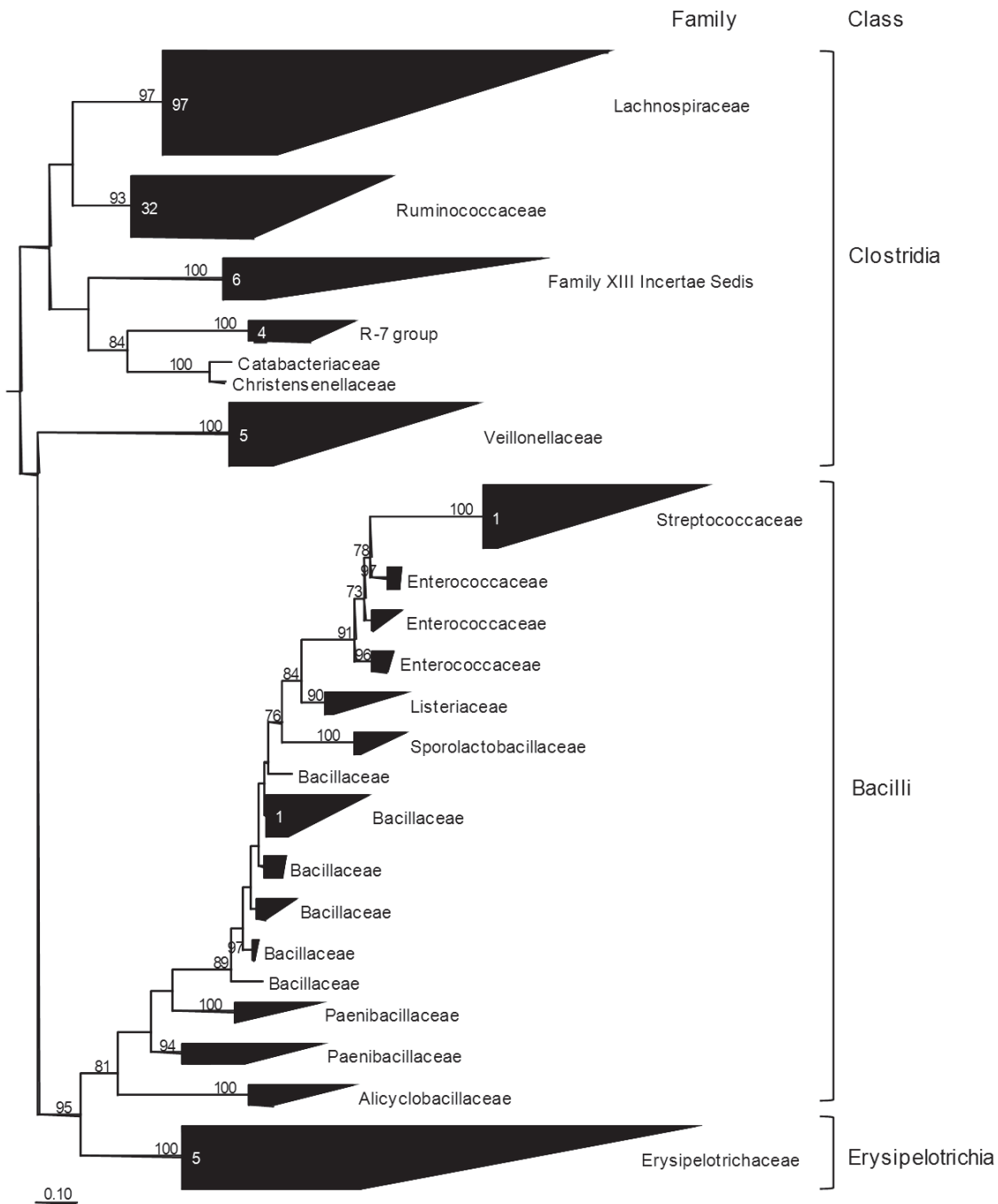


Figure 6-2. Overview of the phylogenetic relationships of the families within the phylum Firmicutes. Collapsed clades that contain isolates are indicated with white numbers indicating the number of isolates that fell into that clade. This tree is an expanded view of the phylum Firmicutes from the tree shown in Figure 6-1. Analyses were performed as described in the legend of Figure 6-1.

6.2.3.1 Lachnospiraceae

The identities of the 97 selected isolates in Lachnospiraceae are summarised in Table 6-1 and in phylogenetic trees are shown in Figure 6-4, Figure 6-5 and Figure 6-6. There are 19 genera in the family Lachnospiraceae listed in the revised roadmap to the phylum Firmicutes (Ludwig *et al.*, 2009b). In addition to these, the genus *Blautia* has recently been formed from reclassified *Ruminococcus* and *Clostridium* species (Liu *et al.*, 2008a). Selected isolates from the current study clustered with 10 of these known genera; *Blautia*, *Butyrivibrio*, *Coprococcus*, *Lachnobacterium*, *Lachnospira*, *Moryella*, *Oribacterium*, *Pseudobutyrvibrio*, *Roseburia* and *Syntrophococcus*.

There are many named species included in the depicted trees that have been noted to be members of Lachnospiraceae that are currently classified in genera that fall outside Lachnospiraceae. These include members of *Clostridium* (*algidixylanolyticum*, *aminovalericum*, *bolteae*, *glycyrrhizinilyticum*, *hathewayi*, *herbivorans*, *lentocellum*, *polysaccharolyticum*, *saccharolyticum*, *xylanolyticum*, and *xylanovorans*), members of *Eubacterium* (*eligens*, *hallii*, *rectale*, *ruminantium*, and *xylanophilum*) and *Ruminococcus torques* (Ludwig *et al.*, 2009b). These species are phylogenetically within Lachnospiraceae but the type species of the genera are phylogenetically in other families. This indicates that considerable revision of taxonomy is still required.

Based on the phylogenetic placement of the isolates in coherent clusters, and the sequence differences between the 16S rRNA genes of the isolates and their closest relatives, the isolates were grouped into genus level clusters and species level clusters. A value of 16S rRNA gene sequence similarity of <95% is commonly used to indicate organisms are likely to be from different genera and <97% for different species (Schloss *et al.*, 2004), but this rule does not strictly fit all known genera. The Lachnospiraceae sequences compared here had a minimum sequence similarity of 80.1%. The minimum sequence similarity (excluding outliers) within each genus varied: 96.7% within *Pseudobutyrvibrio*, 92.3% within *Roseburia*, 91.3% within *Butyrivibrio*, 93.1% within *Syntrophococcus* and 91.4% within *Blautia*. Compared with the currently defined genera represented in this analysis, more conservative similarity values were used for novel groups. Values of <93% was chosen to define sequences that were likely to belong to separate genera and <97% to define sequences that were likely to represent separate species. These values were used in conjunction with the identification of stable

groups (>70% bootstrap support) seen in the ML tree to define genus level clusters (GLC) and species level clusters (sp.). The rationale for choosing 93% similarity to define a genus within this family is illustrated in Figure 6-3. If a cut-off value of 94% similarity was applied, the accepted genus *Roseburia* would be split up into many groups, while at 92% several defined genera (for example *Pseudobutyrvibrio*, *Roseburia*, *Lachnobacterium* and *Lachnospira*) would be clustered together into one GLC. When a cut-off value of 93% was applied, these defined genera were correctly grouped. The assumption was made that the as-yet undefined genera in this family will follow the same pattern. Therefore, a minimum of 93% 16S rRNA gene sequence similarity was used to define a GLC and was applied to all the sequences in the phylum Firmicutes. This minimum value excludes a minority of outlying sequences that stably cluster within a group (with high bootstrap values) but have lower similarity values to the rest of the group. These usually form long branches within the cluster of sequences that make up the group, and the sequences may contain sequencing error, insertions or deletions. This was not investigated further, and did not occur for sequences from any of the selected isolates.

The isolates were grouped into 45 GLC within Lachnospiraceae, including 10 known genera. 32 GLC are new genera (which did not contain a type strain from a recognised genus). The last three GLC (GCL 55 and GLC 58) were probably new genera based on clustering and sequence differences, but contained isolates with valid names. The sequences from the selected isolates within GLC 58 were on average >94% similar to the known *Pseudobutyrvibrio* strains, but the GLC did not have strong bootstrap support and contained isolates that have been classified as *Lachnobacterium* spp. Upon phenotypic characterisation, the isolates in GLC 58 may be moved to *Pseudobutyrvibrio*. GLC 55 contained the type strain of the species *Coprococcus catus* but was clearly divergent from the cluster containing the type species of the genus (*Coprococcus eutactus*). GLC 29 contained the type strain of the species *Eubacterium hallii* which probably should be reclassified as it is clearly divergent from the genus *Eubacterium* and its type species, *Eubacterium limosum*.

Each of the isolates was presumptively pure because they had uniform cell morphology when examined microscopically and produced a useable 16S rRNA gene sequence. However, one isolate (NE2001) was originally identified as being a member

of *Prevotella* (see Table 6-1), but the longer 16S rRNA gene sequence grouped with *Coprococcus*. The near full-length sequence did not appear to be chimeric. The DNA for the partial sequence came from the original culture and the DNA for the near full-length sequence was re-extracted from a revived culture. This indicates the original culture was probably mixed. Perhaps one species (a *Prevotella* sp.) was dominant before freezer storage and that the other species (a *Coprococcus* sp.) grew and was dominant upon revival from freezer storage. All remaining isolates appeared to group consistently regardless whether the short (V1-V3 region; see Table 6-1) or near full-length 16S rRNA gene sequences were analysed. This highlights the need for the purity of each isolate to be confirmed before future work, but shows that the approach taken in this study was valid. The time-consuming step of ensuring culture purity can now be carried out on the isolates that are of special interest, before, for example, phenotypic characterisation and genome sequencing.

6.2.3.1.1 *Butyrivibrio* and *Pseudobutyrvibrio*

About a quarter of the total isolates (210 out of 828) were identified by QIIME analysis as being members of the genus *Butyrivibrio*. The short (V1-V3) 16S rRNA gene sequences from these isolates grouped into 20 clusters of *Butyrivibrio* (see Table 6-1). Twenty six isolates were chosen to represent these 20 clusters and near full-length 16S rRNA gene sequences were obtained. The phylogenetic relationships of these isolates are shown in Figure 6-5. The selected isolates clustered with the type strains of *B. proteoclasticus*, *B. hungatei* and *B. fibrisolvens* and into six new GLC. There was a continuum of related strains from *B. proteoclasticus* to *B. hungatei*, making it difficult to determine species boundaries. This is further compounded by the lack of bootstrap support for most of the branches within the genus *Butyrivibrio*.

A more accurate estimate of the number of *Butyrivibrio* species was expected from the near full-length 16S rRNA gene sequences, as these offer more power to discriminate differences between species than partial sequences (Schloss, 2010). The 26 selected isolates grouped into only nine species-level clusters when the near full-length sequences were analysed using the tree based methods. This suggests the original clusters based on partial 16S rRNA gene sequences may have overestimated the number of *Butyrivibrio* species. However, isolate sequences from each original cluster where

more than one representative was chosen (e.g. *Butyrivibrio* cluster 1, 3 and 10; see Table 6-1) always grouped with the corresponding sequences from that cluster in the analysis of near full-length sequence, showing that it is probable that the original clustering of the short (V1-V3) sequences did not exclude species. It does suggest that a QIIME-based analysis of short reads does have the potential to overestimate species relative to phylogenetic analysis based on near full-length sequences. The species-level groupings found here will need phenotypic characterisation to determine whether they are in fact different and new species. Two isolates originally thought to be part of *Butyrivibrio* clustered with other *Lachnospiraceae* sequences when the near full-length sequences were used, again illustrating potential differences when comparing near full-length 16S rRNA gene sequences as opposed to partial ones.

QIIME analysis of the short length 16S rRNA gene sequences of the 828 total isolates placed 9.6% of the isolates in *Pseudobutyrvibrio*. There only appeared to be one species of *Pseudobutyrvibrio* in NJ phylogenies of the short sequences. Ten of the total isolates were selected to represent *Pseudobutyrvibrio* and nine of these grouped into a single species-level cluster within *Pseudobutyrvibrio*, shown in Figure 6-6. The remaining isolate grouped with *Roseburia*. Two species of *Pseudobutyrvibrio* (*ruminis* and *xylanovorans*) can be found in the *Pseudobutyrvibrio* species level cluster, but it is impossible to distinguish between them on the basis of 16S rRNA gene similarity alone. *Pseudobutyrvibrio ruminis* is the type species of the genus, so the species-level cluster was named after this species.

GLC 58 contained an additional three of the selected isolates, and fell into two new species-level clusters (sp. 39 and sp. 40; Figure 6-4). These also had >94% sequence similarity to the 16S rRNA gene sequences from the type strains of true *Pseudobutyrvibrio* spp., indicating these could be two new species of *Pseudobutyrvibrio*. However, this GLC grouped away from the cluster of true *Pseudobutyrvibrio* spp. This GLC contains isolates named *Lachnobacterium* sp. and unclassified isolates, and its relationship with the recognised genus *Pseudobutyrvibrio* needs to be determined. GLC 58 is not monophyletic, and may represent multiple genera. Alternatively, the genus *Pseudobutyrvibrio* may extend to include GLC 58, and also GLC 24 and GLC 25 (Figure 6-4).

Table 6-1. Identity of isolates within Lachnospiraceae.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / Species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
FCS006	Butyrivibrio cluster S1	1	<i>Butyrivibrio</i> / sp. 3	Yes
AE3003	Butyrivibrio cluster 1	83	<i>Butyrivibrio</i> / <i>Butyrivibrio hungatei</i>	Yes
LB2008	Butyrivibrio cluster 1	(83) ^b	<i>Butyrivibrio</i> / <i>Butyrivibrio hungatei</i>	Yes
XBD2003	Butyrivibrio cluster 1	(83)	<i>Butyrivibrio</i> / <i>Butyrivibrio hungatei</i>	Yes
XBD2006	Butyrivibrio cluster 1	(83)	<i>Butyrivibrio</i> / <i>Butyrivibrio hungatei</i>	Yes
AE2015	Butyrivibrio cluster 2	6	<i>Butyrivibrio</i> / <i>Butyrivibrio proteoclasticus</i>	Yes
V9D2008	Butyrivibrio cluster 3	10	<i>Butyrivibrio</i> / <i>Butyrivibrio hungatei</i>	Yes
VCB2006	Butyrivibrio cluster 3	(10)	<i>Butyrivibrio</i> / <i>Butyrivibrio hungatei</i>	Yes
YAB3001	Butyrivibrio cluster 4	3	<i>Butyrivibrio</i> / <i>Butyrivibrio proteoclasticus</i>	Yes
FD2007	Butyrivibrio cluster 5	3	<i>Butyrivibrio</i> / <i>Butyrivibrio proteoclasticus</i>	Yes
XBB1001	Butyrivibrio cluster 6	3	<i>Butyrivibrio</i> / <i>Butyrivibrio proteoclasticus</i>	Yes
FB2022	Butyrivibrio cluster 7	4	<i>Butyrivibrio</i> / sp. 3	Yes
NC2007	Butyrivibrio cluster 8	3	<i>Butyrivibrio</i> / sp. 3	Yes
FB3011	Butyrivibrio cluster 9	3	<i>Butyrivibrio</i> / sp. 3	Yes
MC2021	Butyrivibrio cluster 10	25	<i>Butyrivibrio</i> / sp. 3	Yes
DAZ1013	Butyrivibrio cluster 10	(25)	<i>Butyrivibrio</i> / sp. 3	Yes
VCD3004	Butyrivibrio cluster 10	(25)	<i>Butyrivibrio</i> / sp. 3	Yes
AE3004	Butyrivibrio cluster 11	18	<i>Butyrivibrio</i> / sp. 4	No
NC2002	Butyrivibrio cluster 11	(18)	<i>Butyrivibrio</i> / sp. 6	No
WCE2006	Butyrivibrio cluster 12	5	<i>Butyrivibrio</i> / sp. 5	No

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / Species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
AE2005	Butyrivibrio cluster 13	19	<i>Butyrivibrio / Butyrivibrio hungatei</i>	Yes
AD3010	Butyrivibrio cluster 14	1	Lachnospiraceae GLC 3 / sp. 11	No
V9D2006	Butyrivibrio cluster 15	1	Lachnospiraceae GLC 7 / sp. 15	No
MC2013	Butyrivibrio cluster 16	1	<i>Butyrivibrio / sp. 1</i>	No
NC3005	Butyrivibrio cluster 17	2	<i>Butyrivibrio / sp. 2</i>	No
MD2001	Butyrivibrio cluster 18	29	<i>Butyrivibrio / Butyrivibrio fibrisolvens</i>	Yes
MA2020	Lachnospiraceae cluster 1	3	Lachnospiraceae GLC 4 / sp. 12	No
ND2006	Lachnospiraceae cluster 2	1	Lachnospiraceae GLC 5 / sp. 13	No
MC2017	Lachnospiraceae cluster 3	2	Lachnospiraceae GLC 6 / sp. 14	Yes
AC3007	Lachnospiraceae cluster 4	2	Lachnospiraceae GLC 2 / sp. 10	No
VCD2007	Lachnospiraceae cluster 5	1	Lachnospiraceae GLC 1 / sp. 8	No
WCD3001	Lachnospiraceae cluster 6	1	Lachnospiraceae GLC 1 / sp. 9	No
ND2018	Lachnospiraceae cluster 7	2	Lachnospiraceae GLC 1 / sp. 7	No
XPB1014	Lachnospiraceae cluster 8	3	Lachnospiraceae GLC 22 / sp. 36	No
VCB2013	Lachnospiraceae cluster 9	4	Lachnospiraceae GLC 22 / sp. 36	No
VCB2004	Lachnospiraceae cluster 10	1	Lachnospiraceae GLC 22 / sp. 36	No
XBB1006	Lachnospiraceae cluster 11	1	Lachnospiraceae GLC 19 / sp. 32	No
AD2015	Lachnospiraceae cluster 12	7	Lachnospiraceae GLC 33 / sp. 59	No
NB2016	Lachnospiraceae cluster 13	1	Lachnospiraceae GLC 33 / sp. 60	No
ND2007	Lachnospiraceae cluster 14	1	Lachnospiraceae GLC 20 / sp. 33	No
FC2014	Lachnospiraceae cluster 15	1	Lachnospiraceae GLC 25 / sp. 44	No
V9D2009	Lachnospiraceae cluster 17	2	Lachnospiraceae GLC 9 / sp. 21	No

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / Species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
NC2004	Lachnospiraceae cluster 19	1	Lachnospiraceae GLC 30 / sp. 56	No
AE3002	Lachnospiraceae cluster 20	2	Lachnospiraceae GLC 28 / sp. 54	Yes
XBB2008	Lachnospiraceae cluster 21	1	Lachnospiraceae GLC 17 / sp. 30	No
FCS020	Lachnospiraceae cluster 22	3	Lachnospiraceae GLC 23 / sp. 37	No
YSD2013	Lachnospiraceae cluster 23	4	Lachnospiraceae GLC 23 / sp. 38	No
WCD3007	Lachnospiraceae cluster 24	1	Lachnospiraceae GLC 32 / sp. 58	No
WCE3003	Lachnospiraceae cluster 26	1	Lachnospiraceae GLC 21 / sp. 34	No
AC2044	Lachnospiraceae cluster 27	1	Lachnospiraceae GLC 14 / sp. 26	No
MA2001	Lachnospiraceae cluster 28	1	Lachnospiraceae GLC 12 / sp. 24	No
XBB1003	Lachnospiraceae cluster 29	11	Lachnospiraceae GLC 10 / sp. 22	Yes
YAB2006	Lachnospiraceae cluster 31	1	Lachnospiraceae GLC 10 / sp. 22	Yes
LB2020	Lachnospiraceae cluster 32	2	GLC 55 / sp. 46	No
XBB2003	Lachnospiraceae cluster 33	13	<i>Coproccoccus</i> / sp. 51	No
AC2029	Lachnospiraceae cluster 34	9	<i>Coproccoccus</i> / sp. 53	No
MD2004	Lachnospiraceae cluster 35	7	<i>Coproccoccus</i> / sp. 49	Yes
LC2019	Lachnospiraceae cluster 35	(7)	<i>Coproccoccus</i> / sp. 50	Yes
AC2031	Lachnospiraceae cluster 36	13	<i>Coproccoccus</i> / sp. 49	Yes
XPB1013	Lachnospiraceae cluster 37	1	Lachnospiraceae GLC 15 / sp. 27	No
VCD3003	Lachnospiraceae cluster 38	1	Lachnospiraceae GLC 21 / sp. 35	No
FE2016	Lachnospiraceae cluster 39	1	Lachnospiraceae GLC 31 / sp. 57	No
NC2008	Lachnospiraceae cluster 40	1	Lachnospiraceae GLC 13 / sp. 25	Yes
V9D2002	Lachnospiraceae cluster 42	1	Lachnospiraceae GLC 11 / sp. 23	No

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / Species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
YAD2002	Lachnospiraceae cluster 43	1	<i>Moryella</i> / sp. 19	No
YGB2004	Lachnospiraceae cluster 44	1	Lachnospiraceae GLC 18 / sp. 31	No
AB2028	Lachnospiraceae cluster 45	3	Lachnospiraceae GLC 16 / sp. 29	No
XPB1003	Lachnospiraceae cluster 46	8	Lachnospiraceae GLC 16 / sp. 28	Yes
ND3007	Lachnospiraceae cluster 47	1	Lachnospiraceae GLC 27 / sp. 48	No
LD2006	Lachnospiraceae cluster 48	7	<i>Syntrophococcus</i> / <i>Eubacterium cellulosolvens</i>	Yes
FE2018	Lachnospiraceae cluster 49	2	Lachnospiraceae GLC 8 / sp. 16	No
FC2009	Lachnospiraceae cluster 50	1	Lachnospiraceae GLC 29 / sp. 55	No
AC2012	Lachnospiraceae cluster 51	18	GLC 58 / sp. 40	Yes
YGD2002	Lachnospiraceae cluster 52	1	GLC 58 / sp. 39	Yes
FD2005	Lachnospiraceae cluster 53	1	GLC 58 / sp. 40	Yes
LB2003	Lachnospiraceae cluster 54	5	<i>Lachnospira</i> / <i>Lachnospira multipara</i>	Yes
ND3006	Lachnospiraceae cluster 55	3	Lachnospiraceae GLC 24 / sp. 41	No
V9D3004	Lachnospiraceae cluster 56	1	Lachnospiraceae GLC 26 / sp. 47	Yes
AC2035	Lachnospiraceae cluster 57	2	Lachnospiraceae GLC 25 / sp. 42	No
XBD2001	Lachnospiraceae cluster 58	4	Lachnospiraceae GLC 25 / sp. 43	No
AE2004	Lachnospiraceae cluster 59	4	<i>Lachnobacterium</i> / <i>Lachnobacterium bovis</i>	Yes
MC2003	Lachnospiraceae cluster S41	1	<i>Lachnospira</i> / <i>Lachnospira multipara</i>	Yes
YSB2008	Lachnospiraceae cluster S9	1	<i>Coprococcus</i> / sp. 52	No
WCE2007	Lachnospiraceae II cluster 1	1	<i>Blautia</i> / sp. 20	No
FC2008	Oribacterium cluster 1	1	<i>Oribacterium</i> / sp. 17	No
VCD1014	Oribacterium cluster 2	13	<i>Oribacterium</i> / sp. 18	Yes

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / Species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
NE2001	Prevotella cluster 21 ^c	1	<i>Coprococcus</i> / sp. 49	Yes
AC2014	Pseudobutyrvibrio cluster 1	1	<i>Roseburia</i> / sp. 45	No
AD2017	Pseudobutyrvibrio cluster 1	85	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
DAZ1006	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
DAZ2006	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
LB2011	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
MA3007	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
MD2005	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
MD2007	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
VCB1013	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes
VCD1002	Pseudobutyrvibrio cluster 1	(85)	<i>Pseudobutyrvibrio</i> / <i>Pseudobutyrvibrio ruminis</i>	Yes

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 total isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in each cluster is shown. Where there is more than one sequence from a cluster, the number is repeated in brackets.

^c This culture was originally identified as a member of *Prevotella*, but after revival of the stored culture a single cell type was obtained with a 16S rRNA gene sequence similar to known *Coprococcus* species.

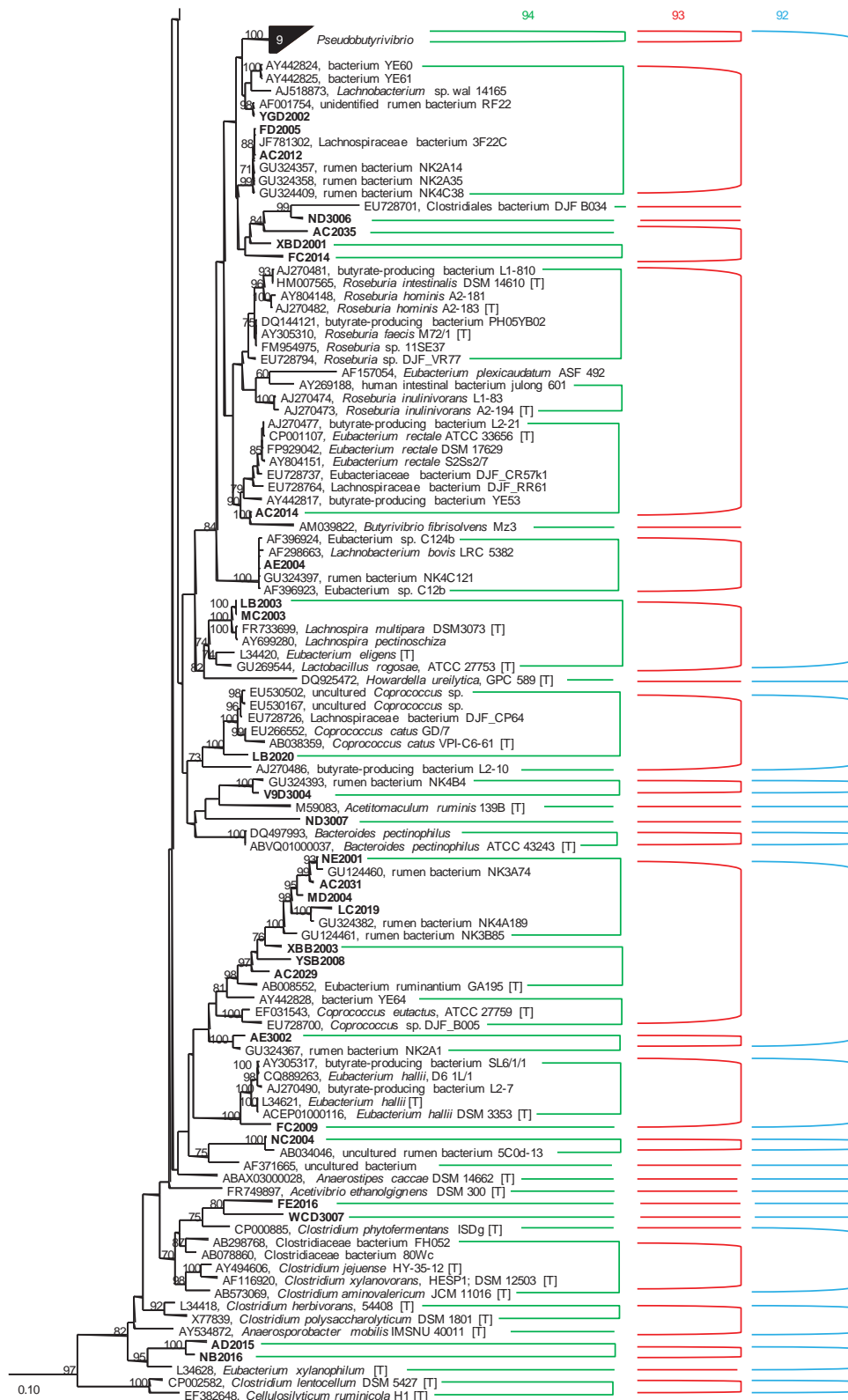


Figure 6-3. Clusters formed at various similarity levels. The tree is an expanded view of Figure 6-1 and illustrates part of the family Lachnospiraceae. [T] denotes a type species. Clusters are indicated at a minimum of 94% (green) 93% (red) and 92% (blue) similarity as described in the text.



0.10

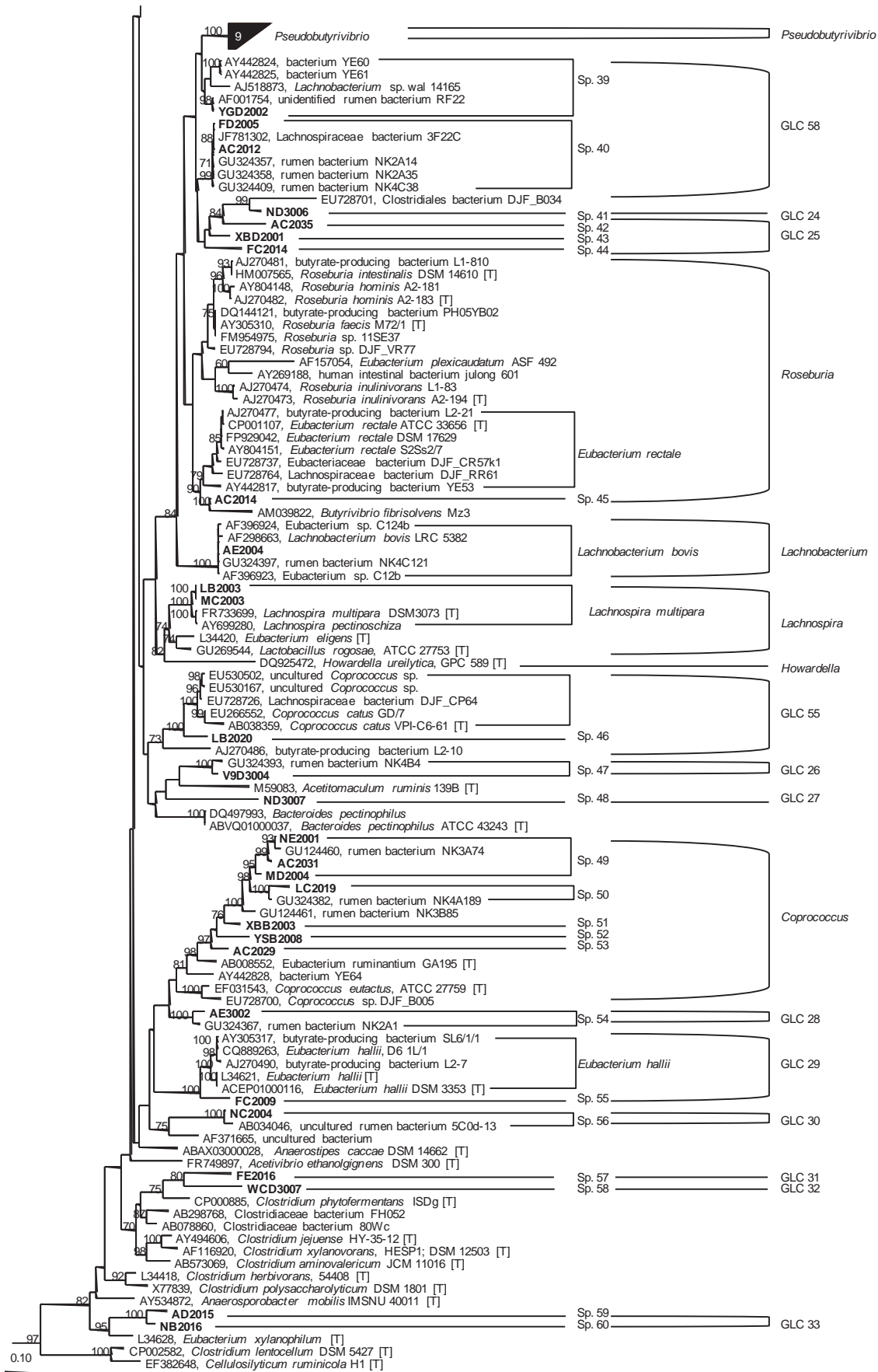


Figure 6-4. Expanded view of Lachnospiraceae.

The full tree is based on a ML analysis in RaxML of a dataset comprising 1075 near full-length high quality 16S rRNA gene sequences from the new isolates, their closest relatives and representatives of the phyla depicted. This figure shows a subset of the tree shown in Figure 6-1. The number of isolates found within each collapsed clade is shown in white. Species-level groups (sp.) are shown to the right of the tree. Genus-level clusters (GLC) are shown to the far right of the tree. [T] denotes a type strain. Bootstrap values >70% are shown at internal nodes. The scale bar indicates the mean number of nucleotide substitutions per site. The tree is rooted with eight reference sequences (not shown): *Sulfurihydrogenibium subterraneum* HGM-K1 (AB071324), *Thermotoga maritima* MSB8 (AE000512), *Aquifex aeolicus* VF5 (AE000657), *Thermodesulfatator indicus* CIR29812 (AF393376), *Thermodesulfobacterium commune* YSRA-1 (AF418169), *Fervidobacterium nodosum* Rt17-B1 (CP000771), *Desulfurobacterium thermolithotrophum* BSA (CP002543) and *Caldimicrobium rimae* DS (EF554596).

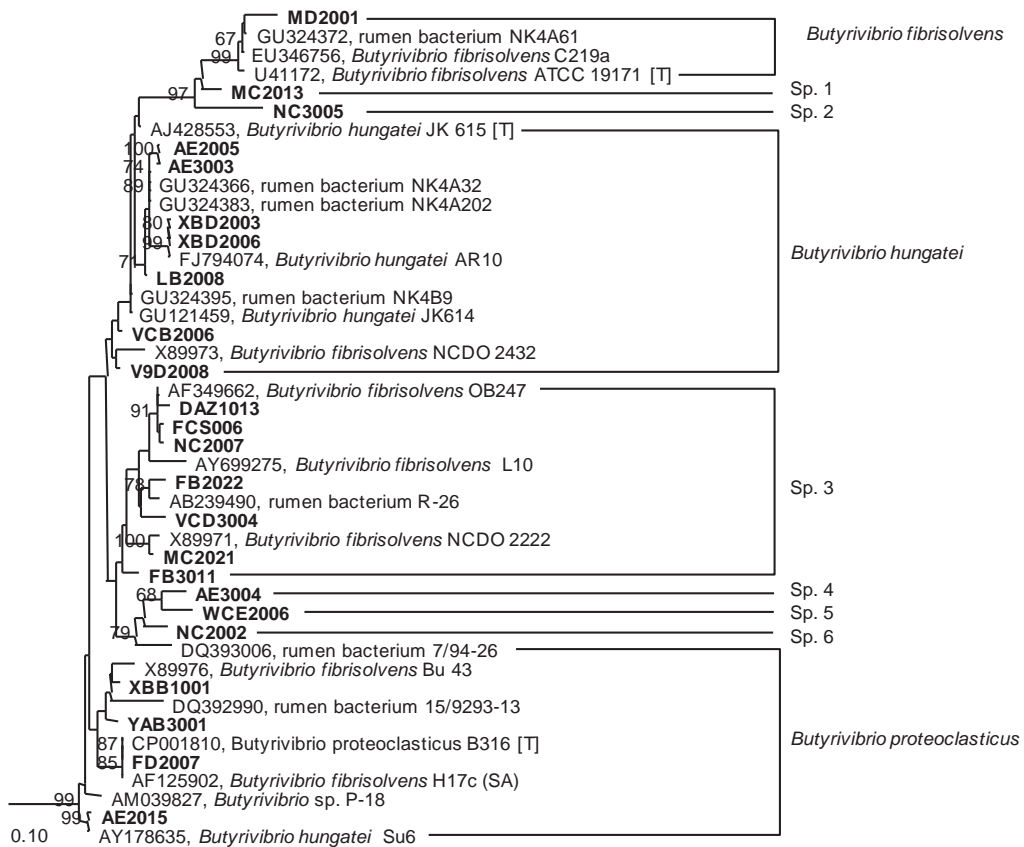


Figure 6-5. Expanded view of *Butyrivibrio* from Figure 6-4. Details are described in the legend to Figure 6-4.

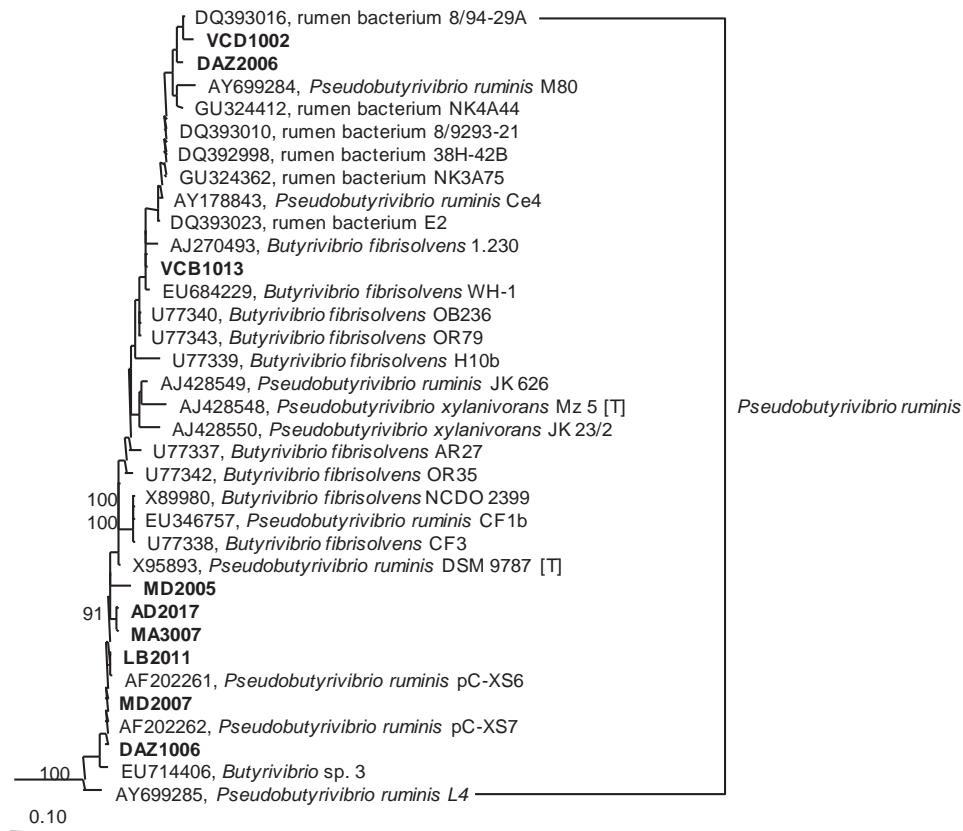


Figure 6-6. Expanded view of *Pseudobutyrvibrio* from Figure 6-4. Details are described in the legend to Figure 6-4.

6.2.3.2 Ruminococcaceae

The identity of the 32 selected isolates that fell in Ruminococcaceae is shown in Table 6-2 and the phylogenetic tree showing the placement of the isolates within Ruminococcaceae is shown in Figure 6-7. Eleven recognised genera in Ruminococcaceae are listed in the Roadmap to the Firmicutes (Ludwig *et al.*, 2009b). In addition, the recently described genus *Saccharofermentans* falls into this family (Chen *et al.*, 2010). The new isolates clustered into *Ruminococcus* and *Saccharofermentans* and four new GLC.

The type genus of Ruminococcaceae is *Ruminococcus*, which includes the type species *Ruminococcus flavefaciens* as well as three other species, *R. albus*, *R. bromii* and *R. callidus* (Ludwig *et al.*, 2009b). By applying the 93% similarity guideline to define a genus-level cluster, *R. flavefaciens* and *R. callidus* formed a GLC of true *Ruminococcus* spp., and *R. albus* formed a separate GLC (GLC 35) separated by a GLC (GLC 34) containing only new and unnamed isolates. The sequences in the *Ruminococcus* GLC were on average 91.1% similar to those in GLC 35 (containing *R. albus*), suggesting that the currently defined genus *Ruminococcus* needs to be re-evaluated and split into two or more genera. Also, by applying the 97% similarity guideline to define a species, not all strains named *R. flavefaciens* (RGL 01, RGL 25, C1a and C52) clustered with the type strain of *R. flavefaciens*. Again this suggests reorganisation of this genus may be warranted. This confirms observations by Jindou *et al.* (2008) who reported two lineages were observed for *R. flavefaciens* based on an analysis of the scaffoldin gene cluster.

Ten selected isolates with near full-length sequences grouped with *R. flavefaciens* in the *Ruminococcus* cluster. In the original analysis of just the V1-V3 regions, these isolates appeared as 10 separate species-level clusters, and so using the partial 16S rRNA gene sequences appeared to overestimate the number of *Ruminococcus* species.

Isolate V9D2013 represented a novel lineage in *Ruminococcaceae* (GLC 36). It showed <90.6% similarity to the type strains of recognised species in this family. Additionally, V9D2013 had no close clone relatives with >94% similarity, and the closest known isolate is rumen bacterium NK4A214 with 91.5% sequence similarity. The closest type strains were from the species *Sporobacter termitidis* (90.6% similarity) and *Papillibacter cinnamivorans* (88.7% similarity).

Thirteen isolates clustered in a stable group (bootstrap 100%) in GLC 37 and a further two with *Saccharofermentans*. Together, these clusters formed a clearly divergent group within *Ruminococcaceae*. The average interspecies similarity of this cluster was 94.8% and the average similarity to the *Ruminococcus* cluster was 83.6%.

GLC 37 contained the uncharacterised rumen isolate R-25 (Koike *et al.*, 2010) and the second GLC contained the recently described *Saccharofermentans acetigenes* P6 (Chen *et al.*, 2010). Isolate R-25 was only 91.7% similar to *S. acetigenes* P6, suggesting they belong in different genera. However, the sequences of isolates XPD2007 and NE3001 were >93% similar to both isolate R-25 and *S. acetigenes* P6, and so appeared to fall between them. There was only low bootstrap support for their relationship with *S. acetigenes* P6. Upon phenotypic characterisation, these strains may be moved to GLC 37. However, it is unlikely isolate R-25 and *S. acetigenes* P6 will be phenotypically similar. R-25 was isolated from rumen-incubated rice straw and is a representative of a previously identified group of uncultured organisms from the fibre-associated fraction of rumen contents group U2; (Koike *et al.*, 2003b). *Saccharofermentans acetigenes* P6 was isolated from anaerobic sludge in a brewery wastewater treatment reactor (Chen *et al.*, 2010). A phenotypic comparison of these two isolates has not yet been made and there is insufficient information available to determine how different from each other they are.

Table 6-2. Identity of isolates within Ruminococcaceae.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
ND2009	Ruminococcus cluster 1	3	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
AE3010	Ruminococcus cluster 2	2	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
XPD3002	Ruminococcus cluster 6	1	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
YRD2003	Ruminococcus cluster 7	1	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
MA2007	Ruminococcus cluster 8	8	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
YAD2003	Ruminococcus cluster 9	6	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
NE2006	Ruminococcus cluster 10	1	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
DAZ1015	Ruminococcus cluster 11	1	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
ND2012	Ruminococcus cluster 12	1	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
V9D2012	Ruminococcus cluster 17	1	Ruminococcaceae GLC 34 / sp. 63	No
MD3002	Ruminococcus cluster 18	9	Ruminococcaceae GLC 34 / sp. 62	No
XPD3003	Ruminococcus cluster 18	(9) ^b	Ruminococcaceae GLC 34 / sp. 61	Yes
FB2012	Ruminococcus cluster 19	1	Ruminococcaceae GLC 35 / sp. 65	No
AD2013	Ruminococcus cluster 20	3	Ruminococcaceae GLC 35 / <i>Ruminococcus albus</i>	Yes
FC2018	Ruminococcus cluster 24	3	Ruminococcaceae GLC 35 / sp. 64	Yes
MC2020	Ruminococcaceae cluster 20	2	<i>Ruminococcus</i> / <i>Ruminococcus flavefaciens</i>	Yes
V9D2013	Ruminococcaceae II cluster 1	1	Ruminococcaceae GLC 36 / sp. 66	No
AB4001	Ruminococcaceae R-25 like cluster 1	47	Ruminococcaceae GLC 37 / sp. 67	Yes
MC4003	Ruminococcaceae R-25 like cluster 1	(47)	Ruminococcaceae GLC 37 / sp. 67	Yes
MB2017	Ruminococcaceae R-25 like cluster 2	1	Ruminococcaceae GLC 37 / sp. 67	Yes

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
YAD3003	Ruminococcaceae R-25 like cluster 3	5	Ruminococcaceae GLC 37 / sp. 67	Yes
AD2012	Ruminococcaceae R-25 like cluster 5	9	Ruminococcaceae GLC 37 / sp. 67	Yes
AE3018	Ruminococcaceae R-25 like cluster 5	(9)	Ruminococcaceae GLC 37 / sp. 67	Yes
MD2010	Ruminococcaceae R-25 like cluster 6	4	Ruminococcaceae GLC 37 / sp. 69	No
NE2014	Ruminococcaceae R-25 like cluster 6	(4)	Ruminococcaceae GLC 37 / sp. 69	No
YRD2005	Ruminococcaceae R-25 like cluster 6	(4)	Ruminococcaceae GLC 37 / sp. 69	No
XPD1004	Ruminococcaceae R-25 like cluster 8	1	Ruminococcaceae GLC 37 / sp. 70	Yes
YRB3002	Ruminococcaceae R-25 like cluster 9	1	Ruminococcaceae GLC 37 / sp. 70	Yes
NE3001	Ruminococcaceae R-25 like cluster 10	6	<i>Saccharofermentans</i> / sp. 71	Yes
XPD2007	Ruminococcaceae R-25 like cluster 12	2	<i>Saccharofermentans</i> / sp. 71	Yes
AC2037	Ruminococcaceae R-25 like cluster 13	5	Ruminococcaceae GLC 37 / sp. 68	Yes
YSB2003	Ruminococcaceae R-25 like cluster 14	1	Ruminococcaceae GLC 37 / sp. 68	Yes

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in the cluster are shown. Where more than one sequence from a cluster is shown the number is repeated in brackets.



Figure 6-7. Expanded view of *Ruminococcus*. Details are described in the legend to Figure 6-4.

6.2.3.3 Clostridiales Family XIII *Incertae Sedis*

Near complete 16S rRNA gene sequences were obtained for all six of the total isolates originally identified as members of Family XIII *incertae sedis*. The identity of these six selected isolates that fell in Clostridiales Family XIII *incertae sedis* is shown in Table 6-3 and the phylogenetic tree showing the placement of the isolates is shown in Figure 6-8. Family XIII *incertae sedis* has two recognised genera, *Anaerovorax* and *Mogibacterium*. There are many validly named species that cluster within this family that may warrant reclassification. The selected isolates clustered within this family (100% bootstrap support) but not with either of the two recognised genera. In this analysis, this family encompassed diverse species with an average sequence similarity of 83.7%. Four new GLC are proposed to encompass the new isolates and there may be 15 separate genera within this family based on the similarity matrix values of the sequences shown here (additional GLC are not shown in the tree in Figure 6-8).

Isolate AD3011 grouped most closely to the two known genera but clustered with clone sequences and was only distantly related to the type strains of *Mogibacterium* and *Anaerovorax*: *Mogibacterium pumilum* (89.3% similar) and *Anaerovorax odorimutans* (90.6% similar). Isolate AB3007 appear to be a representative of a new species, in the same species level group (sp. 72) as the misclassified *Eubacterium* sp. C2 (99.6% similarity). Sp. 72 forms its own GLC but is related (100% bootstrap support) to other GLC (not labelled) containing *Eubacterium nodatum* (91.4% similarity) and *Eubacterium minutum* (92.3% similarity).

6.2.3.4 R-7 group

The R-7 group is a novel group that potentially represents a new family in Clostridia. However, this group had uncertain phylogenetic placement in NJ trees (not shown), often not affiliated with the other families in Clostridia. The R-7 group was always associated with Catabacteriaceae and Christensenellaceae, which are currently considered members of Clostridiales. Further analysis is needed to determine the true placement of this group.

The R-7 group was named after the only previously cultured isolate that fell into this cluster, bacterium R-7 isolated from sheep rumen contents (unpublished GenBank accession AB239481.1). By applying the 93% similarity guidelines, there were two GLC that contained new isolates. Three selected isolates clustered near (>97% sequence similarity) rumen bacterium R-7 and one isolate formed a separate GLC with only clone sequences (89.5% sequence similarity to rumen bacterium R-7). The identity of the four selected isolates is shown in Table 6-4 and the phylogenetic tree showing the placement of the isolates is shown in Figure 6-9. The closest type strains to these new isolates are *Catabacter hongkongensis* (average 84.33% similarity) and *Christensenella minuta* (average 84.85% similarity). The cells of the R-7 group of isolates were short rods/oval or cocci often found in chains. It seems surprising that only one of member of this family has been isolated before now. The four selected isolate sequences represent 44 of the total 838 isolates cultured in this study. Despite being a novel lineage, this group seemed relatively easy to isolate from the plant-adherent bovine rumen fraction, with isolates originating from each of the animals over the course of the year sampled, in eight out of the ten media types used in this study and in at least one animal at each season sampled. The isolation method used, of dilution to a single cell, may create an advantage for isolating R-7 group members.

6.2.3.5 Veillonellaceae

The family Veillonellaceae currently has 26 described genera, including *Selenomonas* (Ludwig *et al.*, 2009b). In this study, *Selenomonas* appeared to be polyphyletic, forming three GLC containing validly named *Selenomonas* species. The type species of the genus, *Selenomonas sputigena*, clustered separately from all other *Selenomonas* species, with an average 90.5% sequence similarity to other *Selenomonas* strains. The closest *Selenomonas* type strain is *Selenomonas diana* (91.7% similarity). This indicates all other *Selenomonas* species might need to be renamed. The second GLC containing *Selenomonas* species encompassed the type strains of *Selenomonas ruminantium*, *Mitsuokella multacida* and *Mitsuokella jalaludinii* along with many isolates, to form GLC 42. The type strains of *Selenomonas diana*, *Selenomonas infelix*, *Selenomonas flueggei*, *Selenomonas noxia*, *Selenomonas artemidis* and *Centipeda*

periodontii formed a GLC designated as *Centipeda*. This GLC contained no selected isolate sequences.

The interspecies sequence similarity within GLC 42 ranged from 91.2-99.6% and the average inter-cluster similarity to GLC *Centipeda* was 90.5%. Further division of GLC 42 was not supported by tree topology and bootstrap analysis. The genus *Mitsuokella* could not be distinguished from *S. ruminantium* by applying the 93% sequence similarity guideline. However *Mitsuokella* can be differentiated from *S. ruminantium* by phenotypic means (Willems *et al.*, 2009). GLC 42 might be considered new genus based on 16S rRNA gene sequence comparisons but phenotypic support is needed to verify this.

The identity of the six selected isolates that fell in Veillonellaceae is shown in Table 6-5 and the phylogenetic tree showing the placement of the isolates is shown in Figure 6-10. The sequences of these six strains isolated in this study fell in GLC 42, which also contained *S. ruminantium* and *Mitsuokella* spp. Three selected isolates (AE3005, AC2024 and FC4001) formed a species level cluster with the type strain of *S. ruminantium* GA192 and isolates of *S. ruminantium*. New isolate AC2024 fell in this cluster but had a long branch length and only shared 95.9% sequence similarity with *S. ruminantium* GA192. Selected isolate ND2010 which was included in new species 79, had 97.4% sequence similarity to the type strain *S. ruminantium* GA192, but an average 96.3% sequence similarity to the *S. ruminantium* species level cluster. Isolate AB3002 formed species level cluster sp. 80 with four other known *S. ruminantium* isolates, and was 95.3% similar to *S. ruminantium* GA192.

These findings support the findings of Sawanon *et al.*(2011), who recognised two distinct clades of *S. ruminantium* isolates that displayed differences in fibre digestion when they were co-cultured with *Fibrobacter succinogenes*. Clade 1 included the *S. ruminantium* type strain GA192 and several other isolates that corresponded to the *S. ruminantium* species level group as defined in this study. Clade 2 included *S. ruminantium* sp. S215, S210, S206 and S109, which corresponded to sp. 80 in Figure 6-10. This suggests that the species level groupings based on 16S rRNA gene sequences may reflect real underlying phenotypic differences.

Table 6-3. Identity of isolates within Clostridia Family XIII *incertae sedis*.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
AB3007	Family XIII <i>incertae sedis</i> cluster 1	1	GLC 56 / sp. 72	Yes
LD2013	Family XIII <i>incertae sedis</i> cluster 5	1	GLC 38 / sp. 75	No
YSD2010	Family XIII <i>incertae sedis</i> cluster 7	1	GLC 38 / sp. 75	No
YAD3004	Family XIII <i>incertae sedis</i> cluster 8	1	GLC 38 / sp. 74	No
XBB3003	Family XIII <i>incertae sedis</i> cluster 9	1	GLC 57 / sp. 73	No
AD3011	Family XIII <i>incertae sedis</i> cluster 17	1	GLC 39 / sp. 76	No

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

Table 6-4. Identity of isolates within R-7 group.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
LC2020	Clostridiales R-7 like cluster 1	31	R-7 GLC 40 / sp. 77	Yes
XBB3002	Clostridiales R-7 like cluster 6	3	R-7 GLC 40 / sp. 77	Yes
WTE2008	Clostridiales R-7 like cluster 26	9	R-7 GLC 40 / sp. 77	Yes
AC2043	Clostridiales R-7 like cluster 38	1	R-7 GLC 41 / sp. 78	No

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

Table 6-5. Identity of isolates within Veillonellaceae.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster	Genus level cluster (sp.)	Genus level cluster (GLC) / species level cluster	Species level cluster includes a previously cultured bacterium
AB3002	Veillonellaceae cluster 4	7	Veillonellaceae	GLC 42 / sp. 80	Yes
FC4001	Veillonellaceae cluster 5	1	Veillonellaceae	GLC 42 / <i>Selenomonas ruminantium</i>	Yes
ND2010	Veillonellaceae cluster 6	2	Veillonellaceae	GLC 42 / sp. 79	Yes
AC2024	Veillonellaceae cluster 7	5	Veillonellaceae	GLC 42 / <i>Selenomonas ruminantium</i>	Yes
AE3005	Veillonellaceae cluster 8	1	Veillonellaceae	GLC 42 / <i>Selenomonas ruminantium</i>	Yes

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

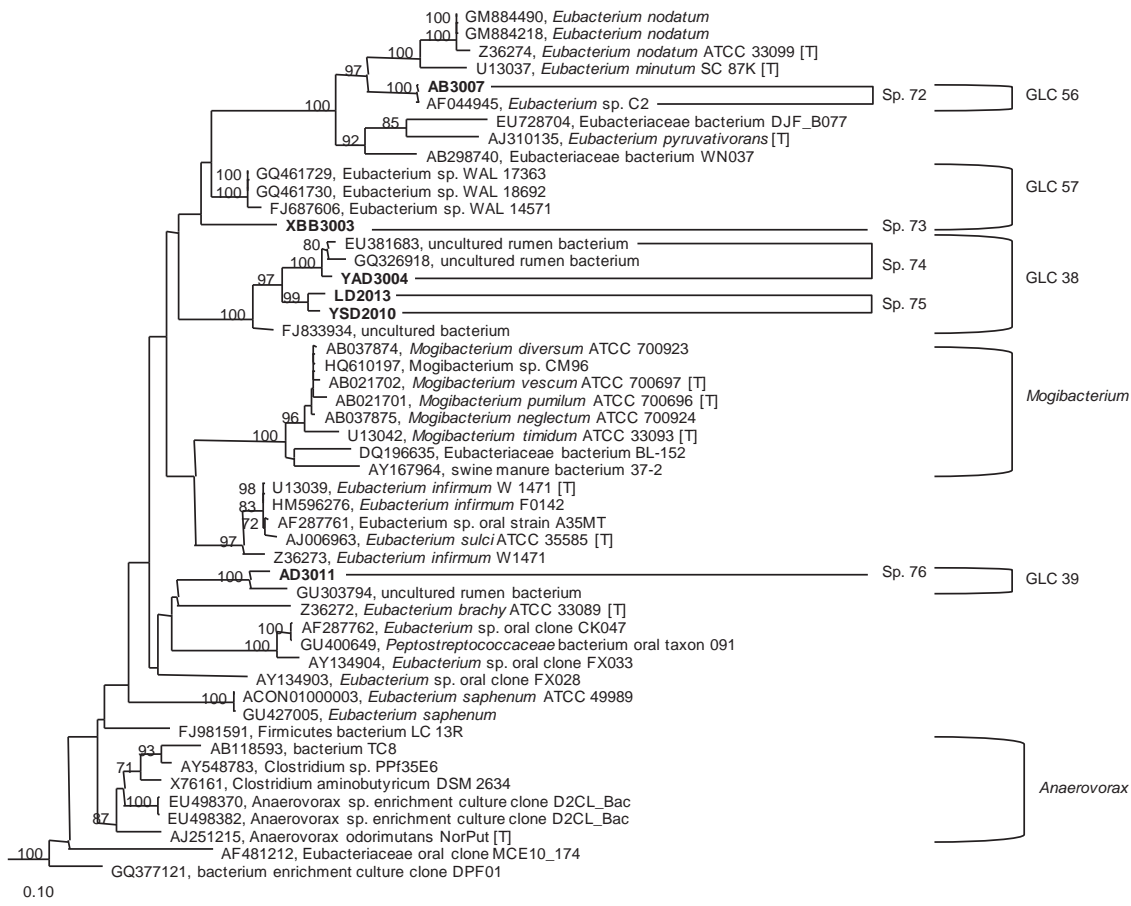


Figure 6-8. Expanded view of Clostridia family XIII *incertae sedis*. Details are described in the legend to Figure 6-4.

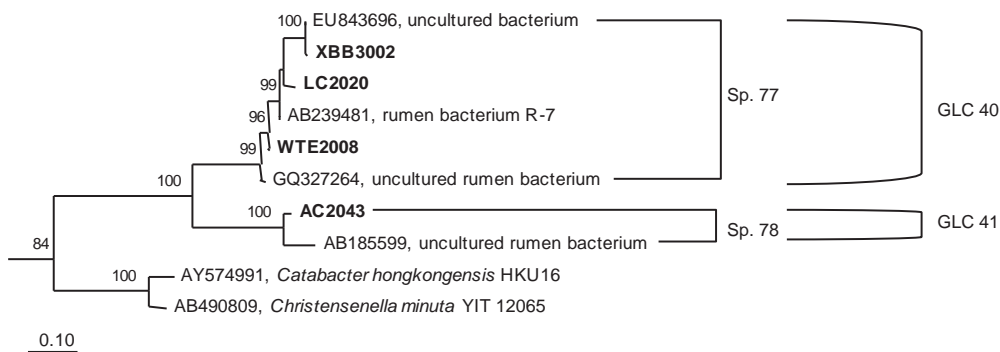


Figure 6-9. Expanded view of the R-7 group. Details are described in the legend to Figure 6-4.

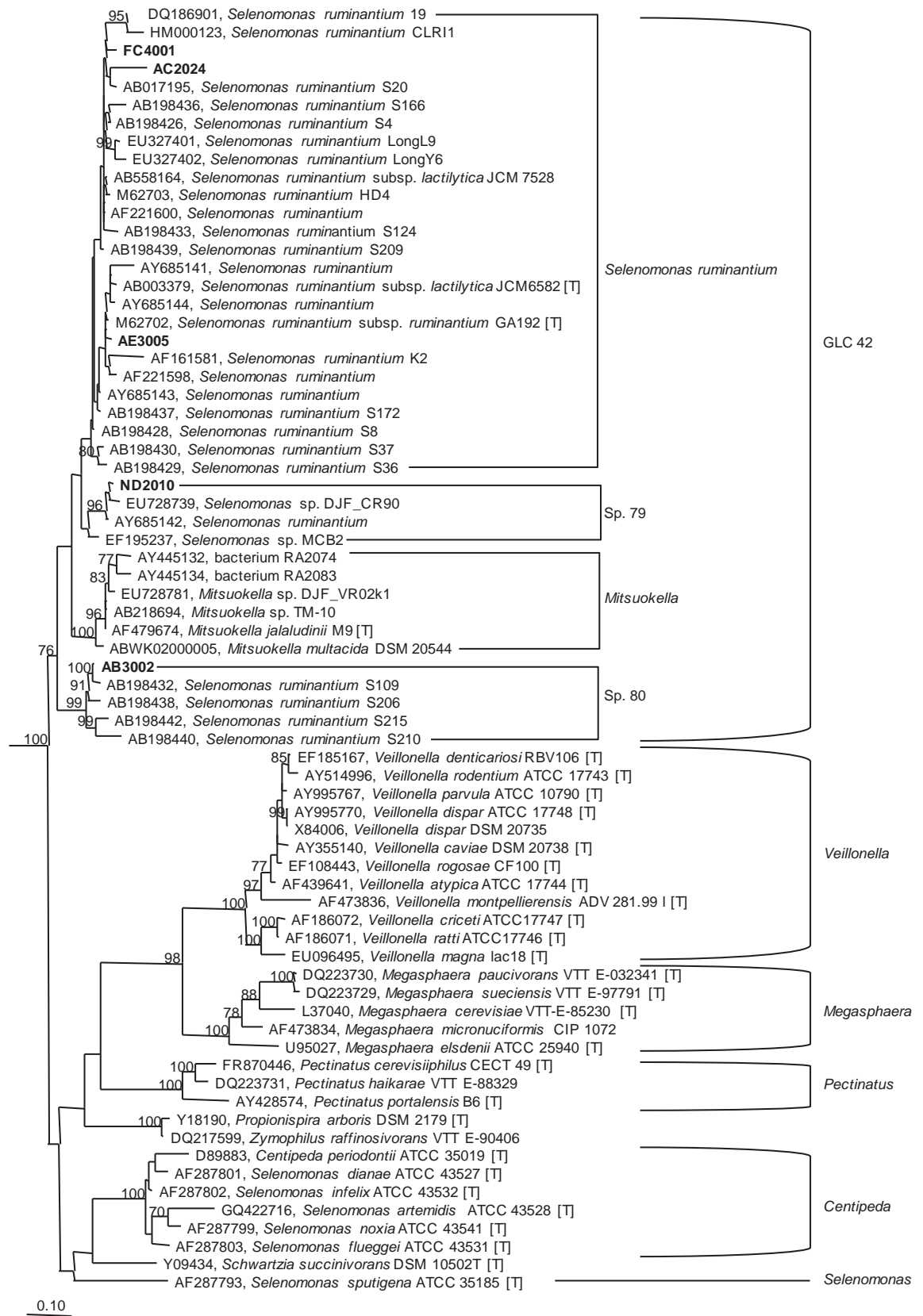


Figure 6-10. Expanded view of Veillonellaceae. Details are described in the legend to Figure 6-4.

6.2.4 Firmicutes, Class Bacilli

Two selected isolates (DAZ1002 and MB2021) fell in the class Bacilli. The identity of these two isolates is shown in Table 6-1 and the phylogenetic placement of MB2021 is shown in Figure 6-11 and of DAZ1002 in Figure 6-12.

MB2021 clustered in the genus *Bacillus* within the family Bacillaceae in the order Bacillales. MB2021 had a 100% similarity match to *Bacillus circulans* and also fell within the same 97% species level cluster as the type strain of *Bacillus nealsonii* (98.3% similarity). The 16S rRNA gene sequence of this isolate was 100% similar to the sequenced V1-V3 region of the 16S rRNA gene of a medium contaminant found during the isolation experiment, but was isolated months before the contamination occurred. It is possible that it is not of rumen origin given there were no pyrosequences with >96% sequence match to this isolate. However, other members of the genus *Bacillus* were found in the pyrosequenced community, albeit in low numbers (~30 sequences). This isolate may have been a transient rumen community member. *B. circulans* and other closely related *Bacillus* species (*B. sibiricus*, *B. benzoovorans* and *B. firmus*) are associated with silage feed (Inglis *et al.*, 1999; Pettersson *et al.*, 2000). Supporting this idea, MB2021 was isolated from a month when the animals were being fed pasture silage.

DAZ1002 was associated with the genus *Streptococcus* within the family Streptococcaceae in the order Lactobacilli. DAZ1002 formed a stable cluster with the type strains of five closely related species of *Streptococcus*: *S. infantarius subsp. infantarius* (99.7% similarity), *S. lutetiensis* (99.9% similarity), *S. luteciae* (99.9% similarity), *S. equinus* (99.7% similarity), *S. gallolyticus subsp. gallolyticus* (98.5% similarity), and *S. gallolyticus subsp. macedonicus* (98.3% similarity). In this case, 16S rRNA gene sequence similarity alone is not enough to distinguish between separate species of *Streptococcus*. Originally four of these *Streptococcus* species (excluding *S. equinus*) were known as *S. bovis* and have been progressively reclassified based on phenotypic characteristics and DNA-DNA homology (Poyart *et al.*, 2002). *S. bovis* and *S. equinus* were found to represent a single species where *S. equinus* had naming precedence. Alternative marker genes such as the *sodA* gene have been used to differentiate these species (Poyart *et al.*, 2002). Phenotypic evidence or comparisons of

the *sodA* gene is needed to place DAZ1002 into one of these species or to determine if it is a representative of a new species.

Table 6-6. Identity of isolates within Bacilli.

Isolate name	Original cluster designation^a	Total no. of isolates in cluster	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
DAZ1002	Streptococcus cluster 1	1	<i>Streptococcus</i> / <i>Streptococcus</i> sp.	Yes
MB2021	Bacillus cluster 1	1	<i>Bacillus</i> / <i>Bacillus circulans</i>	Yes

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

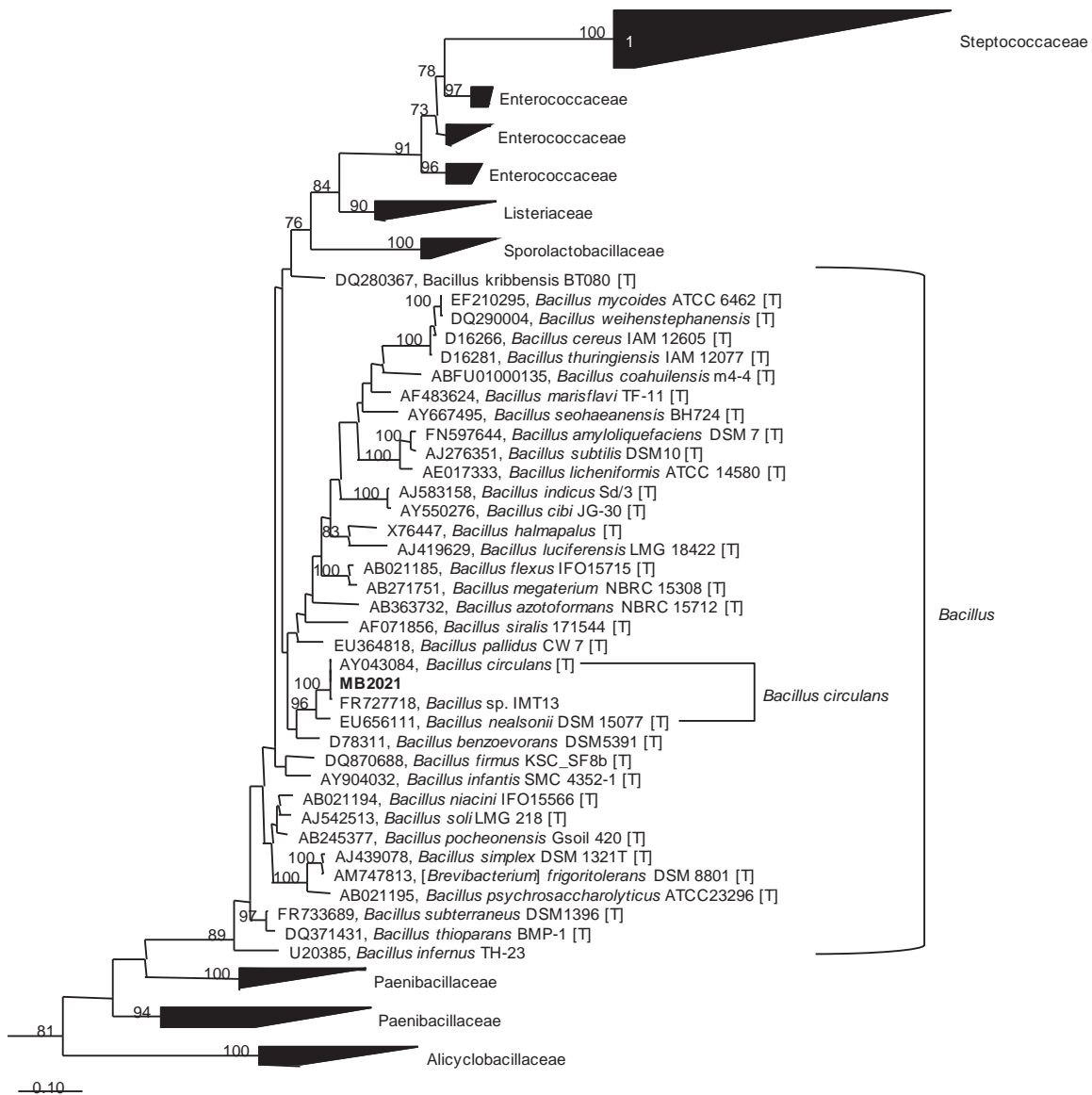


Figure 6-11. Expanded view of Bacilli. Details are described in the legend to Figure 6-4.

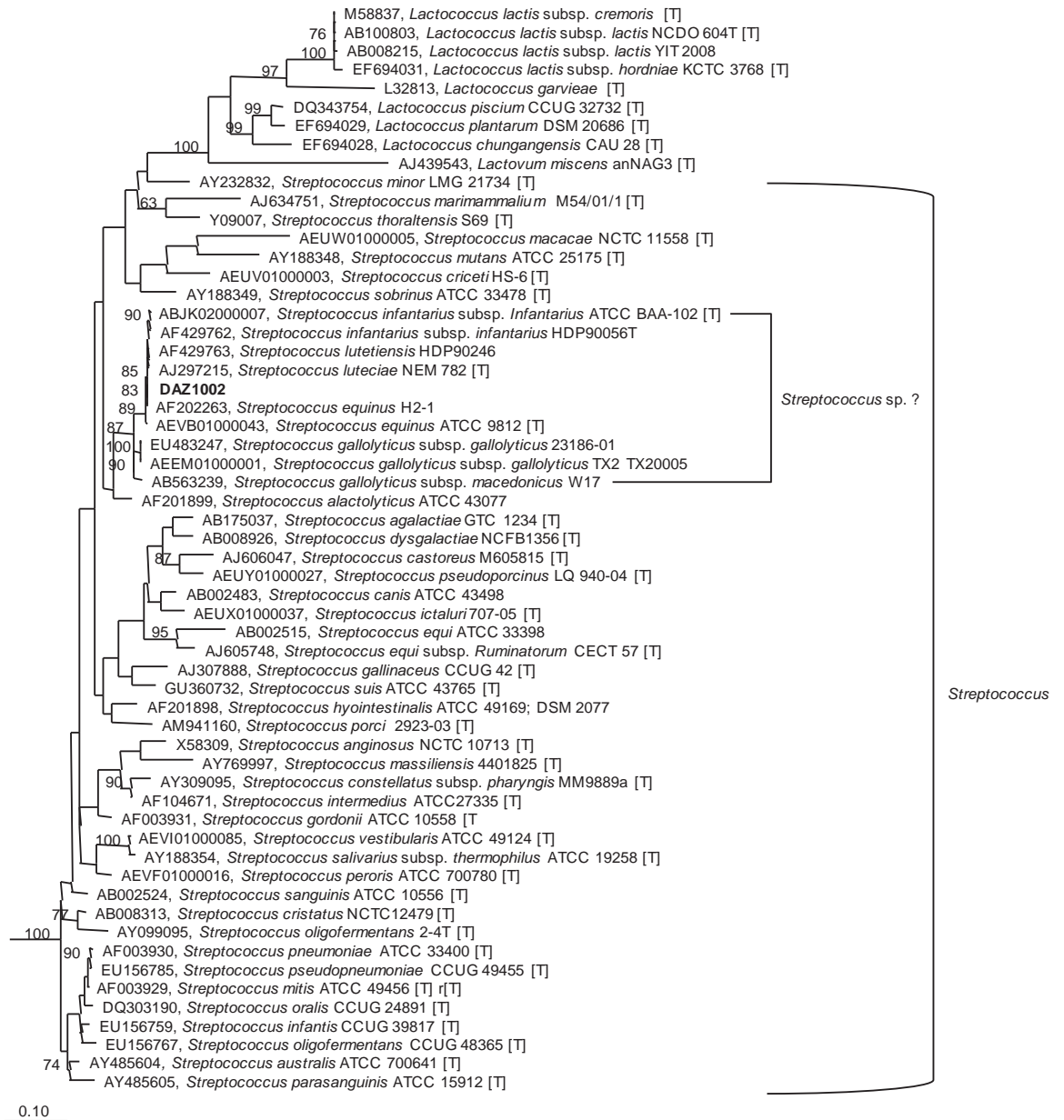


Figure 6-12. Expanded view of Streptococcaceae. Details are described in the legend to Figure 6-4.

6.2.5 Firmicutes, Class Erysipelotrichia

The identity of the five selected isolates in Erysipelotrichia are summarised in Table 6-7 and their phylogenetic placement is shown in the tree in Figure 6-13. The class Erysipelotrichia has recently undergone reorganisation. The class Mollicutes of the phylum Firmicutes previously contained the bacteria now classified in Erysipelotrichia. The class Mollicutes has been elevated to form a new phylum named Tenericutes, but the Erysipelotrichia, previously included within Mollicutes, now form their own class in Firmicutes (Ludwig *et al.*, 2009a). The class Erysipelotrichia currently has a single order, Erysipelotrichales, and one family, Erysipelotrichaceae. This family currently has eight genera listed in the roadmap to the Firmicutes (Ludwig *et al.*, 2009b), plus the two recently described genera *Kandleria* and *Eggerthia*. The type species of genus *Kandleria*, *Kandleria vitulina*, has been recently reclassified from *Lactobacillus vitulinus* (Salveti *et al.*, 2011). Isolates WCE2001 and MC3001 clustered closely with the type strain of *Kandleria vitulina*. Erysipelotrichaceae cluster 1, represented by WCE2001, was one of the largest of the original clusters (of partial 16S rRNA gene sequences) of isolates, with 26 members. WCE2001 shares 100% 16S rRNA gene sequence similarity with *K. vitulina*.

MC3001 was originally identified as a *Butyrivibrio* strain based on the partial 16S rRNA gene sequence obtained from the culture. When the culture was revived, DNA re-extracted and re-sequenced, the resulting sequence was 99.8% similar to WCE2001 and 99.8% similar to *K. vitulina*. This could be a handling error with the initial sequence or perhaps the culture was originally mixed and one strain was more readily revived than the other. The possibility of the isolates being mixed cultures is an acknowledged risk, as the isolates were only putatively pure and did not undergo purification by, for example, separation to single colonies.

Both members of the original cluster 'Erysipelotrichaceae cluster 2' were selected for near full-length 16S rRNA gene sequence analysis. These strains were both isolated on the same medium (RM02 with the addition of dried grass) at the same sampling time, but from different animals. YGB2007 and YGD2005 shared 100% 16S rRNA gene sequence similarity with each other but are distant from other type strains in this family (79.1-83% similarity). Because of this lack of similarity to any other cultured

bacteria, these isolates likely represent a new genus (GLC 43) within the family Erysipelotrichaceae.

Isolate WCE2003 fell in a GLC (GCL 44) related to *Solobacterium* and *Bulleidia*, but its 16S rRNA gene sequence was only moderately similar to those of the type strains of *Solobacterium moorei* (93.8%) and *Bulleidia extracta* (91.2%). By applying the >93% similarity guideline, WCE2003 could be a member of *Solobacterium*, although it fell into a stable group separate from named strains of this genus. The 16S rRNA gene sequences of the type strains of *Solobacterium moorei* and *Bulleidia extracta* are 93.3% similar to each other. Phenotypic typing will be needed to determine the exact novelty of this isolate.

Table 6-7. Identity of isolates within Erysipelotrichia.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
WCE2011	Erysipelotrichaceae cluster 1	26	<i>Kandleria / Kandleria vitulina</i>	Yes
MC3001	Butyrivibrio cluster 8	4 ^c	<i>Kandleria / Kandleria vitulina</i>	Yes
YGB2007	Erysipelotrichaceae cluster 2	2	Erysipelotrichia GLC 43 / sp. 81	No
YGD2005	Erysipelotrichaceae cluster 2	(2)	Erysipelotrichia GLC 43 / sp. 81	No
WCE2003	Erysipelotrichaceae cluster 4	1	Erysipelotrichia GLC 44 / sp. 82	No

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in the cluster are shown. Where more than one sequence from a cluster is shown the number is repeated in brackets.

^c The other three selected isolates in the original cluster among the 828 total isolates were identified as *Butyrivibrio* not *Kandleria vitulina*. Possible reasons are discussed in the text.

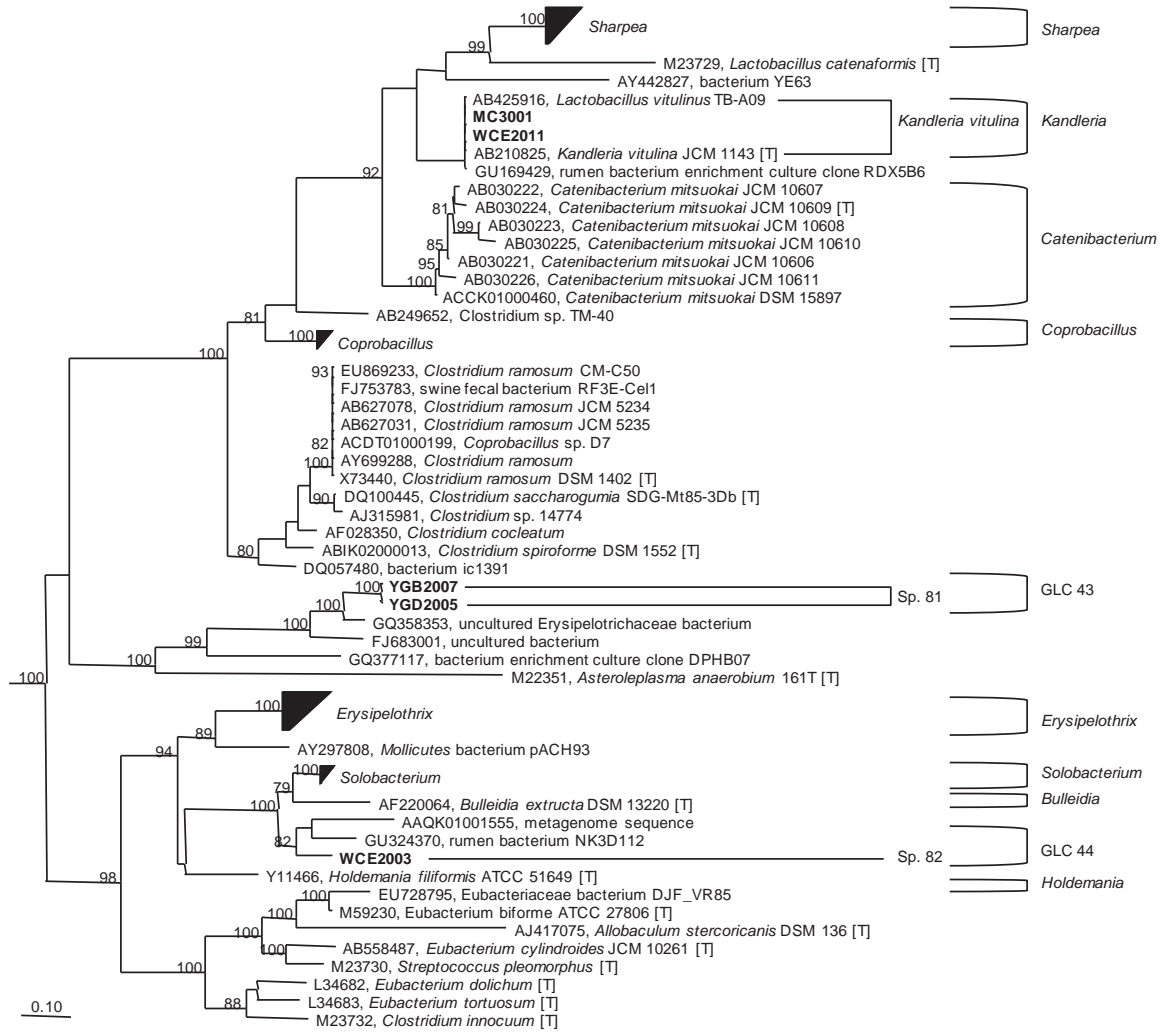


Figure 6-13. Expanded view of Erysipelotrichia. Details are described in the legend to Figure 6-4.

6.2.6 Bacteroidetes

The identity of 21 selected isolates in the phylum Bacteroidetes is summarised in Table 6-8. Three selected isolates were associated with a novel family level group named the RC9 gut group (shown in Figure 6-14) and the remaining isolates were members of Prevotellaceae (shown in Figure 6-15).

The phylum Bacteroidetes has recently gone through a rapid period of reorganisation based on comparisons of the 16S rRNA gene. The new gene-based groups do not always agree with old phenotypically-based taxa (Berger *et al.*, 2005; Jousimies-Somer *et al.*, 2002). An example of this is the genus *Bacteroides*. Originally, the genus *Bacteroides* included gram-negative, non-spore-forming, anaerobic rods that produce succinate, and were divided into three categories based on pigmentation, bile sensitivity and saccharolytic activity. Now, members of *Bacteroides* have been reclassified into several new genera and more changes are expected (Jousimies-Somer *et al.*, 2002). Therefore, it is difficult to determine guidelines of 16S rRNA gene sequence similarity from established genera in this phylum that could be used to define new genera. 16S rRNA gene sequences of type strains within some currently-recognised genera like *Prevotella*, *Porphyromonas* and *Bacteroides* are as little as 85% similar to other members in the same genus. Within the more recently reorganised family Marinilabiliaceae (Ludwig *et al.*, 2010a), the type strains of the genera *Anaerophaga* and *Marinilabilia* share 92.3% 16S rRNA gene sequence similarity. Any similarity value less than 92.3% used as a cut-off would group these two genera together. Therefore, <93% similarity would be an appropriate guideline for defining sequences that are likely to belong to separate genera within this phylum, the same value that was used for the phylum Firmicutes. Similarly, a sequence similarity of <97% was used to determine whether a sequences was likely to belong to a different species.

6.2.6.1 RC9 gut group

Phylogenetic analysis, based on full 16S rRNA gene sequences, revealed that isolates FC2005, WCE2008 and WCE2004 formed a genetically coherent group that was not associated with other validly-named family groups. This group is labelled in the Arb SILVA database as the RC9 gut group, apparently named after sequence RC9 that

was obtained from rumen solids (Tajima *et al.*, 1999). The RC9 gut group comprised of clone sequences and three uncharacterised isolates: *Eubacterium* sp. F1, *Bacteroidales* bacterium P1 and rumen bacterium R-23. Isolates FC2005 and WCE2008 were 99.9% similar to each other and clustered closest to *Eubacterium* sp. F1 (98% sequence similarity). *Eubacterium* sp. F1 is misnamed, and is not a true member of the genus *Eubacterium* as it is only remotely related to the type strain of the type species, *Eubacterium limosum* (72.7% sequence similarity). This finding is supported by previous work that has shown *Eubacterium* sp. F1 groups with species of the phylum Bacteroidetes (Pei *et al.*, 2010). Isolate WCE2004 clustered with cloned sequences in the RC9 gut group and represented a novel GLC and species within this group. The closest cultured relative was *Bacteroidales* bacterium P1 (89.5% similarity). These results supported the initial clustering used to choose representatives for near complete length sequencing, because the two isolates from Bacteroidetes cluster 1 (FC2005 and WCE2008) were still very similar to each other when the full-length 16S rRNA gene sequences were compared, and they were different from the Bacteroidetes cluster 7 isolate (WCE2004), which had 89.2% sequence similarity to FC2005 and 88.9% to WCE2008.

6.2.6.2 Prevotellaceae

The currently-recognised genus *Prevotella* encompasses a large range of 16S rRNA gene sequence diversity, including sequences depicted in Figure 6-15 with as little as 85.2% similarity. Sequence similarities as low as 75.7% have been found in other comparisons (Berger *et al.*, 2005). A genome comparison of *P. bryantii* B₁₄ and *P. ruminicola* 23 demonstrated a lack of synteny across the genomes, exemplifying the high diversity of this genus (Purushe *et al.*, 2010). It has been suggested that the genus *Prevotella* comprises of eleven groups that may warrant reclassification into separate genera if it is supported by additional evidence (Ludwig *et al.*, 2010b). Based on the sequences analysed here, *Prevotella* could be split into 23 separate genera (not all shown on the tree).

Comparisons of 16S rRNA gene similarity revealed three selected isolate sequences associated with the known rumen *Prevotella* species, *P. bryantii* and *P.*

albensis. The remaining 15 selected isolates formed 12 new species-level groups. Eight of the new species-level groups were <97% similar to the sequence of another isolate, and the remaining five were similar to a previously isolated but uncharacterised *Prevotella* species.

Purushe *et al.* (2010) found the *Prevotella* of ruminal origin grouped closely together and away from the oral species. Ten of the selected isolate sequences clustered separately from the oral strains, within GLC 50 (containing both *P. ruminicola* and *P. brevis*), GLC 51 and GCL 52. However, eight isolates, together with *P. bryantii* and *P. albensis*, did not cluster separately from the oral *Prevotella* spp. Several new species-level groups were not closely associated with the known rumen *Prevotella* species, suggesting a greater diversity than evidenced by the current taxonomy.

Table 6-8. Identity of isolates within Bacteroidetes.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
FC2005	Bacteroidetes cluster 1	5	RC9 gut group GLC 53 / sp. 95	Yes
WCE2008	Bacteroidetes cluster 1	(5)	RC9 gut group GLC 53 / sp. 95	Yes
WCE2004	Bacteroidetes cluster 7	1	RC9 gut group GLC 54 / sp. 96	No
FB3001	Prevotella cluster 1	9	Prevotellaceae GLC 47 / <i>Prevotella bryantii</i>	Yes
YRD2002	Prevotella cluster 1	(9)	Prevotellaceae GLC 47 / <i>Prevotella bryantii</i>	Yes
AC2042	Prevotella cluster 3	1	Prevotellaceae GLC 49 / sp. 85	No
AC2036	Prevotella cluster 4	1	Prevotellaceae GLC 46 / <i>Prevotella albensis</i>	Yes
YAB2003	Prevotella cluster 5	1	Prevotellaceae GLC 45 / sp. 83	No
XBB2006	Prevotella cluster 6	3	Prevotellaceae GLC 45 / sp. 83	No
MC2010	Prevotella cluster 7	1	Prevotellaceae GLC 48 / sp. 84	No
VCB2002	Prevotella cluster 8	2	Prevotellaceae GLC 48 / sp. 84	No
NB2015	Prevotella cluster 18	5	Prevotellaceae GLC 52 / sp. 93	Yes
XPB4001	Prevotella cluster 19	5	Prevotellaceae GLC 52 / sp. 93	Yes
VCD1001	Prevotella cluster 20	1	Prevotellaceae GLC 51 / sp. 92	No
LC2012	Prevotella cluster 30	4	Prevotellaceae GLC 50 / sp. 90	Yes
AC2032	Prevotella cluster 34	2	Prevotellaceae GLC 50 / sp. 91	Yes
MB2026	Prevotella cluster 35	1	Prevotellaceae GLC 52 / sp. 94	No
MB2027	Prevotella cluster 38	1	Prevotellaceae GLC 50 / sp. 86	No
MA2016	Prevotella cluster 39	1	Prevotellaceae GLC 50 / sp. 89	Yes
NE3005	Prevotella cluster 40	1	Prevotellaceae GLC 50 / sp. 87	No
FD3004	Prevotella cluster 43	10	Prevotellaceae GLC 50 / sp. 88	No

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in the cluster are shown. Where more than one sequence from a cluster is shown the number is repeated in brackets.

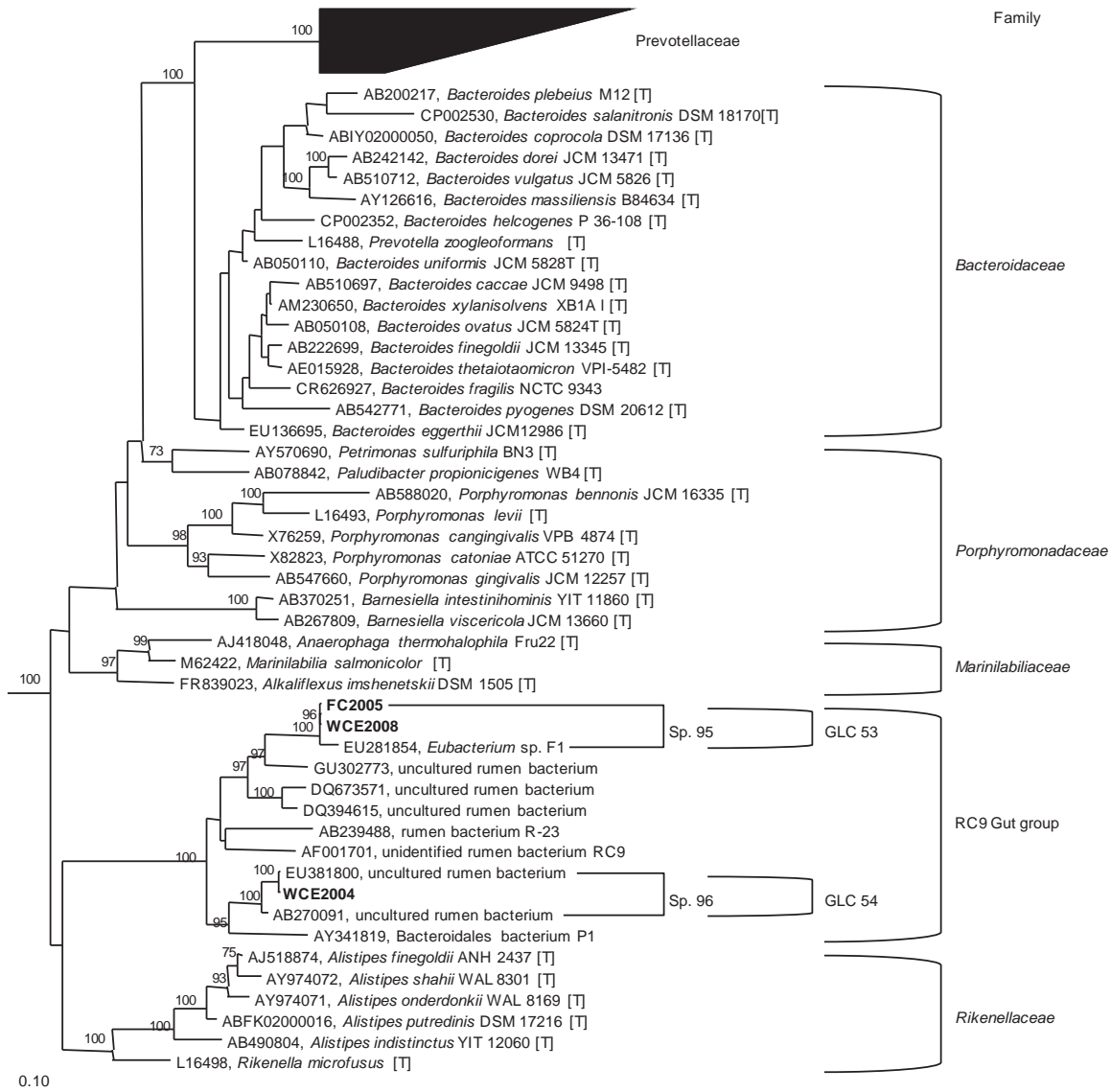


Figure 6-14. Expanded view of Bacteroidetes. Details are described in the legend to Figure 6-4.

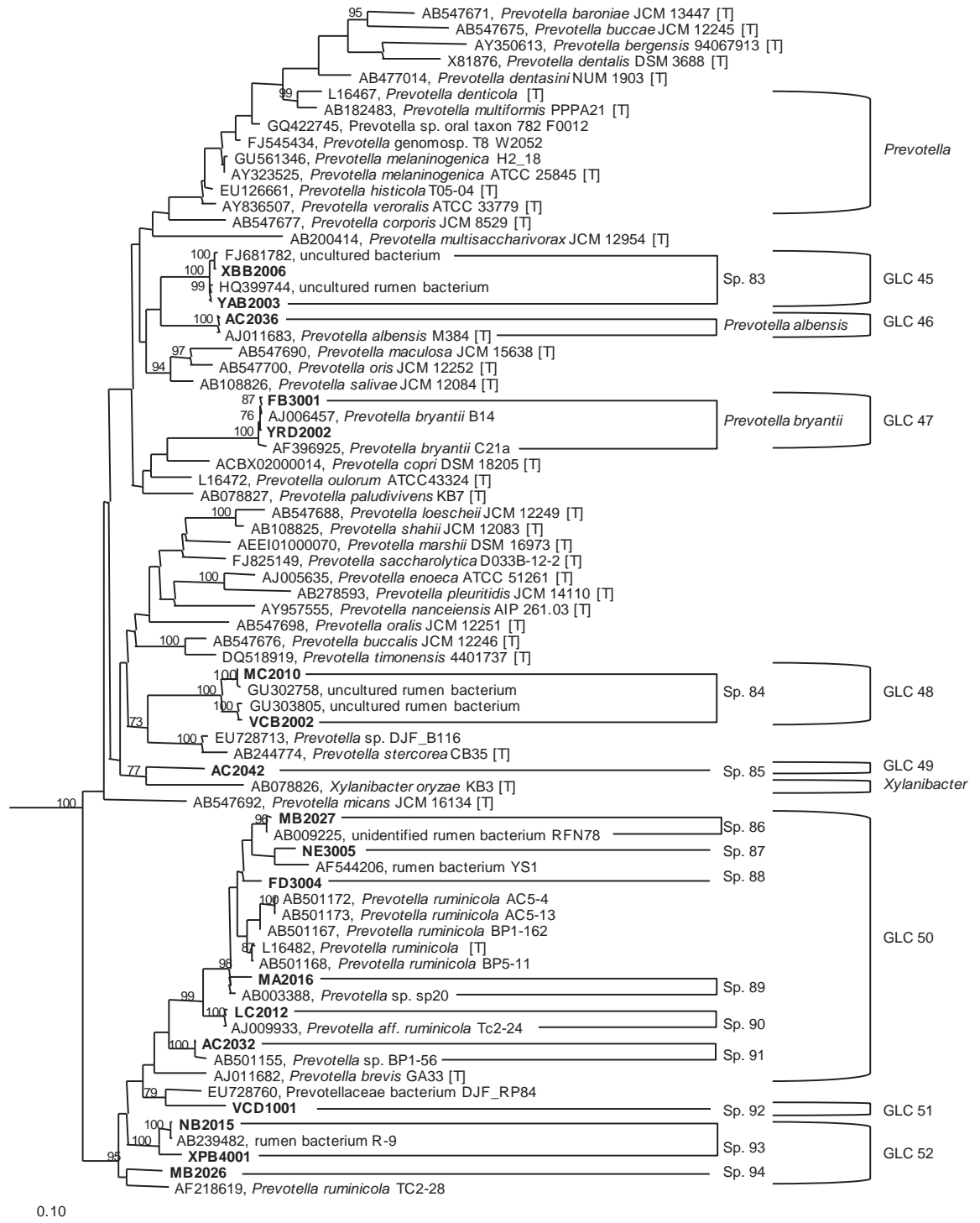


Figure 6-15. Expanded view of Prevotellaceae. Details are described in the legend to Figure 6-4.

6.2.7 Spirochaetes

Spirochetes, members of the phylum Spirochaetes, nearly all have a unique morphology that is instantly recognisable when viewed under the microscope. They are spiral or helix-shaped cells and have a unique cellular ultrastructure. The family Spirochaetaceae contains three genera with isolates, *Spirochaeta*, *Borrelia* and *Treponema*, as well as one genus represented only by a cloned 16S rRNA gene sequence, *Cristispira* (Ludwig *et al.*, 2010b; Paster *et al.*, 1996). The sequences from the selected isolates fell into the existing genus *Treponema*, even though the average inter-sequence difference is 89.3%. The similarity cut-off guideline of 93% for defining a genus does not appear to apply to Spirochaetes as they are currently classified. It has been noted that the average interspecies similarity of 16S rRNA gene sequences of members of the genus *Treponema* is 84.2% (Paster *et al.*, 1991). The genus *Spirochaeta* is also deep branching, with an average interspecies similarity of 87.4%. In contrast, the genus *Borrelia* forms a tight phylogenetic cluster with an average similarity of 97% (Paster *et al.*, 1991). It may be that *Treponema* and *Spirochaeta* actually represent several genera and that these need to be reclassified, but more phenotypic evidence is needed. The depth of the branching of the Spirochaetes means that some Spirochaetes are more closely related to bacteria in other phyla (based on sequence comparisons) than to other Spirochaetes. However, single base signature analysis concludes they are a monophyletic phylum (Paster *et al.*, 1991). Also, the unique cellular ultrastructure shared by all Spirochaetes phenotypically places them in one phylum.

Five of the total 828 isolates were identified as belonging to the phylum Spirochaetes based on partial 16S rRNA gene sequences. All five isolates were selected for full-length 16S rRNA gene sequencing, and analysis confirmed their placement in the phylum Spirochaetes, within the family Spirochaetaceae. The identities of the isolates are summarised in Table 6-9 and their positions in a phylogenetic tree are shown in Figure 6-16. Isolates DAZ1007, XBD1002 and MD2012 clustered closely with the type strain of *Treponema bryantii* (within 97% similarity). Isolate ND3003 clustered closely (99.6% similarity) with *Treponema* sp. AC3. Isolate WCE3006 formed a deep branch on its own within the *Treponema* cluster, and appeared to represent a novel *Treponema* species. Its closest relatives are *Treponema* sp. T (90.8%) and *Treponema succinifaciens* (87.3%), isolated from swine intestines (Cwyk *et al.*, 1979).

6.2.8 Fibrobacteres

All cultures of Fibrobacteres isolated so far are of rumen or gastrointestinal tract origin, although 16S rRNA gene sequences have been recovered from soil and water, suggesting this is a much more widely distributed phylum than suggested from the isolates alone (Spain *et al.*, 2010). Ruminal members of Fibrobacteres are thought to be one of the groups important for cellulose degradation in the rumen (Ransom-Jones *et al.*, 2012). Four selected isolates fell into the phylum Fibrobacteres, as shown in Table 6-10. The partial 16S rRNA gene sequences indicated diversity among the Fibrobacteres isolates, but the short sequences may have under-represented their true diversity. Isolates LB2010 and LC4001 were placed in the same cluster in the analysis of the V1-V3 region of their 16S rRNA gene sequences, but fell into separate clusters when the full-length 16S rRNA gene sequences were analysed, although they are still related to each other at the 97% sequence similarity level.

The phylum Fibrobacteres contains only one named genus, *Fibrobacter*, with isolates (Ludwig *et al.*, 2010b). This makes it difficult to determine a suitable similarity cut-off value for defining a genus. If the guidelines used for Firmicutes and Bacteroidetes (93% for a GLC) were applied to this phylum, the two species of the genus, *F. succinogenes* and *F. intestinalis*, should be classified into two separate genera, and several new species should be described. The sequences from the selected isolates all fall into the species *F. succinogenes* as it is currently defined (within 95% similarity). Previous studies have proposed the presence of sub-species within *F. succinogenes*. Four stable sub-species groups were first described by Amann *et al.* (1992) and later confirmed by Shinkai *et al.* (2007b). Analysing clone sequence information together with isolate sequence information indicated the possibility of further divisions amongst the subspecies groups, splitting groups 1 and 2 into four and two different subgroups, respectively (Kobayashi *et al.*, 2008). In this study, three of the four previously described sub-species groups from Amann *et al.* (1992) contained a selected isolate (groups 2, 3 and 4). If a 97% similarity cut-off was applied, there would be five species within what is currently *F. succinogenes* (Figure 6-17), and the subspecies would be elevated to species. Isolate LB2010 formed a new group (group 5)

previously represented by only cloned sequences. This group is closest to sub-species group 2 as defined by (Amann *et al.*, 1992). Lin *et al* (1994) suggested that *F. succinogenes* sub-species groups 1 and 2 are the most abundant in rumen samples, while Koike *et al.*(2004) found groups 1 or 3 dominant, depending on diet. Although DNA-DNA hybridisation values and comparative rRNA sequencing are sufficient to elevate sub-species within *F. succinogenes* to genus rank, the phenotypic evidence currently does not support this (Amann *et al.*, 1992; Lin *et al.*, 1995). Based on the 16S rRNA gene sequence similarity, in this study these sub-species are considered to be separate species.

Table 6-9. Identity of isolates within Spirochaetes.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
ND3003	Spirochaetales cluster 1	1	<i>Treponema</i> / sp. 102	Yes
WCE3006	Spirochaetales cluster 2	1	<i>Treponema</i> / sp. 103	No
XBD1002	Spirochaetales cluster 3	1	<i>Treponema</i> / <i>Treponema bryantii</i>	Yes
DAZ1007	Spirochaetales cluster 4	2	<i>Treponema</i> / <i>Treponema bryantii</i>	Yes
MD2012	Spirochaetales cluster 4	(2)	<i>Treponema</i> / <i>Treponema bryantii</i>	Yes

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in the cluster are shown. Where more than one sequence from a cluster is shown the number is repeated in brackets.

Table 6-10. Identity of isolates within Fibrobacteres.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
NC3008	Fibrobacter cluster 1	1	<i>Fibrobacter succinogenes</i> subsp. <i>elongates</i> Group 3	Yes
LB2010	Fibrobacter cluster 7	1	<i>Fibrobacter succinogenes</i> Group 5 sp. 97	No
LC4001	Fibrobacter cluster 7	1	<i>Fibrobacter succinogenes</i> Group 2 sp. 98	Yes
NE2017	Fibrobacter cluster 8	2	<i>Fibrobacter succinogenes</i> Group 4 sp. 99	Yes

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in the cluster are shown. Where more than one sequence from a cluster is shown the number is repeated in brackets.

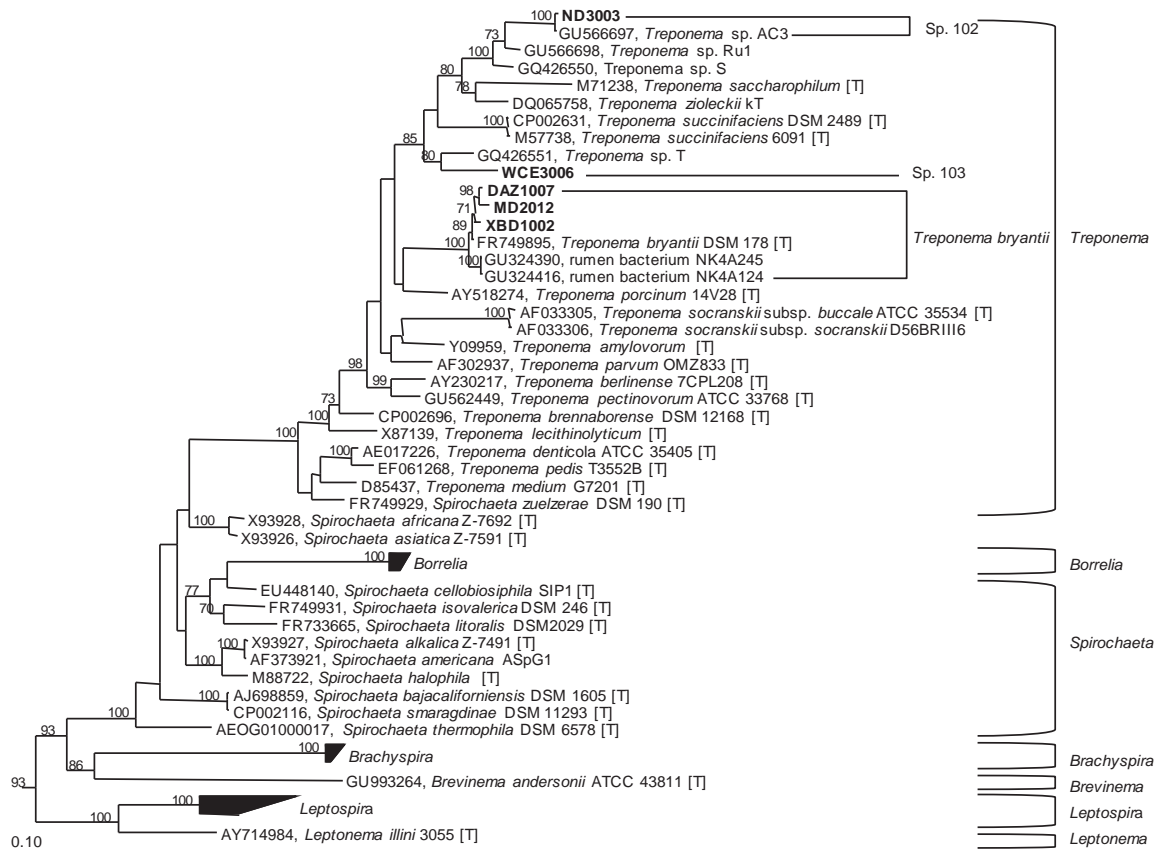


Figure 6-16. Expanded view of Spirochaetes. Details are described in the legend to Figure 6-4.

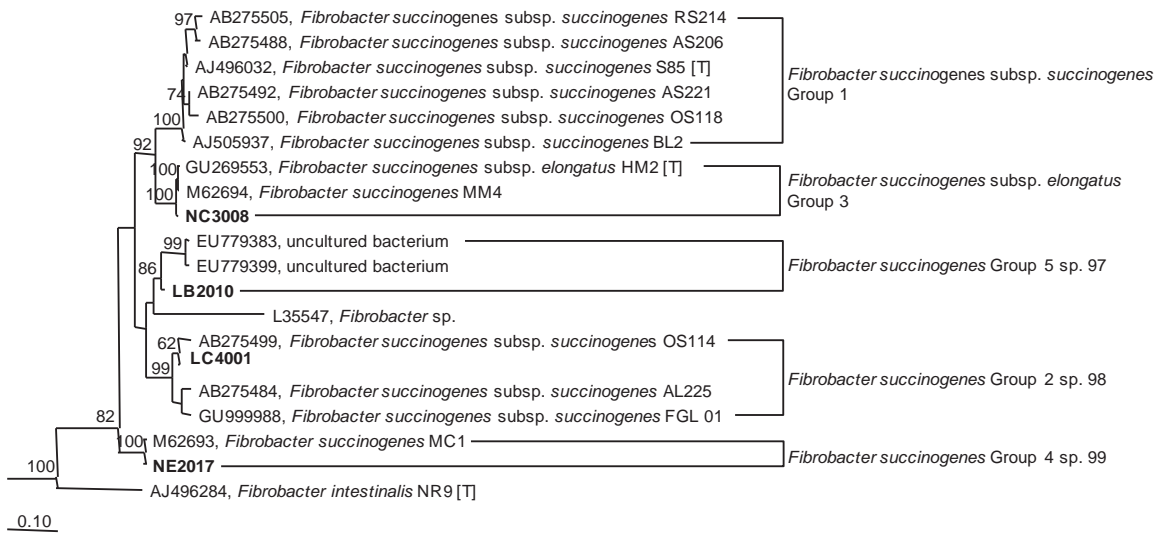


Figure 6-17. Expanded view of Fibrobacteres. Details are described in the legend to Figure 6-4.

6.2.9 Actinobacteria

Five of the selected isolates were members of the phylum Actinobacteria. The identities of the isolates are summarised in Table 6-11 and their placement in a phylogenetic tree is shown in Figure 6-18. Two isolates grouped with *Olsenella* and *Atopobium* in the family Coriobacteriaceae and three isolates with *Propionibacterium* in the family Propionibacteriaceae. Both of these groups, as they are currently named, appeared to be bigeneric when a 93% sequence similarity cutoff is used to define sequences that are likely to belong to separate genera. Genus-level groups are suggested in Figure 6-18, but require phenotypic evidence to verify their existence.

6.2.9.1 Coriobacteriaceae

A phylogenetic analysis based on full 16S rRNA gene sequences revealed that isolates XBB2002 and WCE2013 formed a genetically-coherent group within the bigeneric *Olsenella*–*Atopobium* branch of the family Coriobacteriaceae. XBB2002 was most closely related to the type strains of the recognised *Olsenella* species, *Olsenella uli* (sequence similarity of 96.7%), *Olsenella umbonata* (sequence similarity of 98.2%) and *Olsenella profusa* (sequence similarity of 97.4%). The sequence similarity to the type strain of *Atopobium minutum*, the type species of the genus *Atopobium*, was 92.2%. The 16S rRNA gene sequence of isolate XBB2002 was closest that of *O. umbonata*, whose previously described members shared 100% intraspecies 16S rRNA gene sequence similarity (Kraatz *et al.*, 2011). *O. umbonata* shared 97.2% 16S rRNA gene sequence similarity with its closest named relative, the type strain of *O. profusa*, yet had less than 50% DNA-DNA relatedness, and this was used to delineate it as a new species (Kraatz *et al.*, 2011). Given the closely-related nature of the validly-named strains of *O. umbonata* and its phylogenetic position on the ML tree, XBB2002 probably represents a new species.

Isolate WCE2013 formed a group with clone sequences intermediate to *O. uli* (sequence similarity of 95.1%) and *A. minutum* (sequence similarity of 91.6%). It could therefore belong to either genus or possibly represent a new genus. *Olsenella* is not

distinguishable from *Atopobium* by 16S rRNA gene sequence analysis and the main difference distinguishing these genera is a marked difference in G+C content of genomic DNA (63-64% *Olsenella* and 35-46% *Atopobium*; (Dewhirst *et al.*, 2001). The G+C content of the genomic DNA was not determined for the isolates in this study, so further analysis is needed to correctly place WCE2013 in a genus.

6.2.9.2 Propionibacteriaceae

The 16S RNA gene sequences of selected isolates MB3001 and MB3007 had 100% sequence similarity with the sequence of the type strain of *Propionibacterium acnes*. Strains of *P. acnes* formed a tight phylogenetic group with high similarity scores (99.8%-100%). The genomes of many strains of *P. acnes* have been sequenced and, although not distinguishable by 16S rRNA gene sequence analysis, were not identical (Kilian *et al.*, 2012). Similarly, isolate V9D4008 formed a group with the type strain of *Propionibacterium granulosum* (sequence similarity of 99.6%). The two *P. acnes* isolates and the *P. granulosum* isolate were only 92.2% to 92.3% similar to the type strain of the type species of the genus, *P. freudenreichii*. In this analysis, all strains belonging to *P. acnes* and *P. granulosum* formed a coherent cluster at the 93% similarity level (GLC 59), and *P. freudenreichii*, *P. acidifaciens*, *P. australiense*, and *P. cyclohexanicum* formed another cluster, sharing an average 91.95% similarity with the first cluster. This difference has previously been noted by Charfreitag & Stackebrandt (1989), who reported that that *P. acnes* and *P. freudenreichii* were only remotely related to each other (93% similarity).

Isolate MB3001 was originally identified as a *Prevotella* sp., but the first attempt to sequence its near full-length 16S rRNA gene revealed that this was a mixed culture. The culture was subsequently revived and purified by picking a single colony from a plate. The resulting pure isolate was different to the one that provided the original sequence. It is possible that the *Propionibacterium* sp. was more readily revived from long term storage or grew better on the solid medium.

P. acnes and *P. granulosum* are not commonly associated with the rumen but are associated with human skin and the human gastrointestinal tract and have been previously noted as common contaminants of anaerobic cultures (Johnson *et al.*, 1972).

However, *P. acnes* has previously been isolated from the rumen of a healthy cow (Wu *et al.*, 2009) and *Propionibacterium* spp. were found in high numbers associated with the mucosa of the gastrointestinal tract in calves and lambs (7.2×10^5 - 4.0×10^6 colony-forming units per cm^2 ; (Collado *et al.*, 2007).

Table 6-11. Identity of isolates within Actinobacteria.

Isolate name	Original cluster designation ^a	Total no. of isolates in cluster ^b	Genus level cluster (GLC) / species level cluster (sp.)	Species level cluster includes a previously cultured bacterium
MB3001	Prevotella cluster 37	1	GLC 59 / <i>Propionibacterium acnes</i>	Yes
MB3007	Propionibacterium cluster 1	9	GLC 59 / <i>Propionibacterium acnes</i>	Yes
V9D4008	Propionibacterium cluster 2	1	GLC 59 / <i>Propionibacterium granulatum</i>	Yes
XBB2002	Coriobacteriaceae cluster 3	1	<i>Olsenella/Atopobium</i> / sp. 100	No
WCE2013	Coriobacteriaceae cluster 2	1	<i>Olsenella/Atopobium</i> / sp. 101	No

^a Original cluster designation from NJ tree constructed with partial 16S rRNA gene sequences of the 828 isolates and selected other sequences as described in section 4.2.14.

^b The number of sequences in the cluster are shown.

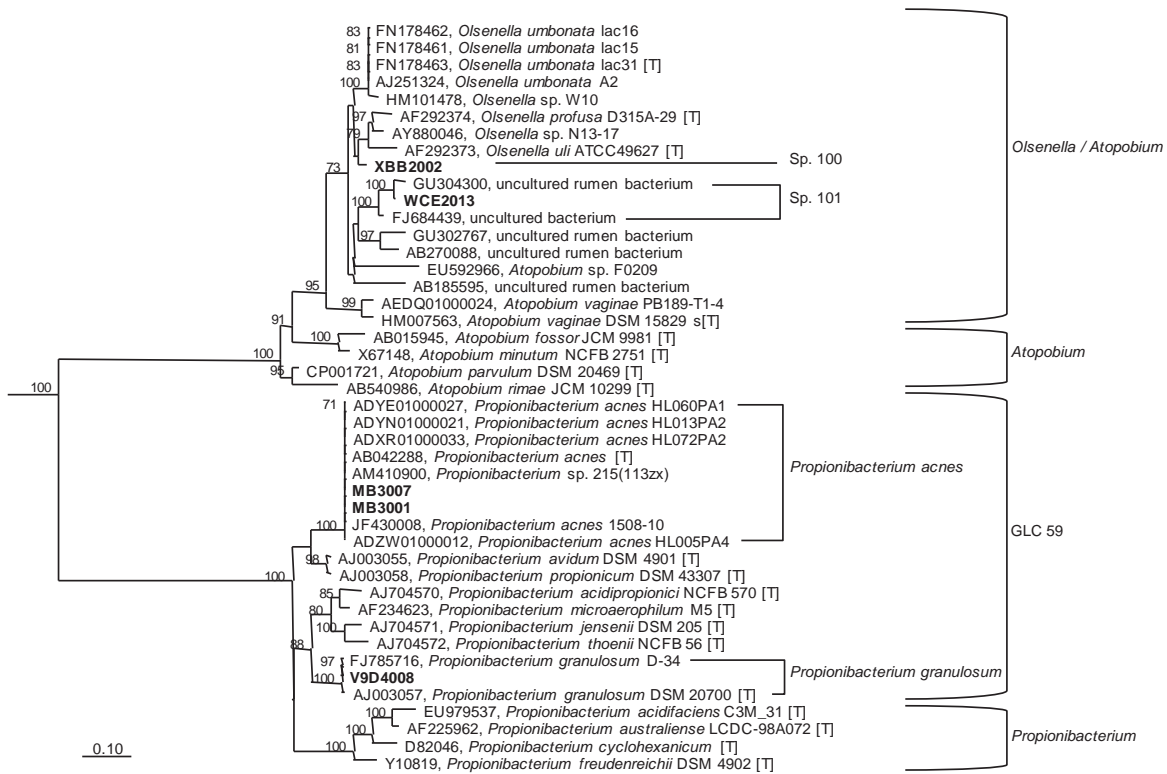


Figure 6-18. Expanded view of Actinobacteria. Details are described in the legend to Figure 6-4.

6.3 Discussion of tree-based comparisons

The number of species-level groups or OTUs predicted from the 828 partial 16S rRNA gene sequences differed depending on the calculation method used (Figure 6-19). Using partial 16S rRNA gene sequences (of the V1-V3 region) and OTU clustering at 96% sequence similarity resulted in a greater number of potential species compared to the tree-based estimation that mainly used 96% sequence similarity as a species boundary. The number of OTUs containing an isolate was also affected by what other sequences were present in the analysis. OTU clustering of the isolate sequences in QIIME at 96% similarity gave between 204 and 232 OTUs. When only the isolates sequences were clustered, this resulted in the lowest estimate of 204 OTUs and when the isolate sequences were analysed together with the pyrosequences (without the removal of chimeric sequences and noise OTUs see section 4.3.12) this resulted in the highest estimate of 232 OTUs that contained isolates. In comparison, only 171 species-level clusters were estimated using a tree-based method on the partial (V1-V3) 16S rRNA gene sequences (with a 96% similarity boundary for species) and 122 species-level clusters with the near full-length 16S rRNA gene sequences (mainly using 97% as a cutoff). These results indicated that species estimates based on partial 16S rRNA gene sequences using clustering algorithms could overestimate the number of species by 1.7-1.9 fold. Care must be taken when interpreting OTUs in pyrosequence studies using partial 16S rRNA gene sequence reads.

Clustering into OTUs may have overestimated the actual number of species, but it did not combine more than one species into a cluster, because when the full-length sequences from two or more isolates from the same original cluster were analysed they nearly always ended up being the same species (the only exception was still >97% similar to its cluster partner but in a neighbouring species-level cluster based on branching pattern in the phylogenetic tree). This also validates the choice for using 96% sequence identity for clustering the partial 16S rRNA gene sequences. Even with the relaxed identity threshold (some recommend 97%), different species were not placed into single OTUs. This means that the isolates without full-length 16S rRNA gene sequences can be placed with some confidence on the basis of being in the same initial clusters as their representative selected isolates for which near-full-length sequence data were obtained.

Of the initial 1037 isolates cultured, 828 isolates were able to be identified using partial (V1-V3) 16S rRNA gene sequence comparisons. The near full-length sequences from 186 selected isolates (representing 801 of the total sequenced isolates) were classified into 14 families including two potentially new families, 77 genera including 59 potentially new genera and 122 species including 103 potentially new species. It should be noted that six of the 59 potentially new genera contained a validly named species but are considered here to be new genera based on their lack of similarity of their 16S rRNA gene sequence to the type species of the genus. The classifications were based upon 16S rRNA gene sequence similarity and still need to be confirmed by phenotypic characterisation. The sequence-based analyses offer a good indication of the diversity captured by cultivation. A summary of the number of species-level groups obtained is shown in Figure 6-19. The isolation techniques used in this study resulted in close to 60% (73) of the species-level groups being novel (<97% sequence similarity to a previously cultured bacterium). This demonstrates clearly that new species, genera and even families of bacteria can be captured using a cultivation approach.

Isolates of 19 known species were also cultured: *Butyrivibrio hungatei*, *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*, *Bacillus circulans*, *Eubacterium cellulosolvens*, *Fibrobacter succinogenes*, *Kandleria vitulina*, *Lachnobacterium bovis*, *Lachnospira multipara*, *Prevotella albensis*, *Prevotella bryantii*, *Propionibacterium granulosum*, *Propionibacterium acnes*, *Pseudobutyrvibrio ruminis*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Selenomonas ruminantium*, *Treponema bryantii* and a *Streptococcus sp.* (either *infantaris*, *lutetiensis*, *luteciae*, *equinus* or *gallolyticus*). All of these with perhaps the exception of the *Propionibacterium* and *B. circulans* are recognised rumen bacteria. This demonstrates that the methods used also cultured a wide range of known species and were not overly selective.

Table 6-12 and Figure 6-20 show the proportion of species-like clusters and isolates sequences in species, genus and family-level clusters. Although there was a large number of a novel species-level clusters (84.4% of species-level clusters were not validly named), these translated to smaller numbers of actual sequences (56.2% of sequences were in a not validly named species-level cluster). This was because the novel species-level clusters tended to contain only one or a few sequences. There were 19 known species in the 122 species-level clusters found, but these represented 43.8%

of the isolates. This is not unexpected, given that about a quarter of the isolates were members of *Butyrivibrio*. It might be expected that because these species were readily isolated in this experiment, that they have already been isolated and described by earlier investigators.

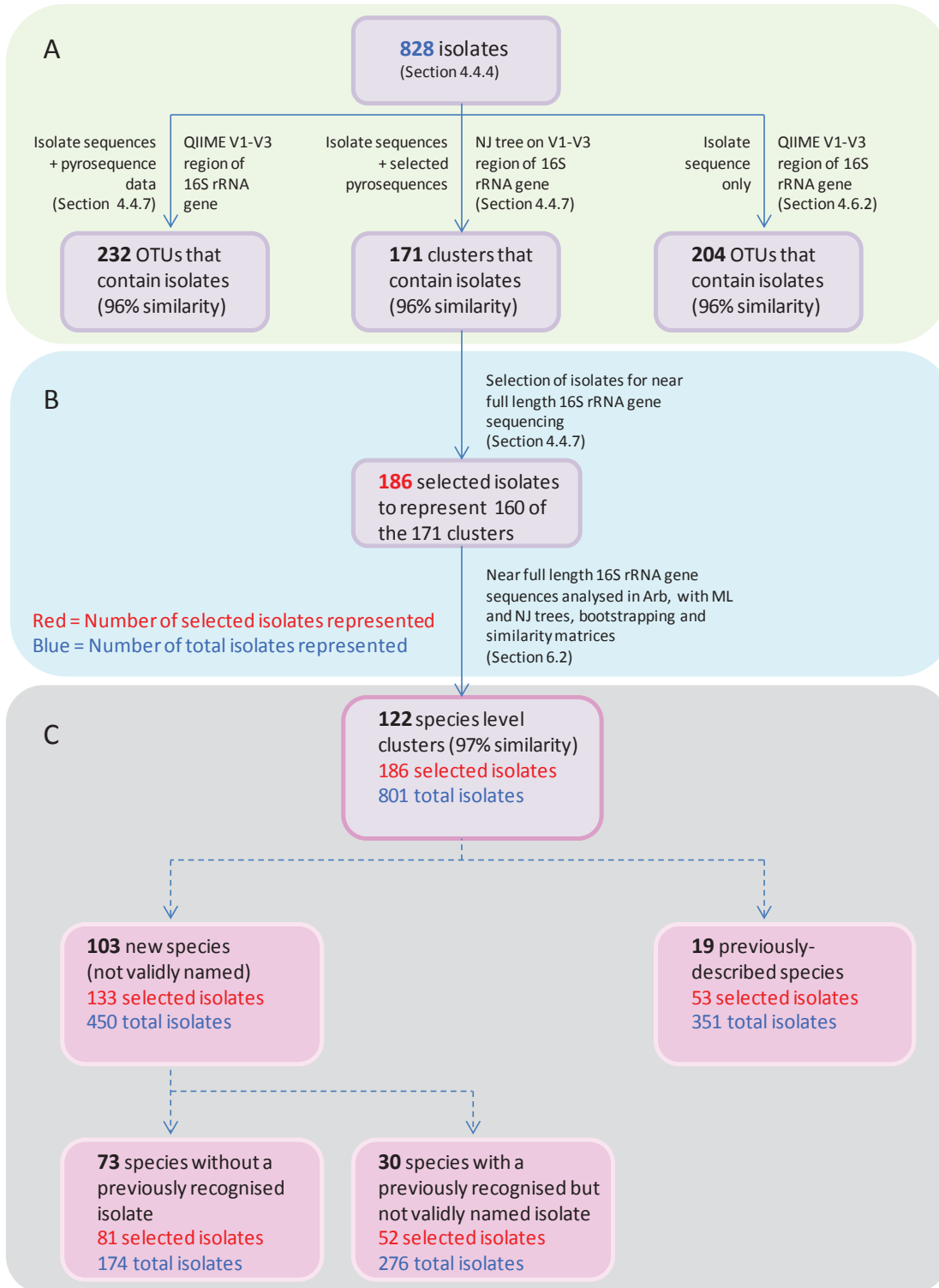


Figure 6-19. Summary of isolate identification.

(A) Isolates were grouped into OTUs using three different methods, based on the partial 16S rRNA gene sequences (V1-V3 regions) from 828 isolates. (B) Representatives of these OTUs were selected for near full-length 16S rRNA gene sequencing. (C) Phylogenetic treeing and sequence similarity comparisons were used to determine the number of species-level clusters that the isolate collection represented and how many of these were novel.

Table 6-12. Number of uncharacterised species-level clusters and isolates at different taxonomic levels.

Novelty of taxa	Family-level cluster		Genus-level cluster		Species-level cluster	
	No. of families	No. of species	No. of genera	No. of species	No. of species	No. of isolates
New	0	0	35	56	73	174
Contains a previous isolate that is not validly named	2	4	24	23	30	276
Contains a validly named isolate	12	118	18	43	19	351

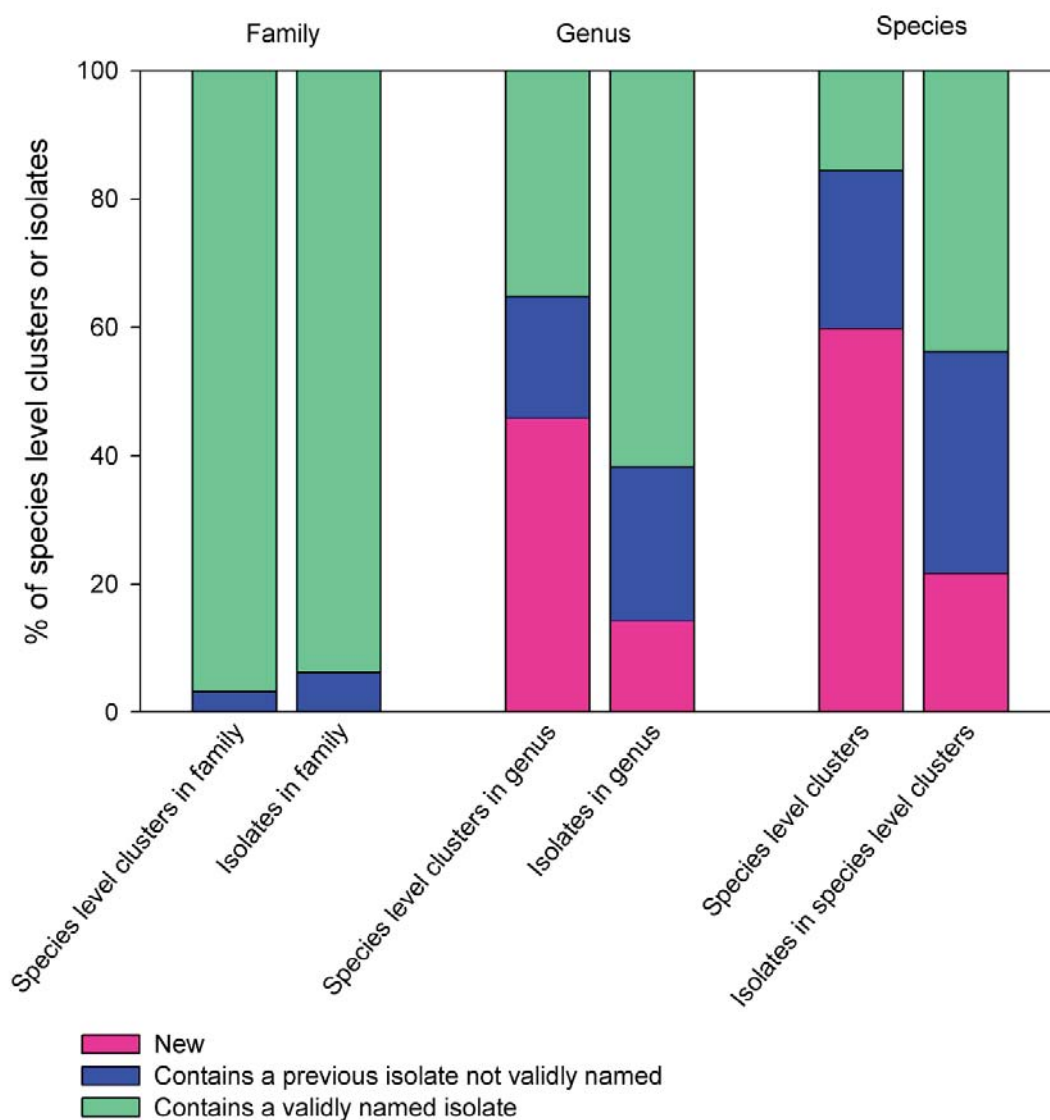


Figure 6-20. Proportions of uncharacterised family-, genus-, and species-level clusters and isolates at different taxonomic levels. Family-, genus-, and species-level clusters were defined by a combination of 16S rRNA gene sequence similarity groupings and two different tree methods.

6.4 Comparison of isolate sequences and pyrosequences by OTU clustering

A feature of this study is that the pyrosequence data were generated from the same samples that the isolates were cultured from. Therefore, this is a true comparison of what was able to be cultured with the composition of the source plant-adherent rumen community. To estimate the success of the isolation, measured by how many species (or proxy species groups) of the plant-adherent rumen community were cultured, both isolate and pyrosequence datasets were combined and analysed by OTU clustering in QIIME. First, chimeric and noise OTUs were removed from the pyrosequence data, so these could be compared with the isolate sequences which were obtained with Sanger sequencing and had been manually checked for errors. Errors in pyrosequence data are known to artificially inflate species estimates (Huse *et al.*, 2010; Kunin *et al.*, 2010a; Quince *et al.*, 2011), but require automated deletion rather than manual curation. The number of OTUs generated from the pyrosequences before and after these cleanup steps are shown in Table 6-13. Filtering for good quality sequences, which involved the removal of any sequences with an ambiguous base call, removing sequences outside the specified size range, and removal of those that had mismatches in either the primer or barcode as well as those with low average quality (<Q27), still resulted in 76487 OTUs after clustering at 96% similarity. This number of OTUs is an order a magnitude greater than recently published estimates of rumen bacterial species numbers of 1000 – 5271 species-level OTUs (Fouts *et al.*, 2012; Hess *et al.*, 2011; Kim *et al.*, 2011c). Applying a quality filter, which used a sliding window of 50 bases, truncating the sequence at the point it dropped below the quality cut-off value of 27, and removing the resulting sequences that then failed the minimum length requirements, improved the quality of the remaining sequences but still resulted in an unrealistic number of OTUs (42633). The CD-HIT-OTU pipeline employs a *de novo* chimera checker and calculates and removes noise OTUs (Li *et al.*, 2012b). This process removed around 2/3rds (66.2%) of the sequences but did not markedly change the overall distribution of sequences assigned to different taxa (Figure 6-21). It did dramatically reduce the number of OTUs, from 42633 to 1539. The resulting cleaned pyrosequence dataset was used in the final analyses. The last step of the CD-HIT-OTU pipeline was to cluster the cleaned sequences at 96% similarity using the CD-HIT algorithm. This resulted in 1539 OTUs.

When the same cleaned pyrosequence dataset was clustered at 96% similarity with the UCLUST algorithm in QIIME, this resulted in 2342 OTUs, demonstrating the clustering method had an impact on the number of OTUs. UCLUST and CD-HIT both use a greedy algorithm to cluster sequences, which identifies a representative sequence for each cluster and recruits new sequences to that cluster if they are sufficiently similar to the representative. Sequences below the similarity threshold form the seed for a new cluster. However, there is no evidence to suggest which method is better, so the choice of algorithm is up to the individual researcher.

The UCLUST algorithm was chosen to analyse the combined pyrosequence and isolate sequence dataset so that this analysis would be consistent with the analysis done in QIIME on the isolate sequences alone. Also, this algorithm can perform well computationally with very large datasets (Edgar, 2010). Additionally, the CD-HIT-OTU pipeline was considered unsuitable to use on the combined pyrosequence and isolate sequence dataset because many (66.4%) of the isolate sequences were regarded as noise and discarded when analysed using this pipeline (not shown). One of the assumptions of CD-HIT-OTU is that massively parallel sequencing has such sequencing depth that it should sequence an actual community member more than once. Therefore OTUs represented by only one sequence, like many of the isolate sequences, are rejected as noise. Therefore, the isolate sequence dataset was combined with the cleaned pyrosequence dataset and clustered with UCLUST. The actual number of OTUs generated from clustering analysis should not be thought of as species but as artificial groups used to examine the data. The number of OTUs will have a big influence on diversity estimates but much less influence on the overall picture of the taxa identified (shown in Figure 6-21), e.g., there are still *Butyrivibrio* whether they are in 50 OTUs or five OTUs.

The OTU clustering analysis was performed on two combinations of datasets (Figure 6-22). The first analysis was of the isolate sequences with the pyrosequences. The second analysis was of the isolate sequences, the pyrosequences, and the sequences from all previously isolated bacteria from all environments deposited in RDP as of February 2013 (345848 good quality >1200 bp sequences). The number of OTUs containing at least one isolate sequence was similar for both analyses (222 and 224 OTUs respectively). Around 94% of the isolate sequences were found in an OTU also

containing at least one pyrosequence (Figure 6-22). There are many possible reasons that the remaining 6% of isolates did not group into an OTU with a pyrosequence. These isolates might be easy to culture but rare community members not detected by pyrosequencing or they could be biased against in the pyrosequencing PCR. If they are a sequence type that is prone to sequencing errors using either Sanger or 454 sequencing technologies, they may end up being sufficiently different and therefore not recruited into a common OTU using the UCLUST algorithm. There is also the possibility they are not genuine members of the plant-adherent rumen community and were contaminants from outside the rumen. The *Propionibacterium* and *Bacillus* isolates may not be genuine plant-adherent rumen community residents. However, the majority of the isolate sequences that didn't cluster into an OTU with a pyrosequence appeared to be valid rumen isolates, and were identified as members of *Pseudobutyrvibrio*, *Butyrvibrio*, *Selenomonas*, *Olsenella* and *Treponema*. Most of the isolate sequences that did not cluster with a pyrosequence (36 OTUs out of 40) in this analysis did cluster with another isolate sequence using the phylogenetic tree based method, which in turn did cluster with a pyrosequence. An example is 86 isolates classified as *Pseudobutyrvibrio*, which appear to be just one species with the phylogenetic tree based analysis (Figure 6-6), but were clustered into 33 OTUs in this analysis, 13 of which did not contain a pyrosequence.

Close to 32% of the pyrosequences were found in an OTU that contained an isolate sequence, indicating that 32% of the sequenced community was cultured at the OTU level. These OTUs represented 7.7% of the OTUs containing a pyrosequence, indicating 7.7% of the sequenced diversity was cultured. Looking at the cultivation success in more detail, around 50% of the largest OTUs (containing 50% of all the pyrosequences in just 105 OTUs) contained an isolate sequence (Figure 6-23). Considering 828 isolate sequences were examined and there are potentially >1000 species in the rumen, this is a good success rate. It is difficult to draw comparison of the cultivation success rate with other studies as no rumen study has compared a large number of isolate sequences with pyrosequences from the same community before. However, the pyrosequence meta analysis by Kim *et al.* (2011c) found 6.5% of the pyrosequences from multiple rumen studies were $\geq 97\%$ a known bacterial sequence.

Here ~32% of the pyrosequences were found in an OTU (96% similarity) with an isolate sequence gained in this study.

The cumulative sequence curve (Figure 6-23) showed an uneven distribution of sequences. Most of the pyrosequences fell into relatively few large OTUs, leaving most of the OTUs with relatively fewer sequences. This pattern is consistent with findings from other pyrosequence surveys of the rumen environment (Fodor *et al.*, 2012; Huse *et al.*, 2008).

To assess how novel the isolates gained in this study were and to estimate how many plant-adherent rumen bacteria have been previously isolated, the isolate sequences, pyrosequences and downloaded RDP dataset of sequences from previously-cultured bacteria from all environments were combined and clustered into OTUs. 55.1% of the isolate sequences cluster into an OTU with a sequence of a previously isolated bacterium, meaning 44.9% of the isolate sequences are novel, i.e., not found in an OTU containing a previously isolated bacterium. A total of 103 OTUs represented previously cultured rumen bacteria (i.e., contain a previously isolated bacterial sequence and a rumen pyrosequence or a rumen isolate sequence) compared to 169 OTUs that represent new isolates gained in this study. This indicates more than 1.6 times the number of OTUs containing a bacterial sequence from all previous culturing efforts were gained in this study. These new isolates will be an important resource for the ongoing efforts to understand processes and community structure in the rumen.

There still remains a large portion of the plant-adherent rumen community without a representative isolate as indicated by the 2144 OTUs that only contain pyrosequences. Interestingly 16.2% of the pyrosequences fall in an OTU with a previously isolated bacterial sequence. This is high compared to the meta-analysis of Kim *et al* (2011c) from several studies on whole rumen contents, in which 6.5% of the pyrosequences are $\geq 97\%$ similar to a known bacterial sequence. These results demonstrate we are still in the early stages of cataloguing rumen bacterial diversity and potentially much more can be gained by further cultivation.

The average number of pyrosequences per OTU was calculated for the pyrosequence containing OTUs within the sections shown in the Venn diagram (Figure 6-22). The OTUs that contained pyrosequence, a new isolate sequence and a previously cultured isolate sequence had the greatest number of pyrosequences per OTU (0.26% of

the total pyrosequences per OTU). It seems that these OTUs are of abundant bacteria that have been cultured before and were isolated again in this study. The OTUs that contained at least one new isolate sequence and pyrosequence had the next largest number of pyrosequences per OTU (0.142% of the total pyrosequences per OTU). This also indicates the numerically dominant plant-adherent rumen community members were cultured. The OTUs that contained a previously isolated bacterial sequence and a pyrosequence but not an isolate sequence from this study had on average fewer pyrosequences per OTU (0.064% of the total pyrosequences per OTU). These OTUs may have been more prevalent in the rumen communities from which they were they were previously isolated. This also offers an explanation to why these have not been isolated again in this study, as they are comparatively rare in the plant-adherent fraction examined in this study. The bacteria that have evaded culturing efforts so far represented by OTUs containing only pyrosequences are on average the rarest members of the plant-adherent rumen community (0.03% of the total pyrosequences per OTU). These sequences may be from culturable bacteria but they are statistically unlikely to be isolated with even a moderate number of cultures. This illustrates the need for even larger scale isolation in order to capture the full diversity present in the rumen.

The data presented here suggests that simple isolation techniques like those used in this study are able to capture much more of the diversity than originally thought. The numerically dominant bacteria present in the plant-adherent fraction from the rumen contents of five New Zealand dairy cows have been cultured. There are still a large number of less prevalent bacteria remaining to be cultured. It should be possible to culture many of the remaining uncultured bacteria if enough attempts are made to make it statistically probable.

Table 6-13. The effect cleanup steps of pyrosequences have on the number of OTUs.

Clustering method	Sequence cleanup steps ^a	OTU similarity cut-off value	Number of input sequences	Number of OTUs	% of OTUs containing ≤ 10 sequences
UCLUST ^b	Average quality score > 25	96%	458785	76776	94.16
UCLUST	Average quality score > 27	96%	457910	76487	94.16
UCLUST	Sliding window quality score > 27	96%	327300	42633	91.13
CD-Hit-OTU ^c	Sliding window quality score > 27	96%	327300 (110585 used for clustering after sequence cleanup)	1539	36.52
	CD-HIT-OTU removes chimeric sequences and OTUs (100% similarity) with <3 sequences				
UCLUST	Sliding window quality score > 27	96%	110585	2342	44.75
	Chimeric sequences and OTUs (100% similarity) with <3 sequences removed				

^a All sequences that contained an ambiguous base call or a mismatch in either the primer or barcode were removed. Sequences that were longer than 600 bases or shorter than 400 bases were excluded prior to any additional cleanup steps.

^b The UCLUST algorithm was implemented in QIIME.

^c The CD-HIT-OTU pipeline clusters the sequences with the CD-HIT algorithm after the chimeric and noise sequence removal steps.

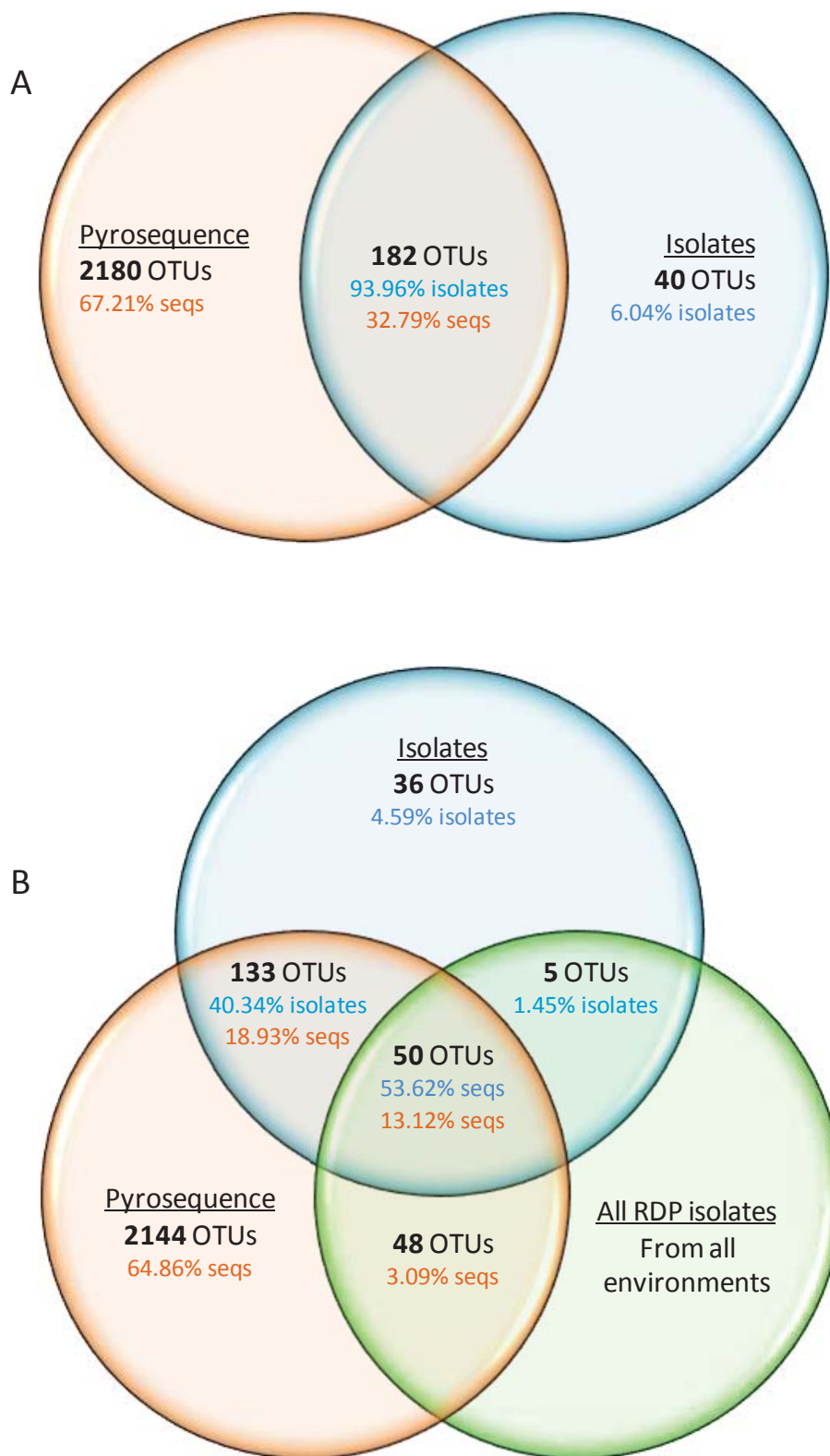


Figure 6-22. Shared OTUs between (A) isolate sequences and pyrosequences from plant-adherent rumen bacteria and (B) isolate sequences, pyrosequences from plant-adherent rumen bacteria all near full-length isolate sequences of good quality from RDP. Sequences were clustered at 96% similarity using UCLUST in QIIME, separately for each analysis.

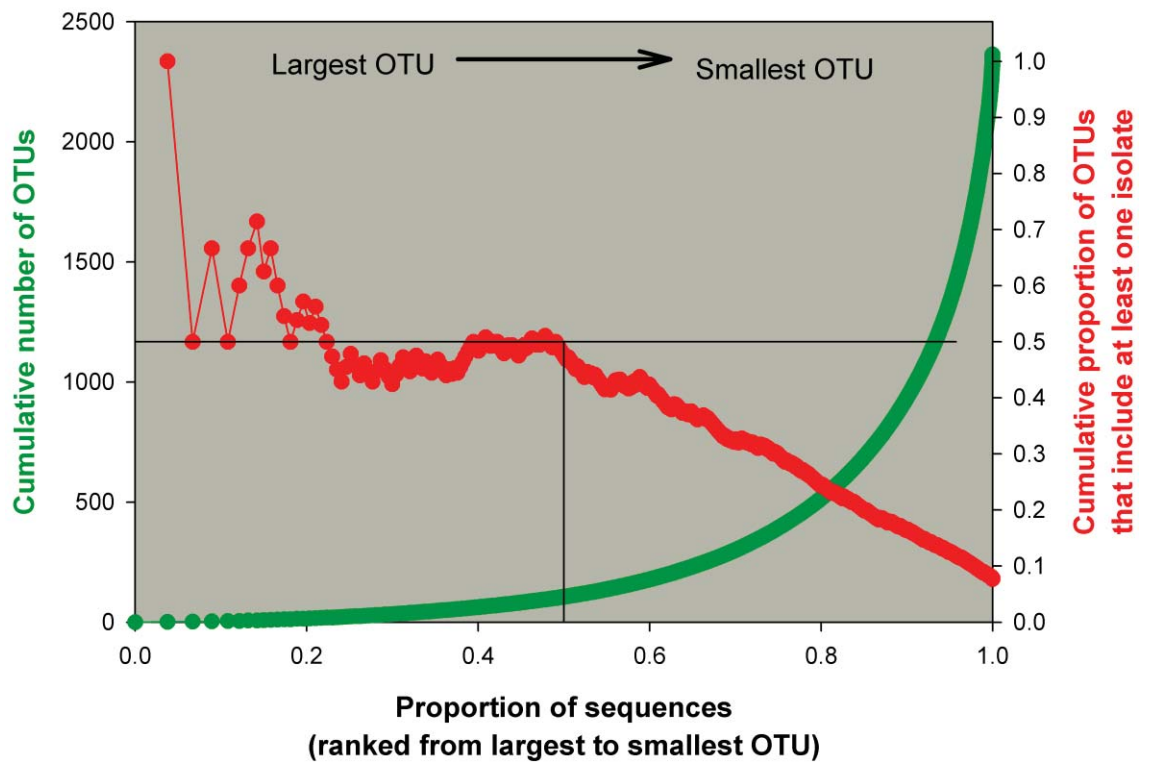


Figure 6-23. Cultivation success. The OTUs were ranked from largest to smallest. The red line indicates the cumulative success of cultivation shown as the proportion of OTUs that contain at least one isolate. The green line indicates the cumulative number of OTUs showing that 50% of the amplicon sequences fell into only 105 OTUs.

Chapter 7 Summary, conclusions and future directions

7.1 Rationale

Ruminants have a symbiotic relationship with the complex community of microbes that resides within their rumen. The ruminants themselves do not produce enzymes capable of degrading plant fibre, which typically makes up 55-70% of ingested pasture (Waghorn *et al.*, 2007). Rumen microbes can digest around 70-80% of the fibre associated with consumed forage (Kusmartono *et al.*, 1997). The short-chain fatty acids, such as acetic, propionic, and butyric acids, that the microbes produce as fermentation end products, together with the microbes themselves, provide 70-85% of the ruminants absorbed nutrients (Bergman, 1990; Waghorn *et al.*, 2007). The type and quantity of fermentation products produced greatly impact on the growth and development of the ruminant and hence its productivity. A greater understanding of the processes and microbes involved in fibre degradation could allow for improvements in animal productivity.

Of the rumen microbes, bacteria are thought to be the main group involved in plant fibre degradation. Bacteria from the rumen have been studied and cultivated for many years, yet only around 10% of bacterial species in the rumen are represented by isolates. One of the key objectives of the work presented in this thesis was to obtain representatives of some of these uncultured bacteria. These new isolates are now available for study to increase our understanding of the processes occurring in the rumen. Due to the complex community of rumen bacteria, comprised of many species, investigators are increasingly using “omics” approaches to investigate rumen communities. Information from such studies is hampered by the lack of reference organisms, again highlighting the need for more cultured representative rumen bacteria. The reference organisms serve as genome contexts within which gene and expression libraries can be understood, while their physiological characteristics and deduced activities can be linked to rumen processes.

7.2 Summary of results

The experiments presented in this thesis were designed to examine the community of plant-adherent bacteria in the rumens of grazing dairy cows, over the course of a year. Bacteria adhering to the digesta (feed material) in the rumen were cultured and also investigated using a culture-independent method based on pyrosequencing of 16S rRNA genes. Rumen contents from four cows that formed part of a working dairy herd were sampled five times over the course of a year. This design allowed for changes in the rumen bacterial community over time and for seasonally different pasture feed to be examined.

Digesta sampled from the rumen has three distinguishable bacterial communities: firmly attached to ingested plant material, loosely associated with the ingested plant material, and those that reside free within the fluid. Bacteria that adhere to the ingested plant material are primarily responsible for significant fibre degradation. Most of the bacteria (67% - 84%) were found to firmly adhere to the ingested plant material. A method was developed to separate the plant-adherent bacteria from planktonic and loosely adherent bacteria in rumen samples (Chapter 3). Rumen contents were washed and strained four times in anoxic buffer to remove all non-adherent and loosely-adherent cells. The washed digesta were then blended to release the attached bacteria from the plant material. DGGE analysis of DNA extracted from blended and unblended samples showed the blending treatment did not affect the observed bacterial community.

The composition of the plant-adherent bacterial community in these animals was determined by pyrosequencing PCR-amplified 16S rRNA genes from DNA extracted from rumen samples. This allowed a comparison of the molecularly-detected bacteria, which should reflect the digesta-adherent bacterial community, with the collection of isolates cultured from the same rumen samples. Pyrosequencing of the V1-V3 region of the 16S rRNA gene revealed that there was a high level of diversity, totalling 1539 OTUs and ranging 653-926 OTUs per sample. Overall, the detected bacterial communities were similar in different animals, sampling months and rumen fractions. However, the communities were statistically different between different sampling times, probably due to differences in the diet due to supplementation and seasonal effect on the pasture. The plant-adherent bacterial community was strongly dominated by members

of the phylum Firmicutes (79.5%) followed the phylum Bacteroidetes (12.1%) and minor contributions from 12 other phyla. The major genera found in this study were similar to those reported in other studies. Many of the pyrosequences could not be classified to a genus and instead could only be assigned to family or higher level groups, highlighting the need for more representatives of these unknown groups to be cultured and studied in order to improve the taxonomic databases.

The cultivation approach used a combination of dilution theory and a medium that mimics the physico-chemical conditions prevailing in the rumen to isolate plant-adherent bacteria. The methods were designed to maximise the number of rumen bacteria obtained. The aim was to have the majority of the cultures inoculated with a single viable cell. Dilution theory was used to calculate suitable dilutions of the plant-adherent rumen fraction to use as inocula, and these were inoculated into a newly described medium (RM02) that is representative of physico-chemical conditions prevailing in the rumen (Kenters *et al.*, 2011). In addition to this medium, a total of nine other media were included (each medium was only used for one sampling time) to increase the possibility of isolating diverse bacteria. Over 1000 anaerobic bacteria were isolated from the plant-adherent fraction of rumen content. There were 626 unique sequence types (V1-V3 of the 16S rRNA gene) among the 828 isolates that yielded good quality sequences, and 200 of these isolates were novel (<96% similarity to a previously cultured bacterium). This method was therefore able to isolate diverse and novel bacteria. In addition, bacteria known to reside in the rumen were also isolated. The isolates were members of the phyla Firmicutes, Bacteroidetes, Fibrobacteres, Actinobacteria and Spirochaetes. RM02 medium alone was sufficient to isolate most taxa of bacteria, but the additional media increased the number of novel bacteria isolated. The selection of nine other media produced larger MPNs compared to RM02, but a smaller proportion of these cultures were able to be maintained through to later analyses. The collection from 'other' media had a greater proportion of novel bacteria but a similar range of genera identified. A few genera were isolated only on the 'other' media. Using more than one type of isolation medium therefore increased the cultured diversity of the sampled community. A medium of 100% clarified rumen fluid supplemented with GenRFV mix was the overall best performing isolation medium. It yielded high MPN values, not many of the cultures grown on this medium were lost

after the initial culture, and a large proportion of the isolates gained on this medium were novel bacteria. Use of this medium also was able to isolate members of *Solobacterium* spp. and *Treponema* spp., genera that were not isolated on any other type of medium. However, this medium was very labour intensive to make and showed considerable batch-to-batch variability and hence would not be suitable for large scale cultivation efforts. RM02 plus one or two other media are likely to be able to successfully isolate most bacteria able to grow in pure culture.

An advantage of this study was the isolates were gained from the exact same samples that were used for the total community pyrosequencing analysis. This allowed a true comparison of what could be cultivated with the total detected community. Using an analysis which grouped 16S rRNA gene sequences into OTUs of similar sequences, about 32% of the pyrosequences were found in an OTU that also contained an isolate sequence. OTUs containing at least one isolate sequence and pyrosequence represented 7.7% of the total OTUs. This is a direct measure of the success of the isolation strategy, with 32% of the plant-adherent community (i.e., sequences related to an isolate) and 7.7% of the observed diversity (i.e., of total OTUs) being cultivated. Comparing the isolate sequences and pyrosequences gained in this study with the sequences from all bacteria isolated from all environments showed that the largest pyrosequence OTUs contained at least one isolate sequence from this study and at least one sequence from a previously-cultured bacterium. Altogether, sequences from previously-isolated bacteria were found in 103 OTUs. More than 1.6 times this number of OTUs (169) contained a sequence from an isolate cultured only in this study and no sequence from a previously-isolated bacterium. Therefore, this one study has more than doubled the number of rumen bacterial groups (as defined by OTUs) with isolates in culture. There is still a great deal of bacterial diversity yet to be cultured, but it seems likely that further large scale isolation attempts will continue to capture more of the uncultured rumen bacteria.

Phylogenetic trees of near full-length (>1300bp) 16S rRNA gene sequences were used to more accurately place the isolates. The isolate sequences were grouped into five phyla, 14 families, 77 genera and 122 species. Over half of the isolates (56%) were found to be members of 103 potentially new species. Potentially new taxonomic groups were discovered even at the family level, with two new family groups, and at the genus level with 59 new genera. Many of the new taxa (30 species, 24 genera and both

families) contained a previously-isolated but not validly named bacteria, stressing the need for these isolates to be characterised and named in order to improve the taxonomic framework. However, the majority of potential new species (73) and genera (35) were represented only by isolates cultured in this study.

7.3 Conclusions

It has been observed before in many environments that the physical counts of bacteria from environmental samples are usually (at least) an order of magnitude greater than the culturable counts (Rappé *et al.*, 2003; Staley *et al.*, 1985). There are many possible reasons why only around 10% of bacteria from the rumen are in culture. Some refer to these bacteria as unculturable but possibly more correctly they should be called yet to be cultured. While it is still possible some of these uncultured bacteria may be difficult to culture in artificial systems due to as yet unknown growth requirements, it now seems likely many could be cultured if enough isolation attempts are made. A great number of isolates would need to be obtained to increase the probability of isolating some of the rare species, even if cells of each species had an equal chance at growing in cultivation.

Past culturing efforts have resulted in around 100 rumen species being isolated and characterised. Only with the use of direct amplification and sequencing of 16S rRNA genes was the full diversity of the rumen revealed to be greatly under-represented by cultured bacteria (Edwards *et al.*, 2004; Kim *et al.*, 2011c; Kong *et al.*, 2010). Since this revelation, little has been done to correct the lack of cultured representatives. A handful of recent studies has reported the isolation of new rumen bacteria, but only a few have systematically attempted to culture the uncultured (Kenters *et al.*, 2011; Koike *et al.*, 2010). Here it was shown that many novel groups are not unculturable or even difficult to culture. For example, the novel family level group R-7 was represented by 44 isolates, just over 5% of all the isolates gained. This suggests that perhaps the reason why so many rumen bacteria remain uncultured is not because they are difficult to grow but because a large scale systematic culturing effort has not been made since the adoption of the 16S rRNA gene for identification. The method used to isolate plant-adherent bacteria in this study may have also been a contributing factor to obtaining novel isolates as well as the scale of the isolation. Dilution theory was used to calculate

the point where the inoculum is diluted until it is likely that a resulting culture would have arisen from a single cell, thus decreasing the bias towards bacteria that grow the most rapidly in the laboratory media. Without competition from faster growing bacteria, the bacteria that are less well suited to growing in laboratory conditions can be isolated. The isolates gained in this study can begin to bridge the gap between the cultured and the uncultured.

While the exact number of different species cultivated from the rumen is not known, more than 100 different validly-named species are known from the rumen, and there may even be >200 if uncharacterised species are included (Kong *et al.*, 2010). Here with 103 potential new species, this one study will greatly contribute to the number of rumen bacterial species in culture. Many of the new species that have been identified in this study contained an already existing but un-named, unclassified, or misclassified isolate. Thirty species-level groups contained at least one new isolate from this study and also at least one previously isolated bacterium. This highlights the need to characterise and validly name all rumen isolates that appear to be a new species. Because the ability to characterise the rumen community by the use of molecularly detected markers such as the 16S rRNA gene sequence relies on an accurate database of reference sequences to make comparisons with, these isolates need to be further examined in order to create a suitable framework in which to place rumen bacterial sequences.

This study has shown that ruminal bacterial diversity can be represented by simple cultivation approaches and previously uncultured bacteria can be isolated from the rumen, using appropriate cultivation techniques and large (many hundreds) numbers of isolates to get a better representation of the plant-adherent community.

7.4 Future perspectives

It would be interesting to use similar methods to the ones presented in this thesis to further examine the succession of bacteria that adhere to and break down plant fibre to further shed light on which bacteria are performing specific steps of feed degradation. Plant fibre is a complex mix of structures that no one bacterial species can degrade alone. When new plant feed is swallowed and introduced into the rumen, bacteria attach to it within minutes (Edwards *et al.*, 2007). Feed particles are reduced in size by the animal (rumination) and by the microbiota (fermentation). When particles have been reduced to a sufficiently small size, they are able to pass out of the rumen. At any one time, there is a mix of particles in the rumen from newly ingested large particles to those <0.1mm in size. Legay-Carmier & Bauchart (1989) measured the number of bacteria attached to feed particles of different sizes and found 72-81% of the attached bacteria were associated with particles <0.1 mm in size. It was noted that these bacteria appeared to be growing in a biofilm that made them particularly difficult to dislodge from the particles. Approximately the same concentrations of bacteria were attached to all fractions of particles over 0.1 mm in size (4, 2, 0.8, 0.4 and 0.1 mm). The small feed particles are likely to represent almost completely degraded plant matter that has already spent some time within the rumen. They could potentially have a different community composition than the larger particles. The washing procedure to obtain the adherent fraction in this study may have been enriching for the initial colonisers of plant material. Many of the small feed particles are able to pass through two layers of cheesecloth and would then form part of the liquid wash fraction. The remaining large particles that are retained with two layers of cheesecloth would be more representative of the feed that has been in the rumen for a shorter period of time and the attached bacteria, the initial colonisers of plant material.

Fractionation of the rumen digesta into various particle sizes would allow investigation to determine if they have different bacterial community compositions. Alternatively the examination of the type of bacteria that adhere to the fresh plant material over different periods of time, hence at different stage of fibre degradation, could reveal if there is a succession of bacterial species breaking down the plant fibre. Fresh chopped forage, representative of the animal's diet, could be contained within a bag with a pore size that would allow bacterial colonisation and incubated in the rumen

for different lengths of time. Pyrosequencing analysis of the different rumen fractions or feed incubated for different times could be used to examine the community compositions. If differences in the communities were observed, then an isolation strategy to culture the different members could be used. This would allow the study of the species that colonise at different times and help understand their roles within the rumen. Use of the cultivation approach used in this study combined with pyrosequencing would not only help to understand the dynamics of plant-adherent bacterial biofilm formation, but would also result in isolates representative of the different fractions and stages of degradation that could be studied in more detail.

This study focused on plant-adherent bacteria that may play important roles in fibre degradation, but other important groups may be missing from the culture collection because they are rumen fluid or associated with the rumen mucosa. Further large-scale culturing efforts from different starting inocula, i.e., different rumen fractions, different ruminant species, ruminants from different countries and farming systems, are likely to capture further rumen bacterial diversity in pure culture.

The new isolates were cultured from the plant-adherent fraction of rumen contents, but it remains to be proven that they are fibre degraders. Experiments to test the new isolates ability to adhere to and degrade plant fibre would help elucidate their role within the rumen. It is possible that not all bacteria that adhere to plant material directly degrade plant fibre. Some community members may exist by cross-feeding from the products of fibre degradation released by other community members. These species may still be playing an important role in fibre degradation by interacting with the fibre degrading species. Co-culture experiments could be used to determine if there are synergistic effects on fibre degradation.

Over a hundred potentially new species were isolated in this study and the logical extension of this discovery is to characterise and name the new species. This will help improve the taxonomic framework and provide isolates that can be used as references. Each new isolate to be characterised needs to be first confirmed as pure by streaking to a single colony on agar plates or by further dilution. A robust examination of the selected isolates' morphological, physiological, biochemical properties, growth requirements and end products produced needs to be made and compared with those of their closest relatives. To be validly named, the characterised isolates need to be

described to a suitable standard and published in the scientific literature, either in *International Journal of Systematic and Evolutionary Microbiology (IJSEM)* or elsewhere followed by inclusion in a list of validly described taxa in *IJSEM*.

It has been suggested that in the future full genome sequences will be needed to describe new taxa (Jones, 2012). To date, over 200 of the new isolates have been selected to have their genomes sequenced as part of the Hungate 1000 Project (www.hungate1000.org.nz). This initiative has the aim to sequence the genomes of 1000 rumen microbes. There are currently fewer than 30 bacteria from the rumen with complete genome sequence available as reference organisms compared to the human microbiome project where genome sequences are available for over 1500 reference organisms (NIH Human Microbiome Project, 2013). These reference genomes can be used to classify sequences gained from ‘omics’ studies. Additionally, the new isolates will now be available to test hypotheses formed from ‘omics’ based studies of rumen bacteria, by allowing verification of activities and properties inferred from bioinformatic analyses of rumen samples.

Appendix 1

Table A-1. Description and phylogenetic placement of isolates obtained in this study.

D – Description of cells from microscopic observation.

Q – QIIME. The taxonomy assigned within QIIME v1.4.0 using the naïve Bayesian classifier of RDP with curated Greengenes data files (gg_otus_4feb2011). The taxonomy was named to the family level or as far as classification was possible: k – domain, p – phylum, c – class, o – order and f – family.

B – The top BLASTN match to an isolate (name followed by accession number) and in the right hand column, its percentage identity match. The 16S rRNA genes sequence (V1-V3) of each isolate was compared using BLASTN to all of the bacterial isolates in RDP (265493 sequences downloaded 23/09/2011).

C – Cluster name. In the right hand column; yes = that isolate was selected to represent that cluster, blank cell = not selected to represent that cluster. Inferred phylogeny of 16S rRNA gene sequences from isolates were constructed using neighbour joining with p-distances in MEGA5. Clusters of 96% similarity were visualised and named.

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AB2001	D: Rods in chains and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.3%
AB2002	D: Small curved rods Q: k__Bacteria	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	94.2%
AB2004A	D: Short fat rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100%
AB2004B	D: Spheres and rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella</i> aff. <i>ruminicola</i> Tc2-24; AJ009933 C: Prevotella cluster 30	99%
AB2005	D: Very long large grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.5%
AB2006	D: Long curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> A46; AF125899 C: Pseudobutyrvibrio cluster 1	99.7%
AB2007	D: Short blunt ended rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 11	96.9%
AB2008	D: Uneven spheres, maybe some extremely thin rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales	B: <i>Prevotella bryantii</i> B14 (DSM 11371); AJ006457 C: Prevotella cluster 2	93.1%
AB2009	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 3	98.5%
AB2010	D: Slightly curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.3%
AB2011	D: Long rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 46	99.8%
AB2012	D: Short rods, some connected in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> Su6; AY178635 C: Butyrivibrio cluster 2	99.4%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AB2013	D: Large cocci in large clusters Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> B14 (DSM 111371); AJ006457 C: Prevotella cluster 1	100.0%
AB2014	D: Large sausage shaped rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S209; AB198439 C: Veillonellaceae cluster 7	98.3%
AB2015	D: Rods in long chains- hair like Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	100.0%
AB2016	D: Thin rods in long chains- hair like Q: k__Bacteria	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	94.4%
AB2018	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
AB2019	D: Thick rods in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 11	95.9%
AB2020	D: Clumping straight rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a EU346756 C: Butyrivibrio cluster 18	99.1%
AB2022	D: Fat rods uneven sizes Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
AB2023	D: Spheres or cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> B14 (DSM 111371); AJ006457 C: Prevotella cluster 1	99.8%
AB2024	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	98.0%
AB2025	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	100.0%
AB2026	D: Uneven sized cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AB2027	D: Long chains of rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 10	99.6%
AB2028	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A179; GU324381 C: Lachnospiraceae cluster 45	92.4%
AB2030	D: Curved rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK2A35; GU324358 C: Lachnospiraceae cluster 57	92.1%
AB2031	D: Rods and some spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-20; AB239486 C: Lachnospiraceae cluster 45	93.6%
AB2032	D: Cocci or spheres and the odd rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 3	98.8%
AB3001	D: Large fat rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
AB3002	D: Very large rods some expanded in the centre Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S150; AB198435 C: Veillonellaceae cluster 4	99.0%
AB3003	D: Small rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A179; GU324381 C: Lachnospiraceae cluster 46	99.1%
AB3005	D: Small oval cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.2%
AB3007	D: Medium sized ovals some branched and linked Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	B: <i>Eubacterium</i> sp. C2; AF044945 C: Family XIII incertae sedis cluster 1	99.8%
AB3008	D: Cocci in chains, a few large cells Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
AB3010	D: Large rods and spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> 23; CP002006 C: Prevotella cluster 43	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AB4001	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1% yes
AC2002	D: Curved rods in chains Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.6%
AC2003A	D: Rods in long chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.4%
AC2003B	D: Short fat rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.8%
AC2004	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	100.0%
AC2005	D: Short rounded end rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 13	95.7%
AC2006	D: Large curved rods with rounded ends Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> GA192; M62702 C: Veillonellaceae cluster 7	98.3%
AC2007	D: Tiny rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 5	97.6%
AC2008	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	97.7%
AC2009	D: Curved rods Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> WH-1; EU684229 C: Pseudobutyrvibrio cluster 1	96.6%
AC2010	D: Cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> RGL 25; GU999991 C: Ruminococcaceae cluster 20	97.3%
AC2011	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A114; GU324377 C: Butyrivibrio cluster 18	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AC2012	D: Very faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.5% yes
AC2013	D: Very faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%
AC2014	D: Spheres small and faint Q: k__Bacteria	B: <i>Eubacterium</i> sp. CB4; AB064884 C: Pseudobutyrvibrio cluster 1	97.2% yes
AC2016	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 29	91.9%
AC2017	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
AC2018	D: Short fat rods in clumps Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	99.8%
AC2019	D: Thin long rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 46	99.8%
AC2020	D: Short rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 13	96.4%
AC2022	D: Spheres and short fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium uniforme</i> X3C39; GU269550 C: Lachnospiraceae cluster 60	99.6%
AC2023	D: Thick rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	98.9%
AC2024	D: Thick curved rods with rounded ends and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S209; AB198439 C: Veillonellaceae cluster 7	98.3% yes
AC2025	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	100.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AC2027a	D: Very large rods and extremely thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.5%
AC2028	D: Thin long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Roseburia hominis</i> A2-183; AJ270482 C: Lachnospiraceae cluster 29	95.2%
AC2029	D: Ovals of uneven sizes Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 34	94.9%
AC2030B	D: Ovals in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 13	95.6%
AC2031	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3A74; GU124460 C: Lachnospiraceae cluster 36	97.6%
AC2032	D: Short blunt ended rods and spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella</i> sp. BP1-56; AB501155 C: Prevotella cluster 34	99.1%
AC2035	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2A35; GU324358 C: Lachnospiraceae cluster 57	91.9%
AC2036	D: Thick rods in chains Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella albensis</i> JCM 12258; AB547669 C: Prevotella cluster 4	99.6%
AC2037	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A86; GU324388 C: Ruminococcaceae R-25 like cluster 13	99.5%
AC2040	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
AC2042	D: Spheres and short rods or cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella buccalis</i> JCM 12246; AB547676 C: Prevotella cluster 3	90.8%
AC2043	D: Very sparse chain of large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Bacterium YES7; AY442821 C: ClostridialesR-7 like cluster 38	89.3%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AC2044	D: Very thin almost invisible rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnobacterium</i> sp. wal 14165; AJ518873 C: Lachnospiraceae cluster 27	95.8% yes
AC3001	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.3%
AC3002	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	97.6%
AC3003	D: Thick rods with some thin rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
AC3004	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> B14 (DSM 11371); AJ006457 C: Prevotella cluster 1	100.0%
AC3005	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%
AC3006	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A86; GU324388 C: Ruminococcaceae R-25 like cluster 13	100.0%
AC3007	D: Long thin twisty rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> H15; EU887842 C: Lachnospiraceae cluster 4	91.0% yes
AC3008	D: Ovals in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	100.0%
AC3009	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 36	92.1%
AC3010	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 35	94.4%
AC4002	D: Oval cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrivibrio cluster 10	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AC4003	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
AC4004	D: Large long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 10	100.0%
AC4005	D: Rods and spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> BP5-11; AB501168 C: Prevotella cluster 43	98.2%
AD2001	D: Thick rods some uneven shapes Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 11	95.9%
AD2002	D: Spheres and short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 11	95.9%
AD2003	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	97.5%
AD2004	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.4%
AD2005	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 36	93.1%
AD2006	D: Small curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
AD2007	D: Rods of uneven lengths thin Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	96.5%
AD2008	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
AD2009	D: Curly short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 8	98.1%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AD2010	D: Thin rods slightly curved Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
AD2011	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	99.8%
AD2012	D: Sparse large spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	97.5% yes
AD2013	D: Large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus albus</i> OR108; AF030452 C: Ruminococcus cluster 20	99.5% yes
AD2014	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.8% yes
AD2015	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Anaerostipes</i> sp. 5_1_63FAA; JF412658 C: Lachnospiraceae cluster 12	91.1% yes
AD2016	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 10	96.3% yes
AD2017	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	98.4% yes
AD2018	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.5% yes
AD2019	D: Diplococci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	98.4% yes
AD2020	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 34	98.9% yes
AD3001	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK2B18; GU324368 C: Ruminococcus cluster 9	100.0% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AD3002	D: Thick slightly curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 11	95.7%
AD3003	D: Curved thin rods hair like Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	100.0%
AD3004	D: Very short rods or cocci in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.3%
AD3005	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.6%
AD3006	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> LB4; AY445603 C: Ruminococcus cluster 1	99.5%
AD3007	D: Faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.5%
AD3009	D: Faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.5%
AD3010	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A144; GU324379 C: Butyrivibrio cluster 14	97.5%
AD3011	D: Branched rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	B: <i>Eubacterium infirmum</i> ; ML049; GU400875 C: Family XIII incertae sedis cluster 17	87.9%
AD3013	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 34	97.6%
AD4001	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella oris</i> F045; GU409727 C: Prevotella cluster 8	98.0%
AE2001	D: Thin rods in chains Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AE2002	D: Rods of uneven lengths Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	93.8%
AE2003	D: Uneven thick rods and small spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 10	98.5%
AE2004	D: Clumps of filaments with spheres attached (some rods) Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B19; GU324407 C: Lachnospiraceae cluster 59	99.8% yes
AE2005	D: Tiny spheres the occasional faint rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	97.4% yes
AE2006	D: Uneven rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium uniforme</i> X3C39; GU269550 C: Lachnospiraceae cluster 60	99.6%
AE2007	D: Rods uneven lengths Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrivibrio cluster 10	99.5%
AE2008	D: Curved rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	98.9%
AE2010	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.3%
AE2011	D: Short ovals and clumps of spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrivibrio cluster 10	99.6%
AE2012	D: Few thick slightly curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_B223; EU728721 C: Lachnospiraceae cluster 55	94.5%
AE2013	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
AE2014	D: Cocci small in pairs or chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2432; X89973 C: Butyrivibrio cluster 4	98.9%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AE2015	D: Long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> Su6; AY178635 C: Butyrivibrio cluster 2	100.0% yes
AE2016	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A114; GU324377 C: Butyrivibrio cluster 18	99.1%
AE2017	D: Curved rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: mixed culture ruminal bacterium PAD71; AF071191 C: Prevotella cluster 43	97.8%
AE2018	D: Curved rods Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> WH-1; EU684229 C: Pseudobutyrvibrio cluster 1	100.0%
AE2019	D: Short thick rods some in small chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	97.8%
AE2020A	D: Spheres and large rods (few) Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> 23; CP002006 C: Prevotella cluster 43	99.6%
AE2021	D: Cocci or very short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.9% yes
AE2022	D: Thick short rods or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%
AE2023	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK2B18; GU324368 C: Ruminococcus cluster 9	99.8%
AE2024	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	99.8%
AE2025	D: Fat rods of uneven length Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	96.5%
AE2026	D: Faint spheres hard to see Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AE2028	D: Curved rods and spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumens bacterium R-9; AB239482 C: Prevotella cluster 18	99.6%
AE2029	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.5%
AE2030	D: Curved rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.4%
AE2032	D: Chains of uneven short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrivibrio cluster 10	96.0%
AE2035	D: Curved rods and spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella aff. ruminicola</i> Te2-24; AJ009933 C: Prevotella cluster 30	99.8%
AE3001	D: Thick rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
AE3002	D: Spheres and rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2A1; GU324367 C: Lachnospiraceae cluster 20	97.4%
AE3003	D: Spheres and rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
AE3004	D: Large ovals some in small chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK205; GU121460 C: Butyrivibrio cluster 11	94.8%
AE3005	D: Large slightly curved thick rods with rounded ends or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> 8; AY463966 C: Veillonellaceae cluster 8	97.1%
AE3006	D: Thick rods in chains some expanded Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 11	97.3%
AE3007	D: Cocci or short rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	99.3%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
AE3008	D: Long chains of rods in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 9	99.8%
AE3009	D: Tiny cocci in clusters Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrivibrio cluster 10	99.8%
AE3010	D: Large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> AR72; AF104841 C: Ruminococcus cluster 2	97.5%
AE3012	D: Short blunt ended rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.8%
AE3015	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Clostridiaceae bacterium SN021; AB298755 C: Lachnospiraceae cluster 25	93.8%
AE3016	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3A74; GU124460 C: Lachnospiraceae cluster 34	97.1%
AE3017	D: Ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.0%
AE3018	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	97.0%
AE4001	D: Tiny cocci some in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.2%
AE4002	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.3%
DAZ1001	D: Chains of rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2221; X89970 C: Butyrivibrio cluster 18	99.6%
DAZ1002	D: Oval cells, singly, in pairs or small chains Q: p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	B: <i>Streptococcus equinus</i> BP5-36; AB563266 C: Streptococcus cluster 1	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
DAZ1003	D: Rods in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	98.4%
DAZ1004	D: Chains of rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS2; AF202260 C: Pseudobutyrvibrio cluster 1	98.0%
DAZ1005	D: Small crescent curved rod with tapered ends Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> L4; AY699285 C: Pseudobutyrvibrio cluster 1	98.0%
DAZ1006	D: Thin rods in short chains Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	93.9% yes
DAZ1007	D: Spirals and spheroplasts Q: p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaeae	B: <i>Treponema bryantii</i> DSM 1788; FR749895 C: Spirochaetales cluster 4	98.0% yes
DAZ1009	D: Chains of ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.4%
DAZ1010	D: Long straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrvibrio cluster 1	99.1%
DAZ1011	D: Rods and cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio hungatei</i> JK614; GU121459 C: Butyrvibrio cluster 1	99.6%
DAZ1012	D: Long thin rods with the occasional thick rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.0%
DAZ1013	D: Chains of rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> OB251; U77341 C: Butyrvibrio cluster 10	97.4% yes
DAZ1015	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> C1a; AM915271 C: Ruminococcus cluster 11	94.4% yes
DAZ2001	D: Rods in chains in large clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> L8; AY699274 C: Butyrvibrio cluster 3	98.1%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
DAZ2002	D: Rods in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 7	90.6%
DAZ2003	D: Short fat rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
DAZ2004	D: Short fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 11	97.4%
DAZ2005	D: Irregular fat rods Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	100.0%
DAZ2006	D: Thin rods in clumps Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.2%
DAZ3001	D: Rods in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium E2; DQ393023 C: Pseudobutyrvibrio cluster 1	98.9%
DAZ3002	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> L4; AY699285 C: Pseudobutyrvibrio cluster 1	98.7%
FB2001	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> A46; AF125899 C: Pseudobutyrvibrio cluster 1	99.8%
FB2002	D: Straight rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A63; GU124466 C: Butyrivibrio cluster 18	99.1%
FB2003	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	98.9%
FB2005	D: Faint curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 22	94.7%
FB2006	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FB2007	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.2%
FB2008	D: Curved rods Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	99.9%
FB2010	D: Short fat rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.8%
FB2012	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus albus</i> AR67; AF104839 C: Ruminococcus cluster 19	92.6%
FB2014	D: Large ovals of various sizes Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.7%
FB2016	D: Faint short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 35	95.3%
FB2017	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.5%
FB2018	D: Thin faint rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 23	95.3%
FB2022	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrvibrio cluster 7	97.0%
FB2023	D: Large grainy rods in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK2B42; GU324359 C: Oribacterium cluster 2	98.8%
FB3001	D: Cocci small Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> C21a; AF396925 C: Prevotella cluster 1	100.0%
FB3002	D: Cocci and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 41	99.2%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FB3004	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> Su6; AY178635 C: Butyrivibrio cluster 1	98.5%
FB3005	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.2%
FB3007	D: Cocci in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK3A76; GU324399 C: Ruminococcus cluster 24	94.2%
FB3011	D: Fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 9	96.4%
FB3013	D: Uneven rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 10	95.3%
FB3014	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	100.0%
FC2001	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	96.5%
FC2002	D: Thick rods and clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B19; GU324407 C: Lachnospiraceae cluster 59	99.8%
FC2003	D: Small rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A136; GU324401 C: Lachnospiraceae cluster S16	99.1%
FC2004	D: Large cocci or ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvens</i> Ce2; AY178842 C: Lachnospiraceae cluster 48	98.6%
FC2005	D: Faint rods in chains Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	B: <i>Eubacterium</i> sp. F1; EU281854 C: Bacteroidetes cluster 1	98.2%
FC2006	D: Rods and spheres or cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	98.9%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FC2007	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
FC2008	D: Large grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_CR52; EU728736 C: Oribacterium cluster 1	94.4% yes
FC2009	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Butyrate-producing bacterium SL6/1/1; AY305317 C: Lachnospiraceae cluster 50	95.3% yes
FC2010	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%
FC2011	D: Very large rods and blobs of various lengths Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.5%
FC2013	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> UC 142; AJ428552 C: Butyrivibrio cluster 5	97.4%
FC2014	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnobacterium</i> sp. wal 14165; AJ518873 C: Lachnospiraceae cluster 15	97.0% yes
FC2015	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	99.1%
FC2016	D: Large thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 35	95.1%
FC2018	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK3A76; GU324399 C: Ruminococcus cluster 24	90.8% yes
FC3002	D: Straight rods in clumps Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	97.5%
FC4001	D: Fat short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S7; AB198427 C: Veillonellaceae cluster 5	97.6% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FC4003	D: Large fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S150; AB198435 C: Veillonellaceae cluster 4	98.7%
FCS002	D: Small curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	96.9%
FCS003	D: Short rods in clumps and chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Coprococcus eutactus</i> ATCC 27759; EF031543 C: Lachnospiraceae cluster 9	95.9%
FCS004	D: Short curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.9%
FCS005	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.7%
FCS006	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: mixed culture ruminal bacterium PAD196; AF071192 C: Butyrvibrio cluster s1	96.1%
FCS007	D: Rounded end rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
FCS011	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> TC2-28; AF218619 C: Prevotella cluster 17	87.2%
FCS012	D: Thin twisty rods Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.4%
FCS013	D: Cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> 23; CP002006 C: Prevotella cluster 43	97.9%
FCS014	D: Large twisty segmented rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-26; AB239490 C: Butyrvibrio cluster 10	98.5%
FCS016	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	98.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FCS017	D: Rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 10	95.0%
FCS018	D: Spheres and blobs Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> 23; CP002006 C: Prevotella cluster 43	98.9%
FCS019	D: Large grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S206; AB198438 C: Veillonellaceae cluster 4	99.3%
FCS020	D: Thick and thin rods long Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: mixed culture ruminal bacterium PAD156; AF070464 C: Lachnospiraceae cluster 22	95.6%
FCS021	D: Thick grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: bacterium L4M2 1-7; AY862595 C: Veillonellaceae cluster 1	98.5%
FCS022	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.6%
FCS025	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.2%
FCS026	D: Small grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.3%
FD2002	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	100.0%
FD2003	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	98.0%
FD2004	D: Slightly curved rods in chains Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	98.7%
FD2005	D: Spheres faint Q: k__Bacteria	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 53	91.2% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FD2006	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	99.6%
FD2007	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio proteoelasticus</i> B316; CP001810 C: Butyrvibrio cluster 5	100.0% yes
FD2008	D: Ovals in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	95.5%
FD2009	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.6%
FD3001	D: Sphere Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella aff. ruminicola</i> Te2-24; AJ009933 C: Prevotella cluster 30	99.6%
FD3002	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	94.3%
FD3003	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.5%
FD3004	D: No description available Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> BP1-162; AB501167 C: Prevotella cluster 43	96.9% yes
FE2001	D: Long thin rods some together Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> L8; AY699274 C: Butyrvibrio cluster 7	97.6%
FE2002	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A63; GU124466 C: Butyrvibrio cluster 18	99.8%
FE2003	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> L8; AY699274 C: Butyrvibrio cluster 3	97.0%
FE2004	D: Large fat grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S150; AB198435 C: Veillonellaceae cluster 4	98.7%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FE2006	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	92.7%
FE2007	D: Small rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A63; GU124466 C: Butyrivibrio cluster 18	100.0%
FE2010	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.5%
FE2011	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.5%
FE2012	D: Nothing Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.3%
FE2014	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrivibrio cluster 10	99.6%
FE2016	D: Sparse large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium phytofermentans</i> ISDg; CP000885 C: Lachnospiraceae cluster 39	91.3% yes
FE2017	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.3%
FE2018	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Ruminococcus gnavus</i> A2; EU139255 C: Lachnospiraceae cluster 49	91.3% yes
FE2020	D: A few spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.0%
FE2022	D: Think rods variable lengths Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium populeti</i> ATCC 35295; X71853 C: Lachnospiraceae cluster 12	91.3%
FE2023	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
FE3003	D: No description available Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.3%
FE3004	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.5%
FE3007	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 6	95.4%
FE3010	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	94.3%
FE4001	D: No description available Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.0%
FE4002	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	96.4%
LB2001	D: Slightly curved rods in short chains Q: k__Bacteria	B: <i>Butyrvibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.8%
LB2003	D: Spheres and the odd rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnospira pectinoschiza</i> M56; AY699283 C: Lachnospiraceae cluster 54	98.9%
LB2004	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 36	95.5%
LB2005	D: Crescent shaped rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: Bacterium L4M2 1-7; AY862595 C: Veillonellaceae cluster 1	99.4%
LB2006	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.9%
LB2007	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrvibrio cluster 1	98.9%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LB2008	D: Straight rods Q: k__Bacteria	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	95.9% yes
LB2009	D: Short rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
LB2010	D: Large fuzzy patches of cell debris Q: p__Fibrobacteres;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae	B: <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> GC5; M62688 C: Fibrobacter cluster 7	96.6% yes
LB2011	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	99.6% yes
LB2012	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 13	96.1%
LB2013	D: Ovals with tapered ends in pairs and large empty grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.5%
LB2014	D: Chain of spheres in uneven sizes Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.7%
LB2020	D: Large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_CP64; EU728726 C: Lachnospiraceae cluster 32	97.8% yes
LB2021	D: Rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> 23; CP002006 C: Prevotella cluster 43	98.9%
LB3001	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.8% 99.8%
LB3003	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus albus</i> 7; CP002403 C: Ruminococcus cluster 20	99.8%
LB3004	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 1	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LB3005	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
LB3006	D: Uneven length rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium populei</i> ATCC 35295; X71853 C: Lachnospiraceae cluster 12	91.4%
LB3007	D: Irregular spheres and rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
LB3008	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.4%
LB4001	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> Su6; AY178635 C: Butyrivibrio cluster 2	100.0%
LB4002	D: Large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> 007; LRC017; AF030447 C: Ruminococcus cluster 9	100.0%
LC2001	D: Cocci or spheres - the occasional rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnospira pectinoschiza</i> M56; AY699283 C: Lachnospiraceae cluster 54	98.9%
LC2002	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 13	95.9%
LC2003	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Butyrivibrio cluster 18	98.5%
LC2005	D: Thick blunt ended rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
LC2006	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 13	95.7%
LC2007	D: Rods in long curly chains Q: k__Bacteria	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	96.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LC2008	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	98.5%
LC2009	D: Spheres and crystals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
LC2010	D: Faint fat rods + debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 11	97.0%
LC2011	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 36	92.7%
LC2012	D: Spheres ad thick rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella aff. ruminicola</i> Tc2-24; AJ009933 C: Prevotella cluster 30	99.1% yes
LC2013	D: Short grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 11	96.8%
LC2014	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Butyrivibrio cluster 18	99.1%
LC2016	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.4%
LC2017	D: Straight rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	99.8%
LC2018	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium R-26; AB239490 C: Butyrivibrio cluster 10	97.4%
LC2019	D: Large thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 35	99.8% yes
LC2020	D: Ovals with tapered ends in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumex bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.8% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LC2023	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 36	94.8%
LC2026	D: Ovals in pairs Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 36	94.2%
LC3001	D: Fat short rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
LC3002	D: Spheres or grainy cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvans</i> Ce2; AY178842 C: Lachnospiraceae cluster 48	99.5%
LC3003	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales;f__	B: Rumex bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.3%
LC3004	D: Rods slightly curved Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium E2; DQ393023 C: Pseudobutyrvibrio cluster 1	99.1%
LC3005	D: Curved rods Q: k__Bacteria	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	95.9%
LC3006	D: Cocci in pairs Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS2; AF202260 C: Pseudobutyrvibrio cluster 1	99.3%
LC3007	D: Curved rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.1%
LC3008	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
LC3009	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 3	98.5%
LC3010	D: Grainy fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 12	95.7%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LC3011	D: Cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 35	94.4%
LC4001	D: Cocci faint Q: p__Fibrobacteres;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae	B: <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> GC5; M62688 C: Fibrobacter cluster 7	98.1%
LD2001	D: Fat grainy short rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
LD2003	D: Short rods in chains Q: k__Bacteria	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	96.2%
LD2004	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.5%
LD2005	D: Spheres and the occasional rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-26; AB239490 C: Butyrivibrio cluster 10	97.0%
LD2006	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvens</i> ; L34613 C: Lachnospiraceae cluster 48	99.1%
LD2008	D: Small rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	98.0%
LD2009	D: Thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
LD2010	D: Small cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	99.4%
LD2011	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
LD2012	D: Small rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	99.4%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LD2013	D: Small rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	B: <i>Eubacterium infirmum</i> YQ024; GU400910 C: Family XIII incertae sedis cluster 5	87.9% yes
LD2014	D: Large long rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumén bacterium R-26; AB239490 C: Butyrivibrio cluster 10	97.9%
LD2016	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumén bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	95.0%
LD2017	D: Small rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumén bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
LD2018	D: Large long rods with expanded regions Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumén bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	96.1%
LD2019	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumén bacterium R-9; AB239482 C: Prevotella cluster 19	94.7%
LD3001	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumén bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 46	99.5%
LD3002	D: Small rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumén bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.4%
LD3003	D: Chains of cocci uneven sizes some large Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumén bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	99.3%
LD3004	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumén bacterium NK4A86; GU324388 C: Ruminococcaceae R-25 like cluster 13	99.5%
LD3005	D: Short rods and cocci oval to round Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumén bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.7%
LE2001	D: Thin rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> ; NCDO 2221; X89970 C: Butyrivibrio cluster 18	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LE2002	D: Short rods and spheres Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> AR27; U77337 C: Pseudobutyrvibrio cluster 1	95.2%
LE2003	D: Short rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU3446756 C: Butyrivibrio cluster 18	98.9%
LE2004	D: Spheres and the odd rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnospira pectinoschiza</i> M56; AY699283 C: Lachnospiraceae cluster 54	98.9%
LE2005	D: Faint spheres and lots of square crystals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 3	98.1%
LE2006	D: Fat grainy rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	96.8%
LE2007	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Rumen bacterium</i> 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	99.1%
LE2008	D: Rods in chains spotty Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.8%
LE2009	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnospira pectinoschiza</i> M56; AY699283 C: Lachnospiraceae cluster 54	98.7%
LE2010	D: Short fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 3	97.8%
LE2011	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 6	97.0%
LE2012	D: Short rods and long curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 3	97.6%
LE2013	D: Short fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 34	94.9%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LE2014	D: Large cocci in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvens</i> Ce2; AY178842 C: Lachnospiraceae cluster 48	99.5%
LE2015	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.3%
LE2016	D: Very large long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.5%
LE2018	D: Small cocci Q: p__Fibrobacteres;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae	B: <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> MC1; M62693 C: Fibrobacter cluster 8	98.8%
LE2019	D: Short rods in chains and clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	96.8%
LE2020	D: Short rods of uneven sizes Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Coprococcus eutactus</i> ATCC 27759; EF031543 C: Lachnospiraceae cluster 9	95.3%
LE2021	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	97.6%
LE2022	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.4%
LE3001	D: Spheres or grainy cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvens</i> NE13; AB372004 C: Lachnospiraceae cluster 48	99.1%
LE3002	D: Faint tiny rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	94.7%
LE3003	D: Very large spheres and rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
LE3004	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
LE4001	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> 007; AF030447 C: Ruminococcus cluster 9	99.8%
MA2001	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: butyrate-producing bacterium L2-50; AJ270491 C: Lachnospiraceae cluster 28	95.4% yes
MA2002	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.3%
MA2003	D: Rods cocci and spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumens bacterium R-9; AB239482 C: Prevotella cluster 18	98.9%
MA2004	D: Clusters of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 36	93.7%
MA2005	D: Motile rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 36	92.8%
MA2006	D: Rods in short chains Q: k__Bacteria	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	97.1%
MA2007	D: Large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	96.4% yes
MA2008	D: Spheres and large rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrvibrio cluster 13	96.1%
MA2009	D: Very large rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.3%
MA2010	D: Either cocci or short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrvibrio cluster 1	100.0%
MA2011	D: Small motile cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.4%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MA2012	D: Thick rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
MA2014	D: Short rods the occasional sphere Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.3%
MA2015	D: Thin rods in long chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A179; GU324381 C: Lachnospiraceae cluster 46	99.1%
MA2016	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> AC5-13; ABS01173 C: Prevotella cluster 39	93.6%
MA2017	D: Curved rods in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.9%
MA2018	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.5%
MA2019	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> CF3; AF125900 C: Pseudobutyrvibrio cluster 1	98.9%
MA2020	D: Cocci in massive clump Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A21; GU324371 C: Lachnospiraceae cluster 1	92.8%
MA2021	D: Irregular short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 12	96.4%
MA2022	D: Ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.8%
MA2023	D: Ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	96.7%
MA3002	D: Irregular spheres, rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B19; GU324407 C: Lachnospiraceae cluster 59	99.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MA3003	D: Irregular long chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 10	95.5%
MA3004	D: Very large irregular rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK2B42; GU324359 C: Orbacterium cluster 2	96.5%
MA3005	D: Spheres and short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 13	96.1%
MA3006	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.3%
MA3007	D: Rods, short chains Q: k__Bacteria	B: Rumen bacterium 8/9293-9; DQ393015 C: Pseudobutyrvibrio cluster 1	96.4% yes
MA3009	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 1	99.1%
MA3010	D: Rods in pairs Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	99.8%
MA3011	D: Spheres and rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.1%
MA3012	D: Clusters of large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> RGL 25; GU999991 C: Ruminococcus cluster 2	95.8%
MA3013	D: Irregular chains of cocci Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	100.0%
MA3014	D: Chains of short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	97.7%
MA4001	D: Rods, short chains Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> ; JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.1%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MA4003	D: Non uniform spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	93.3%
MA4004	D: Spheres and short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	98.2%
MB2001	D: Ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	96.7%
MB2003	D: Sparse tiny motile rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 1	99.6%
MB2004	D: Long rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> Su6; AY178635 C: Butyrivibrio cluster 2	100.0%
MB2005	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 11	97.3%
MB2006	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	98.9%
MB2007	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK2B42; GU324359 C: Oribacterium cluster 2	98.6%
MB2008	D: Rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
MB2009	D: Thin rods in long chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 11	96.1%
MB2011	D: Rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.3%
MB2012	D: Small thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MB2014	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.6%
MB2015	D: Curved motile rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 2	97.0%
MB2017	D: Short fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 2	97.3% yes
MB2018	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus albus</i> OR108; AF030452 C: Ruminococcus cluster 20	99.5%
MB2019	D: Motile grainy rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
MB2020	D: Small thin rods, chains or single Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	98.9%
MB2021	D: Single straight rods Q: p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	B: <i>Bacillaceae bacterium</i> C11; AY504437 C: Bacillus cluster 1	100.0% yes
MB2022	D: Thin rods Q: k__Bacteria	B: <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> FGL 01; GU999988 C: Fibrobacter cluster 9	100.0% yes
MB2023	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 12	97.1%
MB2024	D: Rods twisted in clumps Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	B: <i>Eubacterium</i> sp. F1; EU281854 C: Bacteroidetes cluster 1	96.9%
MB2025	D: Chains of short rods or ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 10	99.6%
MB2026	D: Large cocci or spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> BPI-34; AB501152 C: Prevotella cluster 35	94.1% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MB2027	D: Cocci and short fat rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella</i> sp. BP1-145; AB501165 C: Prevotella cluster 38	94.7% yes
MB3001	D: Clumps of grainy rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> TC2-28; AF218619 C: Prevotella cluster 37	91.5% yes
MB3002	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	98.9%
MB3003	D: Grainy rods in clumps Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	100.0%
MB3004	D: Irregular cocci Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	99.8%
MB3005	D: Diplococci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> type strain: C94=ATCC 19208; AM915269 C: Ruminococcus cluster 9	99.5%
MB3006	D: Short fat rods Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	100.0%
MB3007	D: Grainy rods in clumps Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> WB078; GU413944 C: Propionibacterium cluster 1	99.8% yes
MB4001	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142 GU324402 C: Butyrivibrio cluster 1	99.1%
MC2002	D: Chains of irregular cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.5%
MC2003	D: Rods in large clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnospira pectinoschiza</i> M56; AY699283 C: Lachnospiraceae cluster S41	97.8% yes
MC2004	D: Long rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Orribacterium cluster 2	99.3%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MC2006	D: Cocci in chains, short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.9%
MC2007	D: Fat rods in chains large ovals with tapered ends cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A86; GU324388 C: Ruminococcaceae R-25 like cluster 13	99.5%
MC2008	D: Motile curved rods, small cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
MC2009	D: Large cocci some in chains distorted Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 34	98.6%
MC2010	D: Short rods in pairs, singles look like cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella oris</i> JCM 8540; AB547701 C: Prevotella cluster 7	88.1%
MC2011	D: Cocci of different sizes some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3A74; GU124460 C: Lachnospiraceae cluster 36	97.2%
MC2012	D: Fat rods some thinner in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3A75; GU324362 C: Lachnospiraceae cluster 25	93.1%
MC2013	D: Spheres in clumps the odd rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCD0 2221; X89970 C: Butyrivibrio cluster 16	95.3%
MC2015	D: Spheres, cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> C21a; AF396925 C: Prevotella cluster 1	100.0%
MC2016	D: Clumps of rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas</i> sp. DJF_CR90; EU728739 C: Veillonellaceae cluster 6	99.7%
MC2017	D: Large rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A144; GU324379 C: Lachnospiraceae cluster 3	97.1%
MC2018	D: Cocci in irregular chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MC2019	D: Large cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	96.8%
MC2020	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> AR69; AF104840 C: Ruminococcaceae cluster 20	97.6%
MC2021	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCD0 2222; X89971 C: Butyrivibrio cluster 10	99.6%
MC2022	D: Cocci, spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> B14 (DSM 11371); AJ006457 C: Prevotella cluster 1	100.0%
MC2024	D: Thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A86; GU324388 C: Ruminococcaceae R-25 like cluster 13	98.4%
MC3001	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 8	97.8%
MC3002	D: Spheres, motile small cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> B14 (DSM 11371); AJ006457 C: Prevotella cluster 1	100.0%
MC4001	D: Grainy rods , spheres Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
MC4003	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
MD2001	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.1%
MD2002	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> CF3; AF125900 C: Pseudobutyrvibrio cluster 1	99.6%
MD2003	D: Long thin rods in chains Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
MID2004	D: Motile cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 35	94.4% yes
MID2005	D: Short motile rods Q: p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__	B: Rumen bacterium NK4A44; GU324412 C: Pseudobutyrvibrio cluster 1	99.6% yes
MID2006	D: Short rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	95.0% 99.2%
MID2007	D: Thin rods in chains Q: p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	yes 99.3%
MID2009	D: Spheres in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.3% 93.3%
MID2010	D: Sparse long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 6	yes 97.8%
MID2011	D: Twisty chains of rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 7	96.6% yes
MID2012	D: Long tight spirals Q: p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae	B: Rumen bacterium NK4A124; GU324416 C: Spirochaetales cluster 4	99.3% 97.2%
MID2013	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	97.2% 94.2%
MID2015	D: Short rods or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: Bacterium L4M2 1-7; AY862595 C: Veillonellaceae cluster 1	94.2% yes
MID3002	D: Clumps of large ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	97.8% 97.8%
MID4002	D: Short motile rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NB2002	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.8%
NB2003	D: Large straight rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Clostridiaceae bacterium SN021; AB298755 C: Butyrivibrio cluster 17	92.9%
NB2004	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	97.3%
NB2006	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A61; GU324372 C: Butyrivibrio cluster 18	99.8%
NB2008	D: Short rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 13	97.0%
NB2009	D: Rods of varying lengths Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.4%
NB2010	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio</i> sp. 3; EU714406 C: Pseudobutyrvibrio cluster 1	99.6%
NB2011	D: Faint irregular spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.3%
NB2012	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.3%
NB2013	D: Rods and the occasional odd sphere Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	94.1%
NB2014	D: Diplococci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 34	95.0%
NB2015	D: Large fat short rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidiales;f__Prevotellaceae	B: Rumen bacterium R-9; AB239482 C: Prevotella cluster 18	99.3% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NB2016	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium hadrum</i> DSM 3319; FR749934 C: Lachnospiraceae cluster 13	90.8% yes
NB3001	D: Small spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvens</i> L34613 C: Lachnospiraceae cluster 48	98.4%
NB3002	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
NB3003	D: Rods in chains some spheres and short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 6	96.3%
NB3005	D: Large rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Mixed culture ruminal bacterium PAD196; AF071192 C: Butyrivibrio cluster 10	97.6%
NB3006	D: Rods twisted around each other in a clump Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	99.8%
NB3007	D: Attached to glass-fat rods in small chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_VP30; EU728771 C: Lachnospiraceae cluster 8	93.0%
NB4001	D: Rods slightly curved Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Anaerostipes</i> sp. 5_1_63FAA; JF412658 C: Lachnospiraceae cluster 12	90.8%
NB4002	D: Large thick rods and cocci or fragments Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumens bacterium R-9; AB239482 C: Prevotella cluster 18	99.3%
NC2001	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Butyrate-producing bacterium PH05YA09; DQ144120 C: Lachnospiraceae cluster 9	95.6%
NC2002	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 11	95.7% yes
NC2003	D: Large irregular spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NC2004	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Bacterium YE64; AY442828 C: Lachnospiraceae cluster 19	94.4% yes
NC2005	D: Fat short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 12	96.2%
NC2006	D: Curved rods Q: k__Bacteria	B: <i>Butyrivibrio</i> sp. 3 EU714406 C: Pseudobutyrvibrio cluster 1	91.7%
NC2007	D: Clumps of long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 8	97.9%
NC2008	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A136; GU324401 C: Lachnospiraceae cluster 40	99.8% yes
NC2010	D: Slightly curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A114; GU324377 C: Butyrivibrio cluster 18	99.4%
NC2012	D: Tiny rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
NC2013	D: Clumpy, curved rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 10	97.4%
NC2014	D: Rods and cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnospira pectinoschiza</i> M56; AY699283 C: Lachnospiraceae cluster 54	98.2%
NC2015	D: Attached to glass- strange clusters large long with dark spots Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B19; GU324407 C: Lachnospiraceae cluster 59	99.8%
NC2018	D: Chain of short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	100.0%
NC2021	D: No description available Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidiales;f__Prevotellaceae	B: Rumen bacterium R-9; AB239482 C: Prevotella cluster 19	94.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NC3001	D: Small rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
NC3003	D: Irregular spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> C21a; AF396925 C: Prevotella cluster 1	100.0%
NC3005	D: Very long large rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Butyrivibrio cluster 17	91.3%
NC3007	D: Short motile rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	97.8%
NC3008	D: No description available Q: p__Firmicutes;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae	B: <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> HM2; M62689 C: Fibrobacter cluster 1	98.8%
ND2001	D: Short rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
ND2002	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	98.0%
ND2003	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	99.1%
ND2004	D: Cocci in chains with sheaths Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella</i> sp. oral taxon 289 WWP_SS2_P42; GU409397 C: Prevotella cluster 6	91.8%
ND2005	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.4%
ND2006	D: Large grainy crescent shaped rods and debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A144; GU324379 C: Lachnospiraceae cluster 2	97.2%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
ND2007	D: Stuck to glass-very small curved rods some in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Swine manure pit bacterium PPC24; AF445265 C: Lachnospiraceae cluster 14	94.4% yes
ND2008	D: Fat rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 13	97.0%
ND2009	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> LB4; AY445603 C: Ruminococcus cluster 1	100.0% yes
ND2010	D: Fat rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas</i> sp. DJF_CR90; EU728739 C: Veillonellaceae cluster 6	98.7% yes
ND2012	D: Oval cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> RGL 25; GU999991 C: Ruminococcus cluster 12	95.2% yes
ND2013	D: Faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_B223; EU728721 C: Lachnospiraceae cluster 55	94.6% 93.5%
ND2014	D: Faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 35	93.5%
ND2015	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A21; GU324371 C: Lachnospiraceae cluster 7	90.1% 98.2%
ND2016	D: Cocci some swollen spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.2%
ND2017	D: Cocci faint Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 34	97.1%
ND2018	D: Thick curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Lachnospiraceae cluster 7	87.3% yes
ND2019	D: Small cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	96.5%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
ND2020	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
ND2021	D: Chains of large rods some expanded Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumex bacterium R-9; AB239482 C: Prevotella cluster 19	94.9%
ND3001	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
ND3002	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> CF3; AF125900 C: Pseudobutyrvibrio cluster 1	99.3%
ND3003	D: Spirals Q: p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaeae	B: <i>Treponema</i> sp. AC3; GU566697 C: Spirochaetales cluster 1	99.7%
ND3005	D: Small rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A114; GU324377 C: Butyrivibrio cluster 18	99.8%
ND3006	D: Spheres and grainy small rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_B223; EU728721 C: Lachnospiraceae cluster 55	94.8%
ND3007	D: Very few irregular rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Lachnospiraceae cluster 47	92.0%
ND4001	D: Ovals with tapered ends shaped rods in small chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 34	98.0%
NE2001	D: Large rods and debris Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella</i> sp. BP1-148; AB501166 C: Prevotella cluster 21	94.7%
NE2002	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	99.4%
NE2003	D: Small diplococci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumex bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	98.5%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NE2004	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.9%
NE2005	D: Ovals with tapered ends cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.0%
NE2006	D: cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> AR69; AF104840 C: Ruminococcus cluster 10	97.9%
NE2007	D: Short straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A63; GU124466 C: Butyrvibrio cluster 18	100.0%
NE2008	D: Small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrvibrio cluster 1	100.0%
NE2009	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A179; GU324381 C: Lachnospiraceae cluster 46	99.3%
NE2010	D: Large crescent shaped rods and debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S4; AB198426 C: Veillonellaceae cluster 7	97.4%
NE2011	D: Short straight rods in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> CF3; AF125900 C: Pseudobutyrvibrio cluster 1	99.3%
NE2012	D: Short straight rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio hungatei</i> Su6; AY178635 C: Butyrvibrio cluster 2	100.0%
NE2013	D: Short fat rods in chains + spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.3%
NE2014	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 6	93.5%
NE2015	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	100.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NE2016	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 3	98.8%
NE2017	D: Rods with spheres and clusters of spheres Q: p__Fibrobacteres;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae	B: <i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i> MCl; M62693 C: Fibrobacter cluster 8	98.8%
NE2018	D: The occasional large rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	94.9%
NE2019	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.0%
NE2021	D: Really large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 39	89.7%
NE3001	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 10	95.6%
NE3002	D: Large ovals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium aldenense</i> RMA 9741; DQ279736 C: Lachnospiraceae cluster 8	92.3%
NE3003	D: Rods in chains and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
NE3004	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.5%
NE3005	D: Large thick rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumen bacterium YS1; AF544206 C: Prevotella cluster 40	95.9%
NE3006	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-20; AB239486 C: Lachnospiraceae cluster 45	93.5%
NE3007	D: Rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
NE3008	D: Very sparse long rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 1	99.8%
NE3009	D: Cocci in chains and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	97.9%
NE3010	D: Cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	98.9%
NE4001	D: Small spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: <i>Butyrivibrio</i> cluster 1	98.2%
V9B2001	D: Large thick curved rods and thinner faint ones Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-26; AB239490 C: <i>Butyrivibrio</i> cluster 10	97.4%
V9B2002	D: Large straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: <i>Butyrivibrio</i> cluster 1	99.8%
V9B2004	D: Large grainy rods and smaller faint ones Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: <i>Butyrivibrio</i> cluster 1	99.8%
V9B2005	D: Short rods in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: <i>Butyrivibrio</i> cluster 11	96.8%
V9B2008	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium uniforme</i> X3C39; GU269550 C: Lachnospiraceae cluster 60	99.6%
V9B2011	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 3	96.2%
V9B3003	D: Large cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	97.0%
V9D2001	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: <i>Butyrivibrio</i> cluster 1	98.9%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
V9D2002	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 42	95.5% yes
V9D2003	D: Diplococci Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.6%
V9D2004	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.9%
V9D2006	D: Clumps of spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> H15; EU887842 C: Butyrivibrio cluster 15	90.6% yes
V9D2007	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	97.1%
V9D2008	D: Chains of short rods , grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 3	97.4% yes
V9D2009	D: Long thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Parasporobacterium paucivorans</i> SYR1; AJ272036 C: Lachnospiraceae cluster 17	93.3% yes
V9D2011	D: Small curved rods, the occasional cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnobacterium</i> sp. wal 14165; AJ518873 C: Lachnospiraceae cluster 58	94.3%
V9D2012	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK4B32; GU324392 C: Ruminococcus cluster 17	93.7% yes
V9D2013	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Oscillibacter</i> sp. G2; HM626173 C: Ruminococcaceae II cluster 1	91.4% yes
V9D3003	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 13	96.4%
V9D3004	D: Tiny spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 2/9293-25; DQ382351 C: Lachnospiraceae cluster 56	90.0% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
V9D4007	D: Large rods and debris and thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Coprococcus</i> sp. DJF_B005; EU728700 C: Lachnospiraceae cluster 16	93.5%
V9D4008	D: Large irregular rods Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium granulosum</i> D-34; ATCC 25746; FJ785716 C: Propionibacterium cluster 2	98.6%
VCB1003	D: Chains of grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 3	98.3%
VCB1005	D: Grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	98.7%
VCB1006	D: Grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	99.4%
VCB1009	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	98.4%
VCB1011	D: Tiny curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	99.8%
VCB1013	D: Straight grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.6%
VCB1014	D: Tiny rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	99.6%
VCB1015	D: Short small rods spotty Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.6%
VCB2001	D: Short rods in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrivibrio cluster 9	99.3%
VCB2002	D: Very small cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella oris</i> FU017; GU409732 C: Prevotella cluster 8	87.9% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
VCB2003	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	97.9%
VCB2004	D: Rods in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Roseburia hominis</i> A2-183; AJ270482 C: Lachnospiraceae cluster 10	96.3%
VCB2005	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnobacterium</i> sp. wal 14165; AJ518873 C: Lachnospiraceae cluster 58	94.7%
VCB2006	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 3	97.8%
VCB2012	D: Thin long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A144; GU324379 C: Lachnospiraceae cluster 4	96.9%
VCB2013	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Mixed culture ruminal bacterium PADI56; AF070464 C: Lachnospiraceae cluster 9	94.9%
VCB2015	D: Rods of 2 thicknesses Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Parasporobacterium paucivorans</i> SYR1; AJ272036 C: Lachnospiraceae cluster 17	93.2%
VCB3001	D: Small rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
VCB4001	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 6	95.8%
VCD1001	D: Large rods with dark spot in them Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella</i> sp. BP1-56; AB501155 C: Prevotella cluster 20	91.9%
VCD1002	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	97.8%
VCD1005	D: Small grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	97.8%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
VCD1014	D: Grainy ovals and lot of debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.3% yes
VCD1015	D: Grainy short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	96.6%
VCD1017	D: Short grainy rods some in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A21; GU324371 C: Lachnospiraceae cluster 1	92.9%
VCD2001	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.1%
VCD2002	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.5%
VCD2004	D: Tiny cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumens bacterium R-9; AB239482 C: Prevotella cluster 19	97.1%
VCD2006	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 11	95.2%
VCD2007	D: Long rods with expanded centers Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A114; GU324377 C: Lachnospiraceae cluster 5	89.9% yes
VCD2008	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.3%
VCD3001	D: Grainy short rods Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.6%
VCD3003	D: Short fat rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Clostridiaceae bacterium SN021; AB298755 C: Lachnospiraceae cluster 38	91.5% yes
VCD3004	D: Large rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-26; AB239490 C: Butyrivibrio cluster 10	96.6% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
WCD2001	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio proteoclasticus</i> B316; CP001810 C: Butyrivibrio cluster 11	95.5%
WCD2002	D: Faint rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.7%
WCD2005	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 13	95.9%
WCD2007	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	99.6%
WCD2008	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	98.9%
WCD2010	D: Grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Ruminococcus gnavus</i> A2; EU139255 C: Lachnospiraceae cluster 49	91.5%
WCD2011	D: Chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Bacterium YE257; FJ966227 C: Lachnospiraceae cluster 16	92.2%
WCD3001	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A21; GU324371 C: Lachnospiraceae cluster 6	91.8% yes
WCD3002	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 11	97.0%
WCD3003	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.4%
WCD3004	D: Small rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 10	95.3%
WCD3005	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	98.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
WCD3007	D: Nothing Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Bacterium LKS-AN-6; JF502818 C: Lachnospiraceae cluster 24	95.1% yes
WCD4002	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium</i> sp. A7-15; AB238885 C: Lachnospiraceae cluster 23	94.8%
WCE2001	D: Fat grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S150; AB198435 C: Veillonellaceae cluster 4	97.8%
WCE2002	D: Grainy rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumen bacterium R-9; AB239482 C: Prevotella cluster 18	99.6%
WCE2003	D: Clumps of cocci Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Solobacterium</i> sp. oral taxon A05; IP096; GU428535 C: Erysipelotrichaceae cluster 4	94.5% yes
WCE2004	D: Sphere and curved rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	B: Bacteroidetes bacterium oral taxon 280; GU409277 C: Bacteroidetes cluster 7	90.3% yes
WCE2005	D: Rods slightly curved Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS2; AF202260 C: Pseudobutyrvibrio cluster 1	98.9%
WCE2006	D: Fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrvibrio cluster 12	95.7% yes
WCE2007	D: Large grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Bacterium ic1272; DQ057452 C: Lachnospiraceae II cluster I	90.9% yes
WCE2008	D: Curved rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	B: <i>Eubacterium</i> sp. F1; EU281854 C: Bacteroidetes cluster 1	97.4% yes
WCE2010	D: Grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.6%
WCE2011	D: Grainy rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.8% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
WCE2012	D: Twisty rods spirals Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.2%
WCE2013	D: Cocci in chains Q: p__Actinobacteria;c__Actinobacteria;o__Coriobacteriales;f__Coriobacteriaceae	B: Coriobacteriaceae bacterium M11; AB262696 C: Coriobacteriaceae cluster 2	93.1% yes
WCE2015	D: Nothing Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 10	95.7%
WCE3001	D: Twisty rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Mixed culture ruminal bacterium PAD156; AF070464 C: Lachnospiraceae cluster 22	95.3%
WCE3002	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-26; AB239490 C: Butyrvibrio cluster 10	96.7%
WCE3003	D: Dense debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> 49; EF427365 C: Lachnospiraceae cluster 26	93.0% yes
WCE3004	D: Tiny spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	99.1%
WCE3006	D: Debris Q: p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae	B: <i>Treponema</i> sp. T; GQ426551 C: Spirochaetales cluster 2	90.1% yes
WCE4001	D: Long thin rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	98.2%
WTD2001	D: Straight rods some linked Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A578; GU124465 C: Butyrvibrio cluster 1	100.0%
WTD2005	D: Thick grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.3%
WTD3001	D: Short fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.2%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
WTD3002	D: Slightly curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	95.0%
WTD3007	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 36	92.9%
WTE2001	D: Thick curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> CF3; AF125900 C: Pseudobutyrvibrio cluster 1	98.9%
WTE2002	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> CF3; AF125900 C: Pseudobutyrvibrio cluster 1	99.1%
WTE2003	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	99.1%
WTE2004	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	99.4%
WTE2005	D: Very small cocci and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
WTE2006	D: Tiny spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium uniforme</i> X3C39; GU269550 C: Lachnospiraceae cluster 60	99.3%
WTE2007	D: Fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A578; GU124465 C: Butyrivibrio cluster 11	95.7%
WTE2008	D: Large fat rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	98.3%
WTE2009	D: Faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.3%
WTE3002	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
WTE3003	D: Faint spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.3%
WTE3004	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU3446756 C: Butyrivibrio cluster 18	99.1%
WTE3009	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumex bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.3%
XBB1001	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> L8; AY699274 C: Butyrivibrio cluster 6	95.5%
XBB1002	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Lachnobacterium</i> sp. wal 14165; AJ518873 C: Lachnospiraceae cluster 58	94.4%
XBB1003	D: Straight grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A144; GU324379 C: Lachnospiraceae cluster 29	94.7%
XBB1004	D: Clumps of short grainy rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> BP1-162; AB501167 C: Prevotella cluster 43	97.8%
XBB1006	D: Thick rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium saccharolyticum</i> WM1; CP002109 C: Lachnospiraceae cluster 11	94.0%
XBB2001	D: Rods straight Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0%
XBB2002	D: Large irregular rods Q: p__Actinobacteria;c__Actinobacteria;o__Coriobacteriales;f__Coriobacteriaceae	B: <i>Olsenella</i> sp. lac31; FNI78463 C: Coriobacteriaceae cluster 3	96.8%
XBB2003	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	98.0%
XBB2004	D: rods faint Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
XBB2005	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
XBB2006	D: Cocci and cocci in chains Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella albensis</i> JCM 12258; AB547669 C: Prevotella cluster 6	90.4%
XBB2008	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 21	93.8%
XBB2009	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium populeti</i> ATCC 35295; X71853 C: Lachnospiraceae cluster 12	91.1%
XBB3001	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium NK3A31; GU324398 C: Ruminococcaceae R-25 like cluster 15	98.2%
XBB3002	D: Ovals with tapered ends cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 6	96.1%
XBB3003	D: Clumps of very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	B: <i>Eubacterium</i> sp. WAL 17363; GQ461729 C: Family XIII incertae sedis cluster 9	89.2%
XBB4001	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 29	95.0%
XBD1002	D: Rods and spheres Q: p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae	B: <i>Treponema bryantii</i> type strain: DSM 1788; FR749895 C: Spirochaetales cluster 3	97.6%
XBD1003	D: Tiny spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	B: <i>Eubacterium</i> sp. F1; EU281854 C: Bacteroidetes cluster 1	98.1%
XBD1006	D: Rods and spheres Q: k__Bacteria	B: Rumen bacterium 3/9293-14A; DQ392995 C: Pseudobutyrvibrio cluster 1	95.5%
XBD1007	D: Rods with expanded regions Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium</i> sp. A7-15; AB238885 C: Lachnospiraceae cluster 23	94.9%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
XBD1008	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B81; GU324363 C: Butyrivibrio cluster 18	99.4%
XBD2001	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Bacterium YE61; AY442825 C: Lachnospiraceae cluster 58	93.8% yes
XBD2002	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S150; AB198435 C: Veillonellaceae cluster 4	98.1%
XBD2003	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	97.4% yes
XBD2004	D: Small straight grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> JK614; GU121459 C: Butyrivibrio cluster 1	99.1%
XBD2005	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A21; GU324371 C: Lachnospiraceae cluster 3	99.6%
XBD2006	D: Small faint rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio hungatei</i> AR10; FJ794074 C: Butyrivibrio cluster 1	100.0% yes
XBD2009	D: Diplococci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 36	95.4%
XBD2012	D: Sparse cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	98.2%
XBD4001	D: Grainy sausage rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas ruminantium</i> S150; AB198435 C: Veillonellaceae cluster 4	98.3%
XBD4002	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavifaciens</i> LB4; AY445603 C: Ruminococcus cluster 1	98.8%
XBD4004	D: Very faint rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	B: <i>Eubacterium</i> sp. F1; EU281854 C: Bacteroidetes cluster 1	98.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
XPB1001	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	99.1%
XPB1003	D: Long rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4B65; GU324408 C: Lachnospiraceae cluster 46	99.8% yes
XPB1008	D: Small spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrvibrio cluster 10	98.5% 99.8%
XPB1009	D: Clumps, rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio fibrisolvens</i> NCDO 2222; X89971 C: Butyrvibrio cluster 10	99.8%
XPB1011	D: Thick rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: <i>Selenomonas</i> sp. MCB2; EF195237 C: Veillonellaceae cluster 7	97.4%
XPB1012	D: Spheres large rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococaceae R-25 like cluster 1	99.3%
XPB1013	D: Rods in chains and debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 37	90.2% yes
XPB1014	D: Large thick rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio proteoclasticus</i> B316; CP001810 C: Lachnospiraceae cluster 8	91.8% yes
XPB2001	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrvibrio hungatei</i> AR10; FJ794074 C: Butyrvibrio cluster 1	100.0%
XPB2002	D: Debris Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella corporis</i> JCM 8529; AB547677 C: Prevotella cluster 6	90.6%
XPB2004	D: Chains of short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	98.3%
XPB3001	D: Grainy rods Q: p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	B: <i>Propionibacterium acnes</i> 1570; JF277163 C: Propionibacterium cluster 1	98.4%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
XPB4001	D: Fat curved rods Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumex bacterium R-9; AB239482 C: Prevotella cluster 19	94.9% yes
XPD1003	D: Cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
XPD1004	D: Rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumex bacterium NK4A171; GU324380 C: Ruminococcaceae R-25 like cluster 8	96.1% yes
XPD1005	D: Grainy rods in clusters Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	98.9%
XPD1008	D: Motile cocci and the occasional rod Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A578; GU124465 C: Butyrivibrio cluster 1	100.0%
XPD1009	D: Small motile rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A63; GU124466 C: Butyrivibrio cluster 18	98.9%
XPD1010	D: Stuck to the tube very viscous spheres and large clusters of spheres in sheaths like long rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A21; GU324371 C: Lachnospiraceae cluster 1	93.4%
XPD2001	D: Clumps of rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium R-26; AB239490 C: Butyrivibrio cluster 8	97.7%
XPD2002	D: Cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> M55; AY699273 C: Butyrivibrio cluster 13	96.5%
XPD2003	D: Curved rods motile Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A179; GU324381 C: Lachnospiraceae cluster 46	99.3%
XPD2004	D: Cocci in pairs Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	96.5%
XPD2006	D: Rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> OB251; U77341 C: Butyrivibrio cluster 10	98.0%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
XPB2007	D: Thick rods in pairs Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A65; GU324373 C: Ruminococcaceae R-25 like cluster 12	97.1% yes
XPB3001	D: Short rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	96.7%
XPB3002	D: Cocci in short chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> LP-R1-Adx; AF104835 C: Ruminococcus cluster 6	97.2% yes
XPB3003	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4B32; GU324392 C: Ruminococcus cluster 18	97.5% yes
XPB4001	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella ruminicola</i> BP1-40; AB501153 C: Prevotella cluster 43	99.1%
YAB2001	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.6%
YAB2003	D: Rods of uneven sizes Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella albensis</i> JCM 12258; AB547669 C: Prevotella cluster 5	88.8% yes
YAB2005	D: Short curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	B: Bacterium L4M2 1-7; AY862595 C: Veillonellaceae cluster 1	96.4%
YAB2006	D: Rods in chains twisted around each other Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A5235; GU124463 C: Lachnospiraceae cluster 31	95.1% yes
YAB2008	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster 33	96.7%
YAB2009	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Anaerostipes sp. 5_1_63</i> FAA; JF412658 C: Lachnospiraceae cluster 12	90.7%
YAB3001	D: Tiny rods and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> NCDO 2432; X89973 C: Butyrivibrio cluster 4	97.2% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
YAB3002	D: Cocci and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium xylanophilum</i> ; L34628 C: Lachnospiraceae cluster 12	89.0%
YAB3003	D: Curvy rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Pseudobutyrvibrio ruminis</i> ; pC-XS7; AF202262 C: Pseudobutyrvibrio cluster 1	99.6%
YAB3004	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 3	98.4%
YAD2002	D: Very large rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium aminophilum</i> ; 152R-1b; DQ278862 C: Lachnospiraceae cluster 43	90.5%
YAD2003	D: Cocci in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumens bacterium NK2B18; GU324368 C: Ruminococcus cluster 9	99.3%
YAD2006	D: Cocci in chains and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	96.9%
YAD3003	D: Chains of cocci and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumens bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 3	98.0%
YAD3004	D: Sparse spheres and short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	B: Peptostreptococcaceae bacterium oral taxon 091; VE061; GU400653 C: Family XIII incertae sedis cluster 8	89.1%
YGB2001	D: Tiny rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumens bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.8%
YGB2002	D: Cocci in chains or grainy rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
YGB2004	D: Spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Clostridium jejuense</i> HY-35-12; AY494606 C: Lachnospiraceae cluster 44	90.7%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
YGB2005	D: Large grainy rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK2B42; GU324359 C: Oribacterium cluster 2	99.1%
YGB2006	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 51	99.3%
YGB2007	D: Sparse cocci Q: k__Bacteria	B: <i>Eubacterium</i> sp. CJ70; AB080901 C: Erysipelotrichaceae cluster 2	91.1% yes
YGB2008	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium uniforme</i> X3C39; GU269550 C: Lachnospiraceae cluster 60	98.7%
YGB3002	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumex bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.1%
YGB3003	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK2A1; GU324367 C: Lachnospiraceae cluster 20	97.4%
YGD2001	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	98.9%
YGD2002	D: No description available Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium NK4C38; GU324409 C: Lachnospiraceae cluster 52	97.3% yes
YGD2003	D: Tiny curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumex bacterium 8/9293-6; DQ393014 C: Pseudobutyrvibrio cluster 1	99.8%
YGD2004	D: Tiny cocci in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumex bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 26	98.9%
YGD2005	D: Small and large cocci some in chains Q: k__Bacteria	B: <i>Eubacterium</i> sp. CJ70; AB080901 C: Erysipelotrichaceae cluster 2	91.1% yes
YGD3001	D: Cocci Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: Rumex bacterium NK4C107; GU324396 C: Prevotella cluster 34	99.6%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
YGD3002	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK3A76; GU324399 C: Ruminococcus cluster 24	92.5%
YRB2001	D: Grainy straight rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
YRB2002	D: Grainy straight rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
YRB2003	D: Short blunt ended rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Butyrivibrio cluster 18	99.1%
YRB2005	D: Tiny rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Butyrivibrio cluster 18	99.3%
YRB2006	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	97.0%
YRB3001	D: Straight rods Q: k__Bacteria	B: <i>Butyrivibrio fibrisolvens</i> JL5; AY029616 C: Pseudobutyrvibrio cluster 1	99.8%
YRB3002	D: Chains of large cocci and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A171; GU324380 C: Ruminococcaceae R-25 like cluster 9	96.8% yes
YRB5001	D: Cocci or spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 1	96.5%
YRD2001	D: Faint small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A189; GU324382 C: Lachnospiraceae cluster 36	93.3%
YRD2002	D: Spheres Q: p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	B: <i>Prevotella bryantii</i> C21a; AF396925 C: Prevotella cluster 1	99.8% yes
YRD2003	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> C94; AM915269 C: Ruminococcus cluster 7	95.6% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
YRD2004	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	99.3%
YRD2005	D: Very thin long straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 6	94.7% yes
YRD3001	D: Cocci in chains and spheres Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	100.0%
YSB2001	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> Bu 43; X89976 C: Butyrivibrio cluster 4	99.8%
YSB2002	D: The odd large oval plus debris Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium cellulosolvens</i> ; L34613 C: Lachnospiraceae cluster 48	98.8%
YSB2003	D: Straight rods faint Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A86; GU324388 C: Ruminococcaceae R-25 like cluster 14	95.8% yes
YSB2004	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.6%
YSB2005	D: Curved rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Parasporobacterium paucivorans</i> SYR1; AJ272036 C: Lachnospiraceae cluster 16	93.3%
YSB2006	D: Cocci in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium R-25; AB239489 C: Ruminococcaceae R-25 like cluster 1	99.8%
YSB2007	D: Short rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: Rumen bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	99.1%
YSB2008	D: Very small cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Eubacterium ruminantium</i> GA195; AB008552 C: Lachnospiraceae cluster S9	95.5% yes
YSB3007	D: Large rods in chains Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__	B: Rumen bacterium R-7; AB239481 C: ClostridialesR-7 like cluster 9	93.1% yes

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
YSB3008	D: No description available Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	100.0%
YSB3010	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	96.3%
YSB4001	D: Grainy sausage rods Q: p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	B: <i>Lactobacillus vitulinus</i> TB-A09; AB425916 C: Erysipelotrichaceae cluster 1	99.3%
YSB4010	D: Small straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: <i>Butyrivibrio fibrisolvens</i> C219a; EU346756 C: Butyrivibrio cluster 18	99.1%
YSD2001	D: Tiny cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	98.9%
YSD2004	D: Tiny cocci in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A76; GU324387 C: Ruminococcaceae R-25 like cluster 5	96.6%
YSD2005	D: Curved rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.8%
YSD2009	D: Small rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK4A142; GU324402 C: Butyrivibrio cluster 1	99.7%
YSD2010	D: Long chains of cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	B: <i>Eubacterium infirmum</i> YQ024; GU400910 C: Family XIII incertae sedis cluster 7	88.0%
YSD2013	D: Very faint rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium NK3B85; GU124461 C: Lachnospiraceae cluster 23	95.4%
YSD2014	D: Cocci in chains and clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Lachnospiraceae bacterium DJF_CP64; EU728726 C: Lachnospiraceae cluster 32	97.2%
YSD2015	D: Straight rods Q: p__Firmicutes;c__Clostridia;o__Clostridiales	B: Rumen bacterium NK4A237; GU324386 C: Ruminococcaceae R-25 like cluster 6	94.2%

Isolate name	D: Cell description Q: Taxonomic assignment	B: Top BLAST match C: Cluster designation	B: Match C: Picked
YSD3003	D: Very small cocci in clumps Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	B: Rumen bacterium 7/94-26; DQ393006 C: Butyrivibrio cluster 12	96.1%
YSD3008	D: Cocci Q: p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	B: <i>Ruminococcus flavefaciens</i> Y1; AF104846 C: Ruminococcus cluster 8	96.8%

Appendix 2

```
#PERL script to convert and sort distance matrix from phylogenetic trees
generated in MEGA, tree format to parse in Newick format
#format: sort_matrix.pl [Newick_tree] [matrix]
use strict;

#tree file
my $newick_file = $ARGV[0];
my $matrix_file = $ARGV[1];

#read tree file and parse out order of entries
my $tree_content;
{
    local ($/, *READ);
    open READ, $newick_file;
    $tree_content = <READ>;
    close READ;
}

#$tree_content =~ s/[\\(\\),;\\-]/gs;
$tree_content =~ s/\\:|-?\\d+\\.\\d+\\/\\:\\/gs;
$tree_content =~ s/\\s*\\.\\s*\\/\\:\\/gs;
$tree_content =~ s/\\:+/\\:\\/gs;
my @tree_order = split/\\:/,$tree_content;
my @temp = ();
#cleanup array
foreach my $entry (@tree_order) {
    next if ($entry =~ /^\\[\\(\\)]$/);
    next if ($entry =~ /^\\[\\(\\)]\\;/);
    #print "\\nBefore: ...$entry ....";
```

```

$entry =~ s/^{2,}/^(/;
$entry =~ s/^\./;
if ($entry !~ m/^(\\d+)/) {
    $entry =~ s/^(//;
}
if ($entry =~ m/^(\\') {
    $entry =~ s/^(\\'/;
    $entry =~ s/\\'$//;
}
if ($entry =~ m/^(\\'+\\'$/) {
    $entry =~ s/\\'$//;
    $entry =~ s/^(\\'/;
}
die "Error: $entry ..." if ($entry !~ /^w+/);
push (@temp, $entry);
#print "\nAfter: ...$entry ....";
#my$ttt = <STDIN>;
}
@tree_order = @temp;
#$tree_content =~ s/\\d+\\.\\d{4}/ /gs;
#$tree_content =~ s/\\s+/ /gs;
#die "\n...$tree_content ...";
#my @tree_order = split/\\s+/, $tree_content;

#assume that all entries are bracketed by ";
#my @tree_order = ($tree_content =~ m/^(\\'+)\\'/gs);
#my @tree_order = split:/, $tree_content;

#read matrix file
my $matrix_content;
{
    local ($/, *READ);

```

```

open READ, $matrix_file;
$matrix_content = <READ>;
close READ;
}

my %matrix;
$matrix_content =~ s/^\.*?\n\n//s;

#get order of entries in array and hash
my %entry_matrix;
$matrix_content =~ m/([\.+?])\n\n/s;
my $entries = $1;
$entries =~ s/[\s+]/ /gs;
my @temp = ($entries =~ m/[[\s\d]+?\]\s+\#(\S+)/g);
my $count = 1;
foreach my $entry (@temp) {
    next if ($entry !~ /\w+/);
    $entry_matrix{$entry} = $count;
    $count++;
}

#first establish position of each new entry
my $counter = 1;
my %new_order;
my %new_taxa;
foreach my $entry (@tree_order) {
    #print "\n...$entry ...";#my $ttt = <STDIN>;
    next unless ($entry =~ /\w+/);
    $entry =~ s/\/ //g;
    #new position -> old matrix position
    $new_order{$counter} = $entry_matrix{$entry};
    $new_taxa{$counter} = $entry;
}

```

```

    $counter++;
}

#get matrix
$matrix_content =~ s/([\.+?]\n\n/s;
'reset' =~ m/reset/;
$matrix_content =~ m/([\.+)/s; #\n\nTable/s;
my $temp = $1;
my @dm = split/\n/, $temp;
shift @dm; #get rid of header
my $counter_vertical = 1;
my $counter_horizontal = 1;
my $distance_matrix;
#this replicates the lower half matrix
foreach my $entry (@dm) {
    $entry =~ s/^\s*[[\s\d]+\]\s*//; #get rid of line number, captured by counter

    #set the case for the very first instance in table
    $distance_matrix->{'1'}->{'1'} = '0';
    my @line = split/\s+/, $entry;

    foreach my $distance (@line) {
        if ($counter_vertical == $counter_horizontal) {$distance = '0'};
        $distance_matrix->{$counter_vertical}->{$counter_horizontal} = $distance;
        $counter_horizontal++;
    }
    $distance_matrix->{$counter_vertical}->{$counter_vertical} = '0';
    $counter_horizontal = 1;
    $counter_vertical++;
}
my $max_count = $counter_vertical - 1;
#now mirror matrix

```

```

    foreach my $counter_vertical (keys %{$distance_matrix}) {
        foreach my $counter_horizontal (keys %{$distance_matrix->{$counter_vertical}}) {
            $distance_matrix->{$counter_horizontal}->{$counter_vertical} =
            $distance_matrix->{$counter_vertical}->{$counter_horizontal};
        }
    }

#sort matrix according to newick order and format to import into excel
my @new_matrix;
my $new_line = "";
foreach my $new_position (sort {$a<=>$b} keys %new_order) {
    $new_line .= $new_taxa{$new_position}."\t";
    for (my $i = 1; $i <= $max_count; $i++) {
        #next if ($i == $new_position); #skip self
        $new_line .= $distance_matrix->{$new_order{$new_position}}->{$new_order{$i}}."\t";
    }
    $new_line .= "\n";
}
open WRITE, "+>lower_triangle_matrix.txt";
my $header = join ("\t", @tree_order);
print WRITE "\t".$header."\n".$new_line;
close WRITE;

die;

#my @new_matrix;
#my $new_counter = 1;
#my $new_line = "";
#foreach my $entry (@tree_order) {
# next if ($entry !~ /\w+/);

```

```

# print "\n...$entry ...$entry_matrix {$entry} ...";<STDIN>#
#
# $new_line = $entry."\t";
# #entry refers to vertical counter now stored in %entry matrix
#     foreach my $counter_horizontal (keys %{$distance_matrix-
>{$entry_matrix{$entry}}}) {
#         $new_line .= $distance_matrix->{$entry_matrix{$entry}}-
>{$counter_horizontal}."\t";
#     }
# push (@new_matrix, $new_line);
#}

#create lowe triangle matrix
#open WRITE, "+>lower_triangle_matrix.txt";
#my $header = join ("\t", @tree_order);
#print WRITE $header."\n";
#foreach my $entry (@new_matrix) {
#   $entry =~ s/self.+//;
#   print WRITE $entry."\n";
#}
#close WRITE;

#print matrix into file for testing
open WRITE, "+>symmetrixal.txt";
foreach my $counter_vertical (sort {$a<=>$b} keys %{$distance_matrix}) {
    my $line = "\[$counter_vertical\] ";
    foreach my $counter_horizontal (sort {$a<=>$b} keys %{$distance_matrix-
>{$counter_vertical}}) {
        $line .= $distance_matrix->{$counter_vertical}->{$counter_horizontal}.' ';
    }
    print WRITE $line."\n";
}

```

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