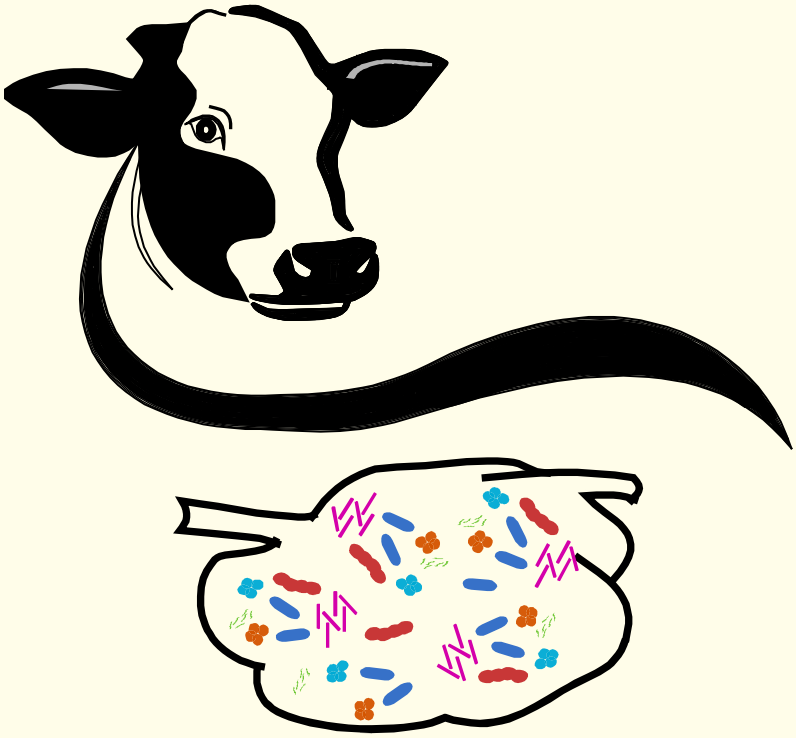
Assessing rumen microbial composition and fibre attachment in dairy cows



Jueeli Deepak Vaidya

Propositions

1) The perfect DNA extraction method does not exist.
   (this thesis)

2) Fibre degrading bacteria are key players in ruminal methanogenesis.
   (this thesis)

3) Microbiome studies need equal attention irrespective of the host.

4) Humans are the main cause of biodiversity loss.

5) Every PhD student should have multiple supervisors.

6) Artificial intelligence lacks the theory of mind.

7) There is underestimation of time and effort for data analysis when compared to laboratory work.

Propositions belonging to the thesis entitled:

"Assessing rumen microbial composition and fibre attachment in dairy cows"

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Assessing rumen microbial composition and fibre attachment in dairy cows

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Chapter 1

General introduction and Thesis outline

Temperatures all over the world have been continuously rising for more than a century. Since 1970, the average surface temperature of the earth has been rising at an average rate of 0.17°C per decade. This climate change due to human activities has fuelled increasing concerns. In 2015, greenhouse gas (GHG) emissions were reduced by 22% when compared with 1990 levels. This reduction was mainly achieved through decreasing fuel combustion as a way to reduce GHG emissions. Currently, the European Union (EU) aims to reduce 20% of its GHG emissions by 2020 and 30% by 2030 as compared with 1990 levels. In order to ameliorate the severe impacts of human activities on local and global climate, there is an agreement from the EU wherein the global warming should be kept below 2 ºC, indicating that the temperature should not increase more than 1.2 ºC above today's values, with the aim to stop the growth of GHG emissions by 2020 (www.ec.europa.eu/eurostat).

METHANE AS A GREENHOUSE GAS

When the sun's energy reaches the earth, some of it is reflected back to space while the rest is absorbed and re-radiated by GHGs, a process often referred to as the GHG effect. Most important GHGs include water vapour, nitrous oxide (N_2O), carbon dioxide (CO_2), methane (CH_4), ozone (O_3) and some manmade chlorofluorocarbons (CFCs). While CO_2 is considered to be the most important GHG associated with climate change (Steinfield et al., 2006), CH_4 is the most abundant non-CO_2 GHG in the atmosphere today (Hansen et al., 2000; Montzka et al., 2011). Furthermore, CH_4 has 25 times the warming effect of CO_2 as it strongly absorbs infrared radiation. As a consequence, CH_4 is thought to contribute 4–9% of the global GHG effect based on its atmospheric concentration and half-life (Forster et al., 2007). There are multiple sources of CH_4 (Dean et al., 2018b), and they can be broadly classified as being either natural or anthropogenic (Table 1). Natural sources are dominated by wetlands, fresh water systems, coastal sediments and oceans, CH_4 hydrates, geological sources and fauna. Anthropogenic sources mainly include agriculture and waste, as well as combustion of biomass and fossil fuels. Enteric fermentation from ruminants, together with manure, accounts for 56% of the methane that is produced from agriculture and waste (Dean et al., 2018b).

Table 1. Identified global natural and anthropogenic CH_4 sources and estimates of annual CH_4 budget as taken from (Dean et al., 2018b). Values indicate the mean and range of global CH_4 (Tg per year) budgets and inventories estimated from observations at the Earth's surface.

Sources	Estimated annual CH_4 Budget in Tg CH_4 per year
Total natural sources	
Natural wetlands	185 (153*–227**)
Other natural sources	199 (104–297)
Other land sources	185 (99–272)
Freshwaters	122 (60–180)
Geological (onshore)	40 (30–56)
Wild animals	10 (5–15)
Termites	9 (3–15)
Wildfires	3 (1–5)
Permafrost soils (direct)	1 (0–1)
Oceanic sources	14 (5–25)
Geological (offshore)	12 (5–20)
Total anthropogenic sources	
Agriculture and waste	195 (178–206)
Enteric fermentation and manure	106 (97–111)
Landfills and waste	59 (52–63)
Rice cultivation	30 (24–36)
Fossil fuels	121 (114–133)
Coal mining	41 (26–50)
Gas, oil, and industry	79 (69–88)
Biomass and biofuel burning	30 (27–35)

Global CH_4 emissions are expected to increase by 7% in 2055, based on the expected farming and consumer lifestyle practices as compared to 1995. This makes the decrease of enteric CH_4 emissions one of the main targets of GHG mitigation of the ruminant livestock production sector, particularly dairy cows (Hristov et al., 2013). CH_4 from ruminants is mainly produced in the rumen (87%) as a result of microbial fermentation of feed plant material under anoxic conditions, and is released into the environment via eructation and breath (Boadi et al., 2004). As the CH_4 is exhaled, it also represents a loss of ingested feed-derived energy which varies from ~2-12% depending on diet (Hook et al., 2010).

RUMEN ANATOMY AND PHYSIOLOGY

Ruminants possess a complex rumen ecosystem with the resident rumen microbial community known to play vital roles in host productivity and health, as well as with respect to the environmental footprint of ruminant livestock production. Unlike in monogastrics, the stomach (or the fore-gut) of the ruminant animal is composed of the reticulum, rumen, omasum and abomasum (Fig. 1). The reticulum and rumen (reticulo-rumen) are joined by a fold of tissue and various pillars. The reticulo-rumen is the largest part of the fore-gut, and fills three-quarters of the abdominal cavity. The pillars help in contraction of various sacs that help in circulating and mixing of the ingested feed (Dehority, 2002) (Fig. 1). Feed when ingested by ruminant cattle gets mixed with saliva. Saliva is known for high concentrations of sodium and potassium bicarbonate and phosphate and helps in buffering the acid produced during ruminal fermentation (Dehority, 2002). The bolus that is subsequently formed can be easily swallowed, and is then transferred to the rumen from the reticulum. After fermentation in the reticulo-rumen, the feed passes into the omasum which serves as a filter pump to sort liquids and fine particles. Additionally, the omasum absorbs water, minerals and nitrogen. The abomasum is where the gastric juices are formed. The hydrochloric acid and the digestive enzymes needed for breakdown of feeds are secreted into the abomasum, and retention time of the feed in the abomasum does not exceed one to two hours. Further breakdown of particles occurs in the small and large intestine, and the residue that flows out from the large intestine enters the colon and gets excreted as manure.

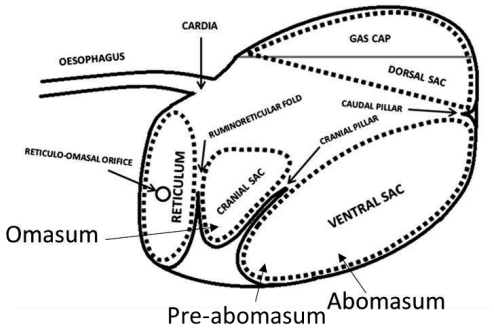

Figure 1. Basic representation of a cow rumen (ecow.co.uk/biology-of-the-rumen/)

The process of rumination involves regurgitation of the ingesta from the reticulo-rumen, re-chewing of solids accompanied by salivary secretion and finally re-swallowing of the bolus. While the digestion of the feed in the mouth is mostly mechanical, the digestion in the rumen is due to microbial activities. Hence, a combined effect of ruminal mastication, host enzymatic action and microbial activity results in breakdown of plant fibres, thereby reducing the particle size. The time spent for rumination depends on the particle size of the ingested feed, with the average retention time varying from 34 - 40 hours depending on the feed (Dehority, 2002; Huhtanen et al., 2016; Processi et al., 2016).

THE ROLE OF RUMEN MICROBIOTA IN ENTERIC METHANOGENESIS

Rumen microbes in general degrade plant derived feed in the rumen that can be either fibrous (cellulose and hemicellulose) or non-fibrous (starches and simple sugars) carbohydrates. Rumen microbes function via complex interactions, which help them to sustain their population and activity. Rumen fermentation is carried out by rumen microbes that hydrolyse the polysaccharides into monomers and further ferment them into volatile fatty acids (VFA), succinate, formate, lactate and gases including ammonia (NH_3), H_2 and CO_2. The VFA and NH_3 are usually absorbed through the walls of the pre-abomasal compartments by diffusion while H_2 and CO_2 are converted to CH_4, which is released via eructation. Additionally a part of the fermentation products is also used by rumen microbes for their nutritional supply (Agarwal et al., 2015). During ruminal fermentation, the metabolites acetate, propionate and butyrate make up more than 95% of VFAs. The stoichiometry of the production of these aforementioned major VFAs determines the amount of CO_2 and CH_4 associated with acetate, propionate or butyrate production. For instance, per mole of glucose fermented to 2 moles of acetate, 2 moles of CO_2 and 4 moles of H_2 are released which enables rumen methanogenic archaea to utilize the 4 moles of H_2 and reduce 1 mole of CO_2 to 1 mole of CH_4. Butyrate production results in 2 moles of H_2 and 2 moles of CO_2 per mole of glucose and thereby requires a net input of reducing equivalents to produce CH_4. Propionate uses H_2 for its production thereby acting as a hydrogen sink. Thus, with propionate formation less CH_4 is produced by the methanogens and consequently more VFA is available to the ruminant for nutrient supply.

Bacteria are the most diverse microbial group in the rumen, and are capable of utilizing a range of different feed components (Stewart et al., 1997). Rumen studies have consistently

identified Firmicutes and Bacteroidetes as the pre-dominant bacterial phyla (De Menezes et al., 2011b; Huws et al., 2016). However, distinct members of these phyla are associated with the liquid or fibrous fractions where they are present in higher or lower relative abundances (Klevenhusen et al., 2017). Rumen microbiota in specific micro-environments play different roles in plant feed degradation. The bacteria (10^{10} per ml) typically form the largest component of the microbial biomass (Mackie, 1997). Bacteria contribute to 80% of the degradation activity. In late studies that used next-generation sequencing technology (NGS), differences in the fluid and the fibrous fractions in dairy cows have been reported (Fouts et al., 2012; Mao et al., 2015; Ji et al., 2017). The predominant taxa in the fibrous content of the rumen belonged to Firmicutes and Bacteroidetes in the study of Mao et al. (2015). At genus level, the proportions of *Prevotella*, unclassified Ruminococcaceae, unclassified Rikenellaceae, unclassified Christensenellaceae and unclassified Bacteroidales were significantly higher in rumen fibrous content than the liquid fraction (Mao et al., 2015). Moreover, in the study of Fouts et al. (2012), seven genera differed significantly between the liquid and the fibrous fractions in dairy cows. Herein, *Prevotella* was over represented in the liquid fraction which is in contrast to the finding of (Mao et al., 2015). In addition, the liquid fraction also had higher relative abundances of *Tannerella* than the solid fraction. Conversely, *Butyrivibrio* and *Blautia* (both members of the order Clostridiales) were present at significantly higher relative abundance in the solid fraction of the rumen. Furthermore, genus-level taxa within the Ruminococcaceae, Lachnospiraceae and unclassified Clostridiales were found more abundant in the liquid fraction compared to the fibrous fraction (Fouts et al., 2012). The fibrous fraction has also been shown to have higher relative abundances of *Succinivibrio* as compared to the liquid fraction in dairy cows (Ji et al., 2017). The difference in fractions can be associated with nutrient digestibility, rumen retention times and biofilm formation. Rumen microorganisms have traditionally been classified in accordance with their main metabolic activity, i.e. fibrolytic which include butyrate, acetate and propionate producers (e.g., *Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes, and Butyrivibrio fibrisolvens*), amylolytic (e.g., *Selenomonas ruminantium, Streptococcus bovis*), proteolytic (e.g., *Prevotella* spp.), lipolytic (e.g., *Anaerovibrio lipolytica*), lactate producers (e.g., *S. bovis and S. ruminantium*), and lactate consumers (e.g., *Megasphaera elsdenii*) (Belanche et al., 2012). Among the numerous ruminal bacteria identified, *Ruminococcus* and *Eubacterium* spp. are identified as H_2 producers while cellulolytic *Fibrobacter* spp. do not produce H_2 and *Bacteroides* spp. are considered to be net H_2 utilizers.

More recently, rumen microbiome analyses have identified 'ruminotypes' depending on CH_4 emission measurements (Kittelmann et al., 2014). For instance, in sheep the low CH_4 ruminotype Q was characterized by high relative abundance of *Quinella ovalis* while the low-CH_4 ruminotype S was associated with high relative abundances of lactate and succinate utilizers including *Fibrobacter* spp., *Kandleria vitulina, Olsenella* spp., *Prevotella bryantii,* and *Sharpea azabuensis*. The high CH_4 ruminotype was characterized by high relative abundances of Lachnospiraceae, Ruminococcaceae, Alphaproteobacteria, Coprococcaceae, *Prevotella* and Bacteroidales suggesting that CH_4 production is dependent on these H_2 producing bacteria. In goats there was increased relative abundance of *Prevotella* and *Selenomonas* spp. when chemical inhibition of methanogens took place (Denman et al., 2015). Furthermore, high CH_4 emitting beef cattle were characterized by low relative abundances of Succinivibrionaceae (Wallace et al., 2015). Conversely, this suggests that Succinivibrionaceae spp. occur at higher relative abundances in low emitting beef cattle. Interestingly, members from this family have also been found in Tammar wallabies, which are also foregut fermenters (Pope et al., 2011). In the study of Pope et al. (2011), metagenome analyses revealed that 77% of the recovered proteobacterial sequences could be assigned to the Wallaby group I (WG-1) within the Succinivibrionaceae. Cultured representatives included in this family are *Succinivibrio, Prevotella* and *Anaerobiospirillum.* Members of Succinivibrionaceae produce succinate as their principal fermentation end-product and use exogenous H_2 to stimulate succinate formation as a way of H_2 sink. Genome analysis of WG-1 revealed that it is closest to *Anaerobiospirillum succiniproducens,* which employs an anaplerotic reaction to produce oxaloacetate (OAA) from phosphoenol pyruvate (PEP), subsequently reducing OAA to produce succinate as the end-product. This H_2-consuming metabolism helps to explain contributions of WG-1 in the foregut of Tammar Wallabies to low CH_4 phenotypes (Pope et al., 2011). It should be noted that individual members of specific genera can be differentially associated with either high or low CH_4 emission, suggesting differences in their metabolism and/or ecology, as was observed from the two studies of cattle and Tammar wallabies where some *Prevotella* related OTUs were associated with high CH_4 emitters and some with low CH_4 emitters.

Another bacterium that has been associated with low CH_4 emissions from dairy cattle is *Faecalibacterium prausnitzii* (M Ross et al., 2013), which is very rarely observed in rumen studies. This bacterium belonging to Firmicutes is a butyrate producer that uses the butyryl CoA : acetyl CoA transferase enzyme to catalyze the last part of the butyrate pathway.

Almost all of the archaea in the rumen are methanogenic. Rumen methanogens utilise mainly H_2 and CO_2 produced by other fermentative rumen microbes (bacteria, protozoa and anaerobic fungi) producing CH_4. Formate and methyl compounds are also available as substrates for the methanogens. Methanogens occupy different niches in the rumen, some being ectosymbionts or endosymbionts of protozoa and anaerobic fungi, whereas others occur free living in the liquid or associated with feed particles (Morgavi, 2010; Valle et al., 2015).

Members of the genus *Methanobrevibacter* are often the most abundant archaea in the bovine rumen (Danielsson et al., 2017). In addition, *Methanosphaera* spp., *Methanimicrococcus* spp. and *Methanobacterium* spp. occur at lower abundances (Hook et al., 2010). Further, a newly proposed methanogenic order, Methanomassillicoccales, has only recently been acknowledged as being the second largest archaeal population in the rumen after *Methanobrevibacter* (Jin et al., 2011). Methylamines and methanol are substrates for Methanomassillicoccales in the rumen (Friedman et al., 2017). *Methanobrevibacter ruminantium, Methanomicrobium mobile, Methanosarcina mazei, Methanosarcina barkeri and Methanobacterium formicicum* have all been isolated from the rumen (Janssen and Kirs, 2008). Among these, *M. ruminantium, M. formicicum* and *M. mobile* utilize H_2/CO_2 and formate to produce CH_4 while *M. mazei* and *M. barkeri* use methyl compounds to produce CH_4. The ruminal environment that provides H_2 and CO_2 contains mostly hydrogenotrophic archaea, rather than acetoclastic methanogens, even though acetate concentrations are high in the rumen. The absence of acetoclastic methanogens is mainly due to the rapid passage rate of the feed. In vivo studies have demonstrated that inhibiting methanogens can lead to decreased acetate-propionate ratios thereby having more reduced VFAs than acetate (Patra et al., 2012; Lopes et al., 2016; Patra et al., 2017). This further underlines the importance to understand the composition of methanogens and their interactions with other rumen microbes in order to provide the necessary basis for strategies to mitigate CH_4 emissions.

Rumen protozoa produce H_2 in a specialized organelle called the hydrogenosome, which is similar to the mitochondrion of aerobic eukaryotes (Morgavi, 2010). Protozoa that are associated with methanogens as endo- or ectosymbionts, provide the methanogens with this H_2 through interspecies H_2 transfer (Morgavi, 2010).

Anaerobic fungi are regarded as degraders of fibrous material due to their ability to produce polysaccharide degrading enzymes. Firstly, the enzymatic hydrolysis results in

opening of the plant fibres which allow the fungi to penetrate complex structural barriers (Ho, 1988; Joblin, 1989; Orpin, 1997). Anaerobic fungi are furthermore known to increase the surface area available for colonisation or attachment of other microbes (bacteria) (Gruninger et al., 2014).

Protozoa ingest rumen bacteria as their main protein source, resulting in increased recycling of ingested N in the rumen and 20-28% decreased amino acid supply to the intestine of the animal. Their role in rumen feed degradation has not yet been unequivocally elucidated, however, reducing the number of protozoa has been shown to enhance feed conversion efficiencies (Williams, 1992). Protozoa can also reduce the number of fungi due to their ability to produce chitinases and to predate on fungal zoospores (Widyastuti et al., 1995).

STRATEGIES TO REDUCE ENTERIC METHANOGENESIS THROUGH BIOTECHNOLOGY, ADDITIVES AND FEEDING

The global concern for CH_4 mitigation is increasing as a result of climate change. With this, concerns regarding rumen CH_4 emissions have led researchers to study different ruminal fermentation pathways as a way to provide the necessary insights for the development of strategies towards decreasing CH_4 emissions. Mitigation strategies can be considered from two related perspectives: (a) improvement of rumen fermentation efficiency and (b) increasing the productivity of animals with respect to milk yield and energy utilization. To this end, efforts are being made in order to increase the productivity by reducing the number of less productive/unproductive animals through dedicated breeding.

Primary strategies proposed to date included altering rumen microbial composition and fermentation using a number of different approaches that are outlined in the following.

Direct fed microbials: *Saccharomyces cereviseae* (yeast)

DFM are commonly used for manipulation of biochemical pathways to decrease rumen methanogenesis, and more specifically for re-direction of H_2 thereby decreasing H_2 production during feed fermentation. Bio-hydrogenation of poly-unsaturated fatty acids utilizes H_2 and serves as a promising approach for lowering CH_4 formation. However, this pathway accounts for only 1 to 2 % of H_2 consumed (Nagaraja et al., 1997). There is a variety of naturally occurring DFM in the rumen that can be classified into bacterial or fungal DFM.

The most commonly used direct fed microbial (DFM) is yeast that has been successful in increasing ruminant productivity (Weimer, 2015). It improves rumen maturity by stabilising rumen pH and increasing activities of fibre degrading microbes (bacteria/anaerobic fungi) (Jeyanathan et al., 2014). One of the main factors that could explain the beneficial effects on fibre degrading bacteria is the capacity of live yeasts to scavenge oxygen, as there can be as high as 16 litres of oxygen entering the bovine rumen daily during feed and water intake, rumination and salivation (Fonty and Chaucheyras-Durand, 2006). Moreover, studies have also shown that live yeasts can reduce the redox potential of the rumen liquid suggesting favourable ecological conditions for anaerobic bacterial growth. Furthermore, germination of zoospores of the anaerobic fungus *Neocallimastix frontalis* and an in vitro filter-paper degradation was stimulated by two strains of *S. cerevisiae* (Fonty and Chaucheyras-Durand, 2006). Studies on live yeast supplementation have shown that it can stimulate the growth of lactate metabolizing bacteria, namely *Megasphaera elsdenii* or *Selenomonas ruminantium,* in vitro. Also, a study on active dry yeasts was performed in dairy cows and sheep, showing that supplementation led to reduced ruminal lactate concentrations, maintaining pH for an efficient rumen function (Fonty and Chaucheyras-Durand, 2006).

Propionate enhancers

Propionate formation is associated with H_2 utilization. In the rumen propionate is produced via the succinate or acrylate pathway. The succinate pathway involves the intermediates malate and fumarate and produces succinate, which can be further de-carboxylated to propionate. The succinate pathway has been shown to be used by lactate producers (e.g. *Selenomonas ruminantium*), fumarate reducers (e.g. *Wolinella succinogenes*), succinate producers (e.g. *Fibrobacter succinogenes*) and succinate utilisers (e.g. *S. ruminantium*). The acrylate pathway catalyzes the conversion of lactate to propionate. *Megasphaera elsdenii* is a major lactate utilizer that produces propionate via the acrylate pathway (Jeyanathan et al., 2014). *Prevotella ruminicola* can also form propionate via the acrylate pathway, however, the amount of propionate formed by this organism is not significant in the rumen (Wallnofer and Baldwin, 1967). Cows supplemented with *M. elsdenii* changed the pattern of rumen fermentation favouring propionate with potential benefits on energy balance and animal productivity. Moreover, a study with a mixed culture of *Propionibacterium jensii* and *Lactobacillus* spp. has shown to decrease CH_4 emissions showing their potential to mitigate rumen CH_4 (Jeyanathan et al., 2014).

Using other inhibitors

Halogenated aliphatic compounds like bromochloromethane (BCM), bromoethanesulfonate (BES), bromoform, carbon tetrachloride, trichloroacetamide and trichloroethyladipate are known to inhibit CH_4 formation. These halogenated compounds serve as electron acceptors and block the function of corrinoid enzymes, thereby inhibiting cobamide dependent methyl group transfer in methanogenesis. A study on BCM as a CH_4 inhibitor observed a negative effect with respect to total bacterial numbers, but growth of methanogens was inhibited in batch and continuous fermenters. These rumen microbial changes were, however, not further investigated (Goel et al., 2009). Chloroform has been shown to decrease CH_4 formation in cattle without affecting feed digestion (Knight et al., 2011).

Reduction of nitrate redirects H_2 away from methanogenesis. Nitrate serves as an alternative electron acceptor to endogenous fumarate in many propionate producing bacteria. Exposure of ruminal bacteria to nitrate has led to a decrease in ruminal cellulolytic activity, which was mainly attributed to the low numbers of cellulolytic and xylanolytic bacteria. Decreased cellulolytic activity can decrease rates of dry matter intake (Latham et al., 2016). Although nitrate may serve as alternative electron acceptors as well as be responsible for direct inhibition of ruminal methanogenesis, presently the use of nitrate as an electron acceptor is avoided as toxicity is associated with its reduction to nitrite (McAllister et al., 1996; Lee and Beauchemin, 2014). Inhibitors like 3-nitroxypropanol have been shown to bind to the methyl co-enzyme M reductase (MCR) active site thereby inactivating MCR activity for CH_4 formation and have been shown to decrease the 16S rRNA gene copy numbers (absolute count) of methanogens (Romero-Perez et al., 2015).

Other inhibitors such as the ionophores monensin and lasalocoid can be used to reduce ruminal CH_4 production (Ellis et al., 2012; Patra et al., 2017). As these compounds are lipophilic ion carriers, they pass through the cell wall of Gram-positive bacteria and penetrate into the cell membrane, thereby serving as Na+/K+ antiporters, dissipating ion gradients for ATP synthesis, nutrient transport and subsequently resulting in delayed cell division and cell death (Tedeschi et al., 2003). Ionophores are shown to inhibit Gram positive bacteria that produce acetate and H_2 and Gram negative bacteria as well that produce formate and H_2 (Patra et al., 2017). Ionophores are thus more related to inhibition of producers of CH_4 precursors (formate and H_2) rather than to a direct effect on methanogens as they are more resistant to

ionophores than H_2 producing bacteria. The reduction in CH_4 production that is brought about by ionophores has often been associated with growth inhibition of ciliate protozoa that produce H_2 (Nagaraja et al., 1997) In addition, the benefits of adding monensin have been associated to a protein sparing effect via direct inhibition of obligate amino-acid fermenting bacteria (Chen and Wolin, 1979; Mbanzamihigo et al., 1996). Additionally, plant secondary metabolites such as saponins, tannins, flavonoids, essential oils (clove, eucalyptus, garlic oil, peppermint, oreganum) have anti-microbial activities and have been used as potential inhibitors of rumen methanogens and CH_4 production (Patra et al., 2017). Moreover, thyme oil or cinnamon oil have been shown to decrease the relative abundances of methanogens (Khorrami et al., 2015).

DIETARY INFLUENCES ON RUMEN MICROBIOTA IN DAIRY COWS

Diet is considered as one of the major factors that influences composition and activity of the rumen microbiota. Forage has always been the most important energy carrier feed in dairy cows. Dairy feed-stuffs consist of 70% carbohydrates, with major constituents being starch and cell wall constituents such as cellulose, hemicellulose and pectin.

Cellulose is the most abundant polysaccharide in fibrous feed, and is composed of linear chains of β-1,4 – linked glucose units. Cellulose in plants does not have side residues as described for hemicellulose (McAllister et al., 1994). Hemicellulose comprises a broad range of hetero-polymers including xylan, glucoronoxylan, arabinoxylan, glucomannan and xyloglucan that are all characterized by a backbone structure of β-1,4 linked xylose residues. Various side chains are linked to the xylose residues such as acetic acid, arabinose, coumaric acid, ferulic acid, glucuronic acid or 4-O-methylglucuronic acid (McAllister et al., 1994). Pectin is a minor fraction in the plant cell wall and is composed of a backbone of α-1,4 linked residues of D-galacturonate (McAllister et al., 1994).

Starches are mainly α-glucans composed of two types of molecules: amylose and amylopectin. It is known that an increasing amount of starch in the ruminant diet leads to decreased CH_4 emission in dairy cows. The CH_4 yield was shown to be reduced when Benchaar et al. (2014) replaced beet pulp (fibrous concentrate) by barley (starchy concentrate). A recent study suggested that a critical dietary concentration of starch is required to alter ruminal methanogenesis (Hassanat et al., 2013).

Table 2 highlights several diets used for dairy cows and the microbial changes observed in bacterial composition with provided dietary interventions. Despite the range of different feeding treatments, similar predominant rumen phyla were identified, however, they differed in their relative abundances (Table 2). The studies mentioned in Table 2 have in common that they all focus on diets differing in starch and fibre ration, and corresponding effects on rumen microbiota composition were analysed using next generation sequencing of 16S ribosomal RNA (rRNA) genes. It should be noted, however, that none of these studies reported CH_4 measurements. Many other studies have shown that a high concentrate based diet results in lower CH_4 production as compared to a mixed forage-concentrate based diet (Rooke et al., 2014; Roehe et al., 2016; Duthie et al., 2017). However, due to animal to animal variation, the ratio of CH_4 to CO_2 as a proxy in these studies did not relate well to daily CH_4 measurements.

ANIMAL ASSOCIATED FACTORS AFFECTING THE RUMEN MICROBIOTA, FERMENTATION PATTERN AND ENTERIC METHANOGENESIS

Animal type (breed, age)

Ruminal CH_4 production can be influenced by numerous animal associated factors such as age, breed and geographical region. These factors affect rumen microbiota composition and function, including CH_4 production. The effect of age on CH_4 production has been investigated earlier in cattle and deer (Molano et al., 2006; Swainson and Clark, 2007), and ìt was considered likely to be associated with animal characteristics like dry matter intake (DMI), body weight (BW) and rumen retention time (Okine et al., 1989; Angela et al., 2000). Another study exploring the bovine rumen microbial community from birth to adulthood (2-year old) concluded that the microbial communities were influenced by age, however, the study did not correlate changes with enteric CH_4 production (Jami et al., 2013).

Table 2. Different dietary interventions and corresponding changes in microbial composition due to fibre/starch ration fed diets to dairy cows.

	Number of cows	***Diet***	***Period***	***Bacterial changes due to dietary interventions***	***Reference***
Cannulated cows	2	2 x 2 (Pasture +Total mixed ration (TMR))	14 days pasture + 14 days TMR	1.Higher abundances of Fibrobacteriaceae in pasture fed TMR 2.Higher abundances of Prevotellaceae, Erysipelotrichaceae, Veillonellaceae in pasture fed cows	De Menezes et al., 2011
Holstein Friesian	12	1. G1:88% purple guinea grass + 2% starch of dried cassava skin and cassava 2. G2: 76% fibre + 10% starch 3. G3: 57.5% fibre + 10% starch	2 months	1.High abundance of Firmicutes (Lachnospiraceae and Ruminococcaceae) in G1 > G2 > G3 2.High abundance of Prevotella in G2 > G1 > G3. 3.High abundances of Proteobacteria (*Desulfobulbus, Ruminobacter, Succinimonas, Pseudomonas*) in G3 > G1 > G2	Thoetkiattikul et al., 2013
Lactating dairy	3	3 x 3 (47% L. chinensis, 11% alfalfa hay and 42% maize silage (LC), 42% cornstalk, 11% alfalfa hay and 47% maize silage (CS), 38% alfalfa hay, 20% L. chinensis and 42% maize silage (AH)	14 day each diet	1.Higher abundances of *Anaerotruncus, Papillibacter, Thermoactinomyces, Actinopolyspora, Bacillus* and *Streptomyces* in CS compared to LC or AH diet. 2.Higher abundances of *Prevotella* and *Pyramidobacter* in CS diet as compared to those fed with AH diet. 3.Highest proportions of *Selenomonas* in AH diet. 4.Higher abundance of unclassified Rikenellaceae in CS diet.	Zhang et al., 2014
Non-lactating Kankrej cows	18	Dry/green roughage:concentrate: K1 (50:50); K2 (75:25) and K3 (100:0)	6 weeks each diet	Most abundant phylum Firmicutes and Bacteroidetes but the abundances of genera varied depending on the primer pairs in this study	Pitta et al., 2014
Chinese Holstein	32	2 groups (Mixed forage diet (MF) and corn stover diet (CSA))	91 days	Succinivibrionaceae lower abundance in CSA as compared to MF	Jin et al., 2016
Holstein heifers	24	Forage:Concentrate (80:20, 60:40, 40:60, and 20:80; the above ratios mentioned as C20, C40, C60, and C80, respectively) with corn silage as the sole forage	4 weeks	1.Concentrates decreased *Enterorhabdus, Blautia, Anaerosporobacter, Fibrobacter, Succinimonas, RFN54, Victivallis, Bilophila, Saccharofermentans*, *Anaeroplasm* and *Acidithiobacillus.* 2.Higher abundances *of Christensenella* and *Turicibacter* with higher concentrate proportions. 3.No archaeal genus was significantly affected by dietary concentrate levels	Zhang et al., 2017

A later study observed higher CH_4 production per DMI in heifers (aged 9 to 10 months) compared with adult cows (aged 45-65 months and 96-120 months), and this difference in CH_4 production per DMI was considered to be influenced by developmental physiological changes related to rumen fermentation (Liu et al., 2017). This study also observed changing correlations among bacterial and archaeal phylotypes with increasing age. To this end, *Prevotella* 2 was strongly correlated with *Methanobrevibacter* in heifers (Liu et al., 2017). In older cows (96–120 months) *Succinivibrio* showed an association with *Methanobrevibacter* (Liu et al., 2017). Network interaction analysis further showed that *Prevotella 2* was replaced by a new network cluster of *Succinivibrio* 1, *Ruminobacter* 1, *Ruminococcus* 1 and *Bacillus* 1 (Liu et al., 2017). This shift may account for the age-related difference in rumen fermentation and CH_4 production. *Prevotella* 2 and *Succinivibrio* both are known to produce propionate, with propionate production competing with methanogens for hydrogen utilization (Denman et al., 2015), potentially explaining the differences in CH_4 production efficiencies at different ages. A few other bacterial taxa that significantly differed with age included *Flavonifractor, Ruminococcus* and *Ruminobacter.* Of these, *Flavonifractor*, which produces acetic and butyric acid as major end products of sugar fermentation (Carlier, 2010), was also positively correlated with *Methanobrevibacter* (Liu et al., 2017) in addition to the previously described *Prevotella* 2 and *Succinivibrio*.

Ruminant studies have also investigated the impact of host genetics in determining key activities of rumen microbiota by looking at the effect of breed type. Differences in the bacterial and archaeal community structure have been observed in Holstein and Jersey cows (King et al., 2011). Based on archaeal 16S rRNA gene clone library analysis, a total of 20 OTUs were common to both the breeds, while 23 OTUs (36 sequences) were found only in the Holstein cows and 12 OTUs (18 sequences) were found only in the Jersey cows. *Methanobrevibacter ruminantium* and *Methanobrevibacter millerae* were present at very similar frequencies in lactating Jersey cows, whereas populations with 95 to 97.9% 16S rRNA gene identity to *Methanobrevibacter* spp. were more abundant (53.0% vs. 47.8%) in Holstein cows compared to Jersey cows (King et al., 2011). Family level classification of bacteria showed higher relative abundance of OTU's belonging to the Lachnospiraceae and p-2534-18B5 (rumen fibrolytic and cellulolytic populations) in Holstein cows as compared to Jersey cows. Additionally, an OTU belonging to the Succinivibrionaceae was more abundant in Jersey cows as compared to the Holstein cows (King et al., 2011). Whereas the study did not assess potential links of microbiota composition to CH_4 emissions, this was done in a recent

study on Aberdeen Angus (AA) and Limousin (LIM) sire breed types (Roehe et al., 2016). Significant differences in daily CH_4 emissions between the breeds (184 g/day for AA vs. 164 g/day for LIM) were concluded to be due to higher feed intake of AA as compared to LIM. The study also observed higher archaea:bacteria ratios in rumen contents of AA animals compared to LIM animals with both forage and concentrate based diets, and suggested the ratio to be a selection criterion for reduction of CH_4 (Roehe et al., 2016).

Host specificity (Animal individuality)

The rumen of cattle contains a core microbiota which includes *Prevotella, Butyrivibrio* and *Ruminococcus*, as well as unclassified *Lachnospiraceae, Ruminococcaceae, Bacteroidales,* and *Clostridiales* (Henderson et al., 2015a), with a large variability in their relative abundance. Other non-core microbial taxa also fluctuate in relative abundance. This is particularly obvious in terms of the rumen bacterial species, although inter-animal variation has also been observed in methanogenic archaeal and protozoal communities (Zhou et al., 2012). This suggests that the host has a controlling effect on its own rumen microbial community (Ribeiro et al., 2017). Additionally, the rumen microbiota is also characterized by functional redundancy and resilience (Weimer, 2015) meaning there is an overlap of functions among different species and that there is resistance or capacity to recover from perturbations.

Moreover, lowered CH_4 emission has most often been related to differences in the size/volume of the rumen (Goopy et al., 2014), and a smaller rumen size has been associated with faster passage rate of the feed (Smuts et al., 1995; Barnett et al., 2012). As a result less feed is digested inside the rumen, resulting in less CH_4. Therefore, one should be careful in interpreting microbiota analyses which can be associated with methanogenesis but not necessarily be the cause of decrease in methanogenesis. Furthermore, differences with respect to the time between regurgitation and sampling can also affect animal to animal variation of the rumen microbiota (Pinares-Patino et al., 2007). The lactation status of a cow can also affect CH_4 production due to increasing nutritional demands, leading to increase in feed intake and meal frequency but shortened duration. This results in shortened rumination and retention times in the rumen causing lower fibre digestibility and lower CH_4 yield as compared to dry cows (Gibb et al., 1999).

METHODS TO STUDY RUMEN MICROBIOTA

Culturing techniques

Traditionally, rumen microbiota was studied via cultivation based techniques. Microbial cultivation has enabled to observe different microbial morphologies, physiology and metabolism involved in plant polymer degradation (Hungate, 1966a). In the late 1970's, researchers also used microscopy to distinguish between different microorganisms based on their different sizes. For instance, *Lampropedia* (1 to 1.5 µm by 1 to 2.5 µm), *Oscillospira* (3 to 6 µm by 10 to 40 µm) and *Quinella* (2.5 to 3 µm by 4 to 9 µm) have different sizes and could be distinguished using microscopy (Clarke, 1979). *Lampropedia* and *Oscillospira* were also seen associated with plant fragments and the epidermial plant cells which were digested partially, although the attraction of these two bacteria to the cuticular surface of the plant leaf has not yet been explained fully (Clarke, 1979). Additionally, culturing techniques using roll tube methods were introduced by Hungate (Hungate et al., 1969) and modified (Eller et al., 1971) for isolation and enumeration of anaerobic microbes. Later Olsen in 1992, developed a modified agar bottle plate for cultivation and isolation of strict anaerobes. The development of plastic anaerobic glove boxes further facilitated colony isolation, which enabled researchers to perform streak and spread plating and dispensing of medium within an anoxic environment. Besides isolating microorganisms, direct counting of microbial cells was introduced in the late 1980's to provide total counts of microbes by roll tubes as well as MPN (most probable number) approaches. However, this was only possible for studying the rumen liquid associated microorganisms but is not suitable for the microorganisms associated with the fibrous content in the rumen (Zhou et al., 2015).

The fibrous content is problematic to study with culture-based techniques due to microbial cells being tightly attached to the biomass, and as a consequence, many of the fibre associated microorganisms remain uncultured (Krause et al., 2003). Fibre-degrading bacteria, such as *F. succinogenes* and *Ruminococcus* spp. have been isolated and have been extensively studied in terms of their fibre degrading ability and mechanisms. Most recently, media supplemented with azo-carboxymethyl cellulose have been utilized to culture cellulolytic rumen bacteria, identifying members of the Firmicutes as the major fibre degrading bacteria. Additionally, members of *Clostridium* cluster IV have been identified to degrade filter paper, whereas *Butyrivibrio* and *Pseudobutyrivibrio* have been identified to degrade xylan and carboxymethyl cellulose, suggesting that these rumen bacteria are involved in fibre degradation (Nyonyo et al., 2014).

Enrichment batch cultivation would normally result in fast growers unless the conditions are selective (for instance different pH or temperature). An alternative to avoid this would be dilution to extinction which would give abundant microbes a chance to grow for instance in biofilms that would allow to enrich for slow growing microbes best adapted to the conditions independent of growth rates. Further steps of isolation that include plating the culture on autoclaved solid agar can produce by-products (by autoclaving the agar) that kill many cells and prevent the cultivation of sensitive microorganisms (Tanaka et al., 2014). As a result nowadays researchers have focussed on using gellan gum instead of agar. However, irrespective of the different strategies used to culture, there is lack in cultivating all – a phenomenon that has been coined as "The Great Plate Count Anomaly" (Tanaka et al., 2014). This limits culture based techniques to serve as a tool to study the total rumen microbiota in detail as it identifies only a smaller subset of microbes.

Molecular techniques

To overcome the limitations associated with cultivation based techniques, researchers started to use culture independent tools, mostly including fingerprinting and sequencing techniques based on the small sub-unit rRNA (16S/18S). Moreover, molecular techniques enable quantification of rumen microbial populations using quantitative PCR (qPCR) with great sensitivity and precision (Singh et al., 2014). qPCR has been used to estimate the size of bacterial, archaeal, anaerobic fungal and protozoal populations (Sylvester, 2004; Denman, 2006; Jeyanathan et al., 2011). Fingerprinting techniques have been used to profile microbial communities, with a wide range of different techniques used that all rely on separation of PCR-amplified DNA fragments that differ in sequence and/or size: temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGGE), single strand conformation polymorphism (SSCP), terminal-restriction fragment length polymorphism (T-RFLP) analysis, automated ribosomal intergenic spacer analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE). Of all these methods, DGGE has probably been most extensively used for animal gastrointestinal tract microbiota analysis. In addition to the separation of amplicons that differ in nucleotide sequence, the intensities of a band can also be used as a semi-quantitative measure for the relative abundance of a certain sequence in the community. Furthermore, individual bands can be excised from the gel and re-amplified for sequencing and for identification of microbes. However, this technique normally allows to detect microbes only above a relative abundance of 1%. In order to further

increase specificity and sensitivity of molecular fingerprinting, phylogenetic microarrays were developed that were generally based on DNA oligonucleotide probes attached to a solid surface, providing more comprehensive insights into the structure and population dynamics of complex microbial eco-systems. One problem with microarrays is that they will only identify microbes for which probes have been included on the array, and thus they are limited in the detection of yet unknown microbial taxa. This limitation of microarrays has brought next-generation technology sequencing methods such as 454-pyrosequencing, and Illumina MiSeq and HiSeq sequencing into attention. These sequencing methods are able to provide novel insights into the composition of microbial communities, including those that reside in the rumen (Zoetendal et al., 2004; Deng et al., 2008) as they provide more comprehensive coverage of yet undescribed microbial taxa. It should be noted, however, that taxonomic annotation at species and even genus level is often problematic as the resolution of these short read sequencing methods is generally not good.

EXPERIMENTAL FACTORS AFFECTING THE ANALYSIS OF RUMEN MICROBIOTA

With an increasing number of ruminal microbial genomes becoming available it is easier to understand their physiology, how these microbes interact within the rumen and their impact on ruminant health and animal performance. To be able to study the microbial composition and function, it is important to validate the methods that are used.

Sampling and Sample storage

Sampling techniques, sample storage, sample handling and collection times have been shown to affect the observed rumen microbial community structure (Geishauser and Gitzel, 1996; Fliegerova et al., 2014). Nevertheless, until now, there has been no standardized rumen sample storage method nor subsequent nucleic acid extraction methods (Granja-Salcedo et al., 2017). In a recent study, rumen samples were collected using oral tubing and the fibrous fraction was collected via a fistula (Henderson et al, 2013). The fraction after squeezing provided a liquid fraction for further comparison. The samples were frozen, freeze-dried and homogenized prior to DNA extraction. With respect to the observed composition of the bacterial community, oral tubing derived samples were characterized by higher relative abundance of Prevotellaceae, whereas Lachnospiraceae (*Butyrivibrio* and *Coprococcus*) were observed at lower relative abundance as compared to the samples collected through the rumen

fistula. Furthermore, higher abundance of *Methanobrevibacter ruminantium* was observed in the samples collected through the fistula (Henderson et al, 2013). In addition to the effect of DNA extraction methods, the influence of sampling (cheese cloth squeezed, centrifuged and filtered) and sample storage at room temperature versus -80º C was investigated by Fliegerova et al. (2014). It was concluded that the bacterial diversity was optimally represented by using a bead beating based DNA extraction method with cheesecloth sieved rumen contents (Fliegerova et al., 2014).

In a recent study, the effect of three storage methods and four storage times on ruminal fibrous content were evaluated based on quality parameters and yield of metagenomic DNA extracted as well as the observed composition of the rumen bacterial community (Granja-Salcedo et al., 2017). In that study, rumen content samples were either pelleted or lyophilized and stored for three, six or 12 months. The pelleted samples were stored at -80 ºC and -20 ºC whereas the lyophilized samples were only stored at -20 ºC. The study concluded that the storage time significantly reduced the yield of DNA extracted. Higher relative abundances of Ruminococcaceae was observed in both, pelleted and lyophilized samples stored at -20 ºC and -80 ºC while Lachnospiraceae remain unchanged in all samples compared to its controls. Relative abundances of Prevotellaceae were higher in pelleted samples stored at -20 and -80 ºC while they were lower in lyophilized samples stored at -20 ºC as compared to its control. Furthermore, the study of (Petri et al., 2013) showed that the sample storage and cryoprotectants (in PBS-glycerol or -80 ºC storage) did not affect the bacterial composition structure.

DNA extraction method

DNA extraction is a critical step in enabling accurate assessment of the complexity of the rumen microbiota. Many studies have observed significant differences in rumen microbial community structure due to biases of the extraction methods used (Yu and Morrison, 2004; Henderson et al, 2013). An important aspect of DNA extraction has always been the amount of DNA yielded, however, there are cases when certain bacteria are harder to lyse than others. Therefore, in order to allow for direct comparison of rumen community structure between different studies, standardizing DNA extraction methods is crucial. In the study of Henderson (2013), the choice of extraction method affected both the observed bacterial composition as well as that of the archaeal fraction. Methanoplasmatales as Rumen Cluster C were found at lower abundance in DNA extracted using a bead beating method as opposed to less physical

methods (PSP®Spin Stool DNA Kit). In turn, efficient lysis of *Methanosphaera* cells was shown to require mechanical disruption (Henderson et al, 2013). Overall the study showed that the method with less physical disruption was associated with lower DNA quality with a bias towards the Gram negative Bacteroidetes (Henderson et al, 2013). Another aspect is that the extraction method can also be affected by the nature of sample. Therefore, depending on the nature of the sample, the mechanical disruption cycles should be optimized.

Textbox 1: Top Institute Food and Nutrition project *Reduced methane emissions from dairy cows*

This research was conducted within the framework of a TI Food and Nutrition (TIFN) funded collaborative interdisciplinary ruminant project focussed on reducing methane emission from dairy cows. The TIFN project included aspects of microbiology, animal nutrition, animal genomics and metabolomics. The TIFN project had five work packages (WP):

1. Indicator for methane emission in milk
2. Explore the (genetic) variation in methane emission in the Dutch dairy cow population
3. Characterizing rumen microbiota composition and studying fibre attachment from dairy cows
4. Understand the interplay between cow (genetics), rumen microbiota and feed by performing an experiment in climate respiration chambers.
5. Developing a model of methane emission in dairy cows

AIM AND OUTLINE OF THE THESIS

The aim of the research described in this thesis is to characterize rumen microbial composition from Holstein Friesian dairy cows, including potential biases associated with DNA extraction, differences in composition between different rumen fractions as well as the impact of diet. In addition, this thesis also looked at the fibre fermentation, including the enrichment, isolation and identification of potential fibre attached rumen bacteria.

Chapter 1 gives an introduction on the role of microbiota in rumen feed degradation with a particular emphasis on CH_4 as well as the different microbiota associated with rumen fluid and the fibrous fraction. The pros and the cons of culture dependent and culture independent methods are discussed, along with strategies used to date for improving the rumen fermentation efficiency and mitigating CH_4. Current understanding of experimental, dietary and animal associated factors that influence rumen microbiota, fermentation patterns and enteric methanogenesis are also reviewed.

Chapter 2 addresses the impact of DNA extraction methods on the observed rumen microbial community structure from four Holstein-Friesian dairy cows fed different ratios of grass and maize silage. To this end, both the liquid and the fibrous fraction were sampled in order to obtain a more comprehensive picture of ruminal microbial community composition. Focus is given to DNA extraction methods routinely used at the Laboratory of Microbiology, Wageningen University, for matrices ranging from mammalian intestinal content and faeces, marine invertebrates to soil and sediment samples. The resulting microbial composition using four DNA extraction methods was assessed using 454 pyrosequencing of barcoded PCR amplicons targeting bacteria, archaea and anaerobic fungi.

Subsequently, the impact of diet (different ratios of grass and maize silage) and time after introduction of the different diets on the microbial community structure has been evaluated in **Chapter 3**. The aim of this study was (1) to investigate the effect of replacing fibre-rich grass silage with starch-rich maize silage on the rumen bacterial and archaeal diversity using samples collected after 10 and 17 days of the diets being fed, and (2) to place the findings in context of ruminal fermentation and previously reported CH_4 emission findings (van Gastelen et al., 2015).

Besides increasing the knowledge on ruminal microbial composition using culture independent techniques, there has also been a focus on enriching ruminal fibre associated populations responsible for fibre degradation and/or attachment in **chapter 4**. Pre-autoclaved ruminal fibres were used as a matrix for attachment and as carbon source to identify fibre attached or associated populations. We enriched fibre associated populations, however, we were not able to demonstrate the enriched consortia's distinct role in fibre attachment.

In **chapter 5**, an effort to isolate a fibre attached microorganism was made by using pre-autoclaved ruminal fibre as a source of carbon for the growth of biomass. An effective method was to use methyl cellulose as a way to first detach the biomass from the fibres and use the suspension for a classical enrichment and isolation approach. A novel bacterium, *Propionibacterium ruminifibrarum* strain JV5^{T} was isolated, that was most closely related to *Propionibacterium australiense*.

The final chapter of this thesis, **chapter 6**, provides an integrated overview and discussion of results obtained in the research described in this thesis as well as findings from other studies. In addition, this chapter provides future directions of research in the rumen microbial manipulations to mitigate CH_4 and improve ruminal fermentation.

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Chapter 2

The effect of DNA extraction methods on observed microbial communities from fibrous and liquid rumen fractions of dairy cows

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ABSTRACT

DNA based methods have been widely used to study the complexity of the rumen microbiota, and it is well known that the method of DNA extraction is a critical step in enabling accurate assessment of this complexity. Rumen fluid (RF) and fibrous content (FC) fractions differ substantially in terms of their physical nature and associated microorganisms. The aim of this study was therefore to assess the effect of four DNA extraction methods (RBB, PBB, FDSS, PQIAmini) differing in cell lysis and/or DNA recovery methods on the observed microbial diversity in RF and FC fractions using samples from four rumen cannulated dairy cows fed 100 % grass silage (GS100), 67 % GS and 33 % maize silage (GS67MS33), 33 % GS and 67 % MS (GS33MS67), or 100 % MS (MS100). An ANOVA statistical test was applied on DNA quality and yield measurements, and it was found that the DNA yield was significantly affected by extraction method ($p < 0.001$) and fraction ($p < 0.001$). The 260/280 ratio was not affected by extraction ($p = 0.08$) but was affected by fraction ($p = 0.03$). On the other hand, the 260/230 ratio was affected by extraction method ($p < 0.001$) but not affected by fraction ($p = 0.8$). However, all four extraction procedures yielded DNA suitable for further analysis of bacterial, archaeal and anaerobic fungal communities using quantitative PCR and pyrosequencing of relevant taxonomic markers. Redundancy analysis (RDA) of bacterial 16S rRNA gene sequence data at the family level showed that there was a significant effect of rumen fraction ($p = 0.012$), and that PBB ($p = 0.012$) and FDSS ($p = 0.024$) also significantly contributed to explaining the observed variation in bacterial community composition. Whilst the DNA extraction method affected the apparent bacterial community composition, no single extraction method could be concluded to be ineffective. No obvious effect of DNA extraction method on the anaerobic fungi or archaea was observed, although fraction effects were evident for both. In summary, the comprehensive assessment of observed communities of bacteria, archaea and anaerobic fungi described here provides insight into a rational basis for selecting an optimal methodology to obtain a representative picture of the rumen microbiota.

KEYWORDS

DNA extraction methods, Rumen fluid, Fibrous content, Bacteria, Archaea, Fungi, 454 Pyrosequencing, Principal co-ordinate analysis, qPCR

INTRODUCTION

The bovine rumen is a complex microbial eco-system consisting of bacteria, archaea, protozoa and anaerobic fungi (Neocallimastigomycota). These microbes interact with each other to break down ruminant feed components, such as plant fibres. Bacteria are the predominant microorganisms in the rumen and hydrolyse feed-derived plant polysaccharides into short chain fatty acids (SCFAs), amino acids and gases, namely H_2 and CO_2 (Russell, 1981). The majority of the SCFAs are rapidly absorbed by the animal host for energy. Anaerobic fungi (Neocallimastigomycota) form a significant part of the rumen microbiota and play an important role in fibre digestion (Bauchop, 1979; Liggenstoffer et al., 2010; Gruninger et al., 2014). These anaerobic fungi were overlooked in early rumen studies due to their intimate association with the plant material during their extensive vegetative life cycle phase, with only the transient zoospores characteristic of their motile life cycle phase being detectable in the rumen fluid (RF) (Gruninger et al., 2014). Although ruminal methanogenic archaea cannot utilise dietary plant polysaccharides directly and comprise only approximately 0.3 – 3% of the total microbial biomass in the rumen, their functional relevance to rumen metabolism is significant. Archaea form methane (CH_4) by utilizing CO_2, H_2, formate, and methanol, which are produced during fermentation of dietary material by other rumen microbes (Hungate, 1966c; Marvin-Sikkema, 1990; Teunissen, 1992). Methane is a potent greenhouse gas and represents a loss of dietary energy to the ruminant (Meale, 2012).

The study of rumen microbial diversity is essential for in-depth understanding of the complex microbial interactions that shape the rumen ecosystem. This understanding can then be used to beneficially improve ruminant productivity, whilst decreasing the environmental footprint of ruminant livestock production (Zhou et al., 2009). Previously, much of the pioneering work by Robert Hungate was performed using traditional microbiological methods, involving isolation and characterization of pure strains to assess the diversity and functionality of rumen microbial communities. These strains, however, represented only a relatively small proportion of the total rumen microbial diversity (Hungate, 1966b). The importance of using culture independent studies to allow identification of uncultured and novel taxa within the rumen microbiota was previously confirmed (Edwards J.E., 2004; Creevey et al., 2014). Archaea which utilize the products from bacteria, are difficult to culture (Paul, 2012). For anaerobic fungi, only a limited number of the identified genera have been recovered in culture to date (Haitjema et al., 2014).

Although culture independent methods overcome some biases associated with culture dependent methods, they also introduce a new set of biases related to extraction and PCR. Several studies have shown that methods used to extract DNA from rumen-derived samples had a significant effect on the apparent microbial diversity observed using various different molecular techniques targeting the 16S ribosomal RNA (rRNA) gene. These techniques include single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), quantitative PCR (qPCR) and next generation technology amplicon sequencing (Yu and Morrison, 2004; Henderson et al, 2013; Villegas-Rivera et al., 2013).

In terms of DNA extraction, RF and fibrous content (FC) fractions represent very different types of physical matrices for processing. A recent study by Henderson et al. (2013) showed that the bacterial communities associated with these two fractions differed from each other. For example, the predominant bacterial phyla in the rumen observed were Firmicutes and Bacteroidetes (Fouts et al., 2012), but the relative abundance of these phyla differed between the RF and FC fractions (Henderson et al., 2013). In the liquid fraction, the predominant bacterial community member was *Prevotella,* belonging to the Bacteroidetes phylum. In contrast, bacterial taxa belonging to the phyla Fibrobacteres and Firmicutes, particularly *Butyrivibrio, Succiniclasticum* and Lachnospiraceae*,* were relatively more abundant in the solid fraction. However, when the effects of different DNA extraction methods and two rumen digesta sampling methods were compared to each other, the choice of DNA extraction method affected the apparent microbial community structure significantly more than the sampling method (Henderson et al, 2013). Another study by Fliegerová et al. (2014) observed the clustering of microbial communities based on the type of RF processing (cheesecloth squeezed, centrifuged or filtered), storage conditions and DNA extraction method.

Differences in observed bacterial patterns due to extraction methods are often caused by the differences in cell lysis efficiency associated with the characteristic cell wall structure of Gram positive and Gram negative bacteria (Fliegerova et al., 2014). However, information on the biases associated with DNA extraction of the rumen FC relative to RF is limited. As primary fibre-degrading microbes are mainly attached to the dietary plant material (Dehority, 1991), it is important to assess the effect of DNA extraction methods on the observed FC and RF microbiota, and to what extent the extracts generated are reflective of the actual microbiota.

In this study, we evaluated the effect of four DNA extraction methods that differ in cell lysis and/or DNA recovery procedures on the outcome of microbiota compositional analysis of both RF and FC fractions. Description and discussion of the fraction effect was, therefore, also performed in order to place the DNA extraction method effects in context. Sample fractions were collected from four rumen cannulated dairy cows each fed different roughage-based diets that were previously shown to result in differences in methane emission (van Gastelen et al., 2015). Quality and quantity of the extracted genomic DNA was evaluated prior to assessment of bacterial, archaeal and fungal communities with quantitative PCR (qPCR) and 454 based pyrosequencing of barcoded 16S rRNA gene and ITS PCR amplicons.

MATERIALS AND METHODS

Animals and diet

The samples used in this study were a subset of a larger study, of which the details have been described elsewhere (van Gastelen et al., 2015). This study was conducted in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research. Briefly, in the larger study 12 rumen cannulated cows were grouped into three blocks according to lactation stage, parity and milk production. The cows within each block were subsequently randomly assigned to one of four dietary treatments. All dietary treatments had a roughage to concentrate ratio of 80:20 based on dry matter. On a dry matter basis, the roughage consisted of either 100 % grass silage (GS100), 67 % GS and 33 % maize silage (GS67MS33), 33 % GS and 67 % MS (GS33MS67), or 100 % MS (MS100). One block of four cows was randomly selected from the above mentioned larger study to sample the RF and FC fractions in order to assess the effect of DNA extraction method on rumen microbiota analysis.

Sample collection, preservation and preparation

After 12 days of adaptation to the diet, the four rumen cannulated cows, i.e. one per dietary treatment, were sampled for RF and FC 3 hours after morning feeding. RF was directly collected using a suction tube through the rumen fistula, and collected in 3 equal (~200 ml) amounts from the front and middle of the ventral sac and from the cranial sac. After collection, the RF samples were pooled, thoroughly mixed, divided into aliquots of ~50 ml, and immediately frozen on dry ice. The solid (fibrous) fraction was collected via the rumen cannula, and then firmly squeezed by hand. All samples were collected within a time span of 30 min, after which they were

transported to the laboratory and stored at -80 °C until DNA extraction. In order to facilitate DNA extraction in 2 ml lysis tubes, approximately 7.5 g of fibrous content (FC) was ground using a mortar and pestle with liquid nitrogen, after which 0.2 g FC was weighed and used for extraction of DNA. RF samples were thawed, 1 ml aliquots centrifuged for 5 min at 9,000 × *g*, and the cell pellets used as the starting material for DNA extractions.

DNA extraction

Four different DNA extraction methods were compared in this study to represent different types and combinations of cell lysis mechanisms and/or DNA recovery procedures. All extractions were performed by one person. Each DNA extraction method was performed with eight samples, i.e. a RF and FC sample derived from four different cows, each of which were fed different diets. All extractions were performed once, with the exception of the sample from the cow fed the GS100 diet for which duplicate DNA extractions were performed. DNA extraction was performed using 0.2 g of ground FC or the cell pellet from 1 ml of RF.

Repeated bead beating (RBB) - Genomic DNA was extracted using the repeated bead beating plus column method, which was previously developed for bovine faeces and rumen digesta (Yu and Morrison, 2004). Briefly, the prepared sample was mixed with 0.5 g of zirconium beads (0.1 mm; Biospec products), 4 glass beads (2.5 mm; Biospec products) and 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, 4 % (w/v) SDS) in 2 ml lysis tubes with screw caps (BIOplastics BV) and then processed as the published protocol. The final genomic DNA was eluted in 100 µl AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0).

Phenol dependent bead beating (PBB) - Prepared samples were mixed with 940 µl TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0), followed by addition of 50 µl 10 % (w/v) SDS and 10 µl Proteinase K (20 mg/ml), and then incubated at 55°C for 1 hour. The mixture was then transferred to a 2 ml lysis tube containing 4 glass beads and 0.5 g zirconium beads (as used for the RBB protocol). Subsequently, 150 µl of buffered phenol (pH 7-8; Sigma Aldrich) was added, followed by bead beating for 3 min using the bead beater (Precellys 24, Bertin technologies) at 5.5 m/s and cooled immediately on ice. The aqueous phase containing the nucleic acids was further mixed with 150 µl chloroform-isoamyl alcohol (24:1), and excess phenol was removed through centrifugation at 14,000 × *g* for 10 min at 4ºC. The upper aqueous phase was removed and transferred to a new tube. The extraction with buffered phenol and chloroform-isoamyl

alcohol was repeated. The nucleic acids were then precipitated from the combined aqueous fractions by adding 0.1 volume of 3M sodium acetate and 1 volume of isopropanol, and incubating at 4 ºC for 30 min followed by centrifugation. The pellets were washed once with 70% (v/v) ethanol and allowed to air-dry before being rehydrated in 100 µl of TE buffer.

Fast SPIN DNA kit for Soil (FDSS) - Genomic DNA extraction was performed using the FastDNA SPIN kit for soil (MP Bio medicals, Solon, OH, USA) following the manufacturer's instructions. Cell lysis in this kit was performed with sodium phosphate buffer and MT buffer in Lysing matrix E tube using the Precellys 24 bead beater for 40 s at a speed of 6.0 m/s, and the DNA purification was done using a binding matrix. DNA was eluted in 50 µl of DES (DNase/Pyrogen free water) that was provided with the kit.

PQIAmini - Genomic DNA was extracted following the method described by (Zoetendal et al., 2006) with minor modifications (van den Bogert et al., 2013). Briefly, prepared samples were mixed with 500 µl of TE buffer, and the genomic DNA was extracted from the re-suspended sample according to the Macaloid-based DNA isolation protocol with the use of Phase Lock Gel heavy tubes (5 Prime GmbH) and phenol during the phase separation step. To remove contaminating RNA, 250 µl of the aqueous phase was pre-treated with 3 µl RNAse A (10 mg/ml; QIAGEN GmbH) at 37 °C for 15 min. Subsequent steps employed a modified version of the QIAamp DNA Stool Mini Kit (QIAGEN) protocol (Leimena et al., 2013). Initially, 22.5 µl proteinase K (20 mg/ml; Ambion) and 300 µl buffer AL from the QIAmp kit were added to the DNA extract followed by incubation at 70 °C for 10 min. The rest of the protocol was performed following the protocol guidelines. DNA was finally eluted in 30 µl of nuclease free water.

Quality control of DNA extracts

The quality and quantity of the DNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies). The integrity of the DNA was visualized using agarose gel electrophoresis with a 1% (w/v) agarose gel containing 1x SYBR® Safe DNA gel stain (Invitrogen).

qPCR analysis

DNA extracted from RF and FC samples was used for quantification of bacteria, archaea and anaerobic fungi by qPCR. The amplification of bacterial and archaeal 16S rRNA genes, and

anaerobic fungal 5.8S rRNA genes was performed in a Bio-Rad CFX96 system (Bio-Rad Laboratories). All qPCR reactions were performed in triplicate. The resulting qPCR data was then processed, and principal component analysis (PCA) was performed using Canoco 5.0 (Ter Braak, 2012). The R software (version 3.0.2) was used for plotting and visualization purposes.

Bacteria and Archaea qPCR - To quantify bacterial 16S rRNA genes, the forward and reverse qPCR primers BAC 1369F (5' CGGTGAATACGTTCYCGG 3') and PROK 1492R (5' GGWTACCTTGTTACGACTT 3') were used (Suzuki et al., 2001). Archaeal 16S rRNA gene copies were quantified using primers 787F (5' ATTAGATACCCSBGTAGTCC 3') and 1059R (5' GCCATGCACCWCCTC 3') (Yu et al., 2005). The reproducibility of the bacterial qPCR assay (primers BAC 1369F and PROK 1492R) has been recently successfully confirmed for rumen fluid samples in our lab (van Lingen et al., 2017). The reproducibility of the archaeal qPCR assay (primers 787F and 1059R) has been shown in a study focussing on bioreactor performance from methanogenic communities in microbial electrolysis cells (Lu, 2012), and the archaeal primers have been tested for their coverage by (Yu et al., 2005). For bacteria and archaea the qPCR reaction mixture (25 µl) contained 12.5 µl 2X iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 200 nM forward primer, 200 nM reverse primer, 10.5 µl nuclease free water, and 1 µl of 0.2 ng/µl (for bacteria) or 2 ng/µl template DNA (for archaea). The thermal cycling conditions for the bacterial and archaeal primer pairs included a pre-denaturing step at 95 °C for 10 min, followed by 35 cycles of 95 °C for 20 s, annealing at 56.3 °C (for bacteria) or 60 °C (for archaea) for 30 s and extension at 72 °C for 30 s. The fluorescent products were detected at the last step of each cycle. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.5 °C/s increments from 60 to 95 °C, with continuous fluorescence collection.

Anaerobic fungi qPCR - The quantification of ruminal anaerobic fungi was carried out using the Neocallimastigales probe-based qPCR assay as previously described (Edwards et al., 2008). Briefly, primers Neo qPCR For (5' TTG ACA ATG GAT CTC TTG GTT CTC 3') and Neo qPCR Rev (5' GTG CAA TAT GCG TTC GAA GAT T 3') primers were used, targeting a conserved region (110 bp) of the 5.8S rRNA gene, along with a TaqMan probe (Neo: 5' FAM-CAA AAT GCG ATA AGT ART GTG AAT TGC AGA ATA CG –TAMRA-3'). The reaction mixture (25 µl) contained 1 × TaqMan Universal PCR Probe Mix (Applied Biosystems), 750 nM of each primer, 200 nM of the probe and 1 µl of 2 ng/µl template DNA. The thermal cycling

programme was 50 °C for 2 min, 95 °C for 10 min (initial denaturation), followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (primer annealing and extension). At the end of each cycle, the accumulation of PCR products was detected by monitoring the fluorescence signal from the probe.

Standard curve preparation - Standard curves were generated using purified PCR products as a template. The bacterial 16S rRNA gene PCR product was obtained with universal bacterial primers 27F and 1492R (Suzuki et al., 2000), using DNA extracted from *Ruminococcus albus* SY3 (kindly provided by Prof. R. John Wallace from the Rowett Research Institute (now part of the University of Aberdeen)). The archaeal 16S rRNA gene PCR product was obtained with universal archaeal primers 25F and 1492R (Dojka et al., 1998; Suzuki et al., 2000), using DNA extracted from *Methanosarcina mazei* MC3 (DSM-2907). The anaerobic fungal 5.8S rRNA gene PCR product was obtained with the Neo qPCR Rev and Neo qPCR Rev primers using DNA extracted from a FC sample from the cow which was fed GS100. All the PCR products were purified with a Purelink PCR Purification kit (Invitrogen), with high-cut off binding buffer B3, and the concentration was measured using Nanodrop. The DNA concentration and amplicon size was used to calculate the number of amplicon copies, and then 10-fold serial dilutions in water were made from 10^8 to 10^2 amplicon copies/µl.

Amplification of target regions for pyrosequencing

Bacterial community assessment - Bacterial community composition was assessed as described previously (van den Bogert et al., 2013). Briefly, a PCR was performed to obtain barcoded amplicons from the V1-V2 region of the 16S rRNA gene, using the 27F-DegS forward primer (5' GTTYGATYMTGGCTCAG 3') (van den Bogert et al., 2011) appended with the pyrosequencing titanium sequencing adapter A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3) and an 8 nt sample specific barcode (Hamady et al., 2008) and an equimolar mix of two reverse primers 338R I – (5' GCWGCCTCCCGTAGGAGT 3') and 338R II – (5' GCWGCCACCCGTAGGTGT 3') that were appended with the pyrosequencing titanium adapter B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3') at the 5' end (Guss et al., 2011). The reverse primers are based on three previously published EUB 338 probes (Daims et al., 1999b). PCRs were performed using a thermocycler (G storm) in a total volume of 100 µl containing 20 µl 5 × HF buffer (Finnzymes), 2 µl PCR Grade Nucleotide Mix (2 mM each), 2 units Phusion®

Hot Start II High Fidelity DNA polymerase (Finnzymes), 500 nM of both the barcoded forward and reverse primer, 65 µl nuclease free water and 2 µl of 20 ng/µl template DNA. The PCR program consisted of an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 56 °C for 20 s and 72 °C for 20 s, with a final extension step at 72 °C for 10 min. Expected PCR product size (311 bp) was confirmed by agarose gel electrophoresis using 5 µl of PCR product on a 1% (w/v) agarose gel containing 1x SYBR® Safe. Non-template negative control PCR reactions were performed alongside each PCR amplification, and were confirmed to yield no product. PCR products were purified with the High PCR Pure Clean-up Micro kit (Roche) followed by quantification using the Qubit dsDNA BR assay kit (Invitrogen). Purified PCR products were mixed in equimolar amounts (400 ng per sample), and the pooled amplicons were purified using a DNA gel extraction kit (Millipore) according to manufacturer's guidelines. The pooled amplicons were then quantified using the Qubit dsDNA BR assay kit, and the sequences determined with a 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC-Biotech, Konstanz, Germany).

Archaeal community assessment - A method adapted from Jaeggi et al. (2014) was used for archaeal composition analysis. Briefly, barcoded amplicons of 16S rRNA genes were generated by PCR using the 340F forward primer (5′-CCCTAYGGGGYGCASCAG-3′) (Gantner et al., 2011) that was 5'- extended with the titanium adaptor A and an 8 nt sample specific barcode, and the 1000R reverse primer (5′-GGCCATGCACYWCYTCTC-3′ (Gantner et al., 2011)) that was appended with the titanium adaptor B at the 5'-end. PCRs were performed in a total volume of 50 µl containing 20 ng of template DNA, 200 nM of each of the forward and reverse primer, 1 U KOD Hot Start DNA Polymerase (Novagen), 5 µl KOD-buffer (10×), 3 µl $MgSO_4$ (25 mM), 5 µl dNTP mix (2 mM each), and 33 µl nuclease free water. PCR conditions were: initial denaturation step at 98 °C for 30 s followed by 25 cycles of 98 °C for 10 s, 52 °C for 20 s, and 72 °C for 20 s, and a final extension step of 72 °C for 10 min. PCR product size (660 bp) was confirmed by agarose gel electrophoresis using 5 µl of PCR product on a 1 % (w/v) agarose gel containing 1x SYBR® Safe. Non-template negative control PCR reactions were performed alongside each PCR amplification and were confirmed to yield no product. The PCR amplicon (approx. 660 bp) was subsequently purified using the MSB Spin PCRapace kit (Invitek), and the concentration was determined using the Qubit dsDNA BR assay kit. Purified PCR products were mixed in equimolar amounts by pooling 200 ng of the purified PCR products of each sample. The pooled sample was purified using the Purelink PCR Purification kit, with high-cut off

binding buffer B3, and pyrosequenced on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC-Biotech, Konstanz, Germany).

Fungal community assessment - PCR was performed to obtain barcoded amplicons from the fungal ITS1 region, using the ITS1FA.001 (5'-CTTGGTCATTTAGAGGAAGTAA-3′) forward primer appended at the 5'-end with titanium sequencing adapter B and the reverse primer (5'-TCCTCCGCTTATTGATATGC-3′) appended with titanium sequencing adapter A and a 6 nt sample specific barcode. PCRs were performed using a thermocycler (Biometra) in a total volume of 50 µl containing 5 µl 10x KOD buffer, 5 µl dNTP mix (2 mM each), 3 µl $MgSO_4$ (25mM), 1 µl KOD polymerase, 400 nM of both the forward and the reverse primer, nuclease free water, and 20-50 ng of template DNA. The PCR program consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 51°C for 10 s, and elongation at 70°C for 15 s, with a final extension step at 70°C for 5 min. Expected PCR product size (variable between 350-750 bp) was confirmed by agarose gel electrophoresis using 5 µl of PCR product on a 1 % (w/v) agarose gel containing ethidium bromide. Non-template negative control PCR reactions were performed alongside each PCR amplification, and were confirmed to yield no product. PCR products were purified with MSB spin PCRapace kit followed by quantification using Nanodrop. Purified PCR products were mixed in equimolar amounts (200 ng per sample), and the pooled amplicons were purified using MSB spin PCRapace kit according to manufacturer's guidelines. The pooled amplicons were then quantified by Nanodrop and pyrosequenced on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC-Biotech, Germany).

Pyrosequencing data analysis

The pyrosequencing data analysis for bacteria and archaea was carried out with a workflow employing the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010) using settings as recommended in the QIIME 1.2 tutorial. De-multiplexing and initial sequence quality filtering were done with the "split_libraries.py" script provided by QIIME using the default settings. OTU picking, alignment and taxonomic classification were done using the workflow script "pick_otus_through_otu_table.py" provided by QIIME using the default settings. Reads were filtered for chimeric sequences using Chimera Slayer (Haas et al., 2011), and clustering of Operational Taxonomic Units (OTUs) was performed with a similarity threshold of 97%. Additional data handling was done using in-house developed Python and Perl

scripts. Taxonomic classification of bacteria and archaea was done using Ribosomal Database Project (RDP) classifier version 2.2 (Wang et al., 2007) using the database GreenGenes set gg_97_otus_6oct2010 as provided with QIIME 1.2. In order to obtain the most likely genus-level identification, sequences were compared to the corresponding RDP reference set using NCBI BLAST (Altschul et al., 1990). Data analysis for fungi was done using a workflow based on QIIME 1.8, using the BLAST method for taxonomic classification of ITS reads against the UNITE database (Abarenkov et al., 2010), using the training set of 07-04-2014. Shannon's index and Chao1 richness index were calculated as implemented in QIIME using bacterial OTU-level data. Principal coordinate analysis (PCoA) analysis of weighted and unweighted UniFrac distances between samples was performed using QIIME with both the bacterial and archaeal OTU-level data. Redundancy analysis (RDA) was performed using Canoco 5 (Smilauer, 2014) to assess the relationship between family-level like phylogenetic groupings of OTU and DNA extraction methods or rumen fractions. The raw sequence data for the bacterial, archaeal and fungal composition analysis is deposited as a project available at https://github.com/jdvaidya/rumenmicrobiotadata. In addition, the sequences are also deposited in ENA under accession number PRJEB22996.

Statistical Analysis

The significance of potential differences in the relative abundances of bacterial taxa between the different sample groups (e.g. different extraction methods, different rumen fractions) was assessed using the non-parametric rank Mann-Whitney test as implemented in Sci-Phy (Jones, 2001). Significance of explanatory variables included in constrained analyses (RDA) was assessed using an unrestricted Monte Carlo permutation test with a total of 999 permutations, and results were visualized in an ordination biplot obtained from Canoco 5. P-values were corrected for multiple testing using Bonferroni correction and those lower than 0.05 were regarded as significant.

Two 1-way ANOVA model were fitted separately to DNA yield and quality (260/280 and 260/230) measurements with extraction method (4 levels: RBB, PBB, FDSS and PQIAmini, see Section 2.3 for detailed explanation of the extraction methods) and rumen fraction (2 levels: fibrous and liquid) as factors, using R software (version 3.0.2). Data was log-transformed before analysis to correct for skewness. The rationale behind the use of two separate 1-way ANOVA instead of a 2-way ANOVA is that we did not consider the Extraction method x Fraction

interaction term, due to large sample heterogeneity (i.e. each of the four cows were fed a different diet).

RESULTS.

Quality and quantity of genomic DNA from four extraction methods

Both RF and FC samples yielded high molecular weight (> 3kb) DNA as confirmed by agarose gel electrophoresis (Fig S1). The integrity of the DNA was best for the RBB method, as less DNA degradation was observed compared to the other three methods. Statistical analysis using ANOVA confirmed that the DNA yield was significantly affected by extraction method ($p < 0.001$) and fraction ($p < 0.001$). For the RF samples the highest quantities of DNA were obtained with PBB, which on average yielded 9.0, 3.0 and, 3.5 times more DNA than the RBB, FDSS and PQIAmini methods, respectively (Table 1). PBB also yielded the highest quantities of DNA with the FC samples, and yielded 9.5, 2.5 and 2.2 times more DNA than RBB, FDSS and PQIAmini methods, respectively (Table 1). Assessment of purity of DNA found that the 260/280 ratio was not affected by extraction ($p = 0.08$) but was affected by fraction ($p = 0.03$). On the other hand, the 260/230 ratio was affected by extraction method ($p < 0.001$) but not affected by fraction ($p = 0.8$). Some DNA extracts of the RF and FC samples had a ratio of absorbance at 260 nm and 280 nm ($A_{260/280}$) that was below 1.8, indicating the presence of contaminants (typically proteins and/or phenol) that absorb at a slightly higher wavelength than DNA (Table 1). This was most evident with the PBB method DNA extracts from the FC samples. The $A_{260/230}$ was lower than 2.0 (maximal value for pure DNA) for virtually all of the samples, but in particular for the FDSS DNA extracts. However, all DNA extraction methods provided DNA of sufficient quality and quantity to proceed with PCR based approaches as described in the following sections.

qPCR analysis of bacteria, archaea and anaerobic fungi

All DNA extracts from RF and FC samples were used for qPCR analysis of total bacteria, archaea and anaerobic fungi (Fig. S2). The PCA of the qPCR data revealed separate clustering of the FC and RF fractions in PC1 (Fig. 1A). These two clusters were separated by anaerobic fungal 5.8S rRNA gene concentrations along the first principal component axis (PC1). There was also evidence of clustering of the extraction methods in the second principal component axis (PC2), with the RBB and FDSS methods clustered to the top half of the plot and PBB and PQIAmini to the bottom (Fig. 1A). Archaeal 16S rRNA gene concentrations were associated with the separation of these two clusters in PC2 (Fig. 1B).

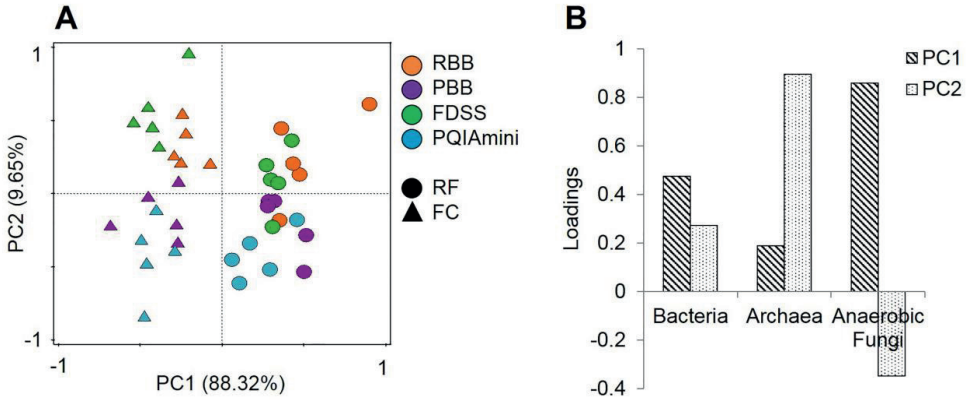

Figure 1. (A) Principal component analysis (PCA) of the combined bacterial (16S rRNA gene), archaeal (16S rRNA gene) and anaerobic fungal (5.8S rRNA gene) qPCR data for rumen fluid (RF, ○) and fibrous content (FC, △) samples. The GS100 diet has duplicate DNA extracts presented as individual datapoints. The percentages provided at the axes indicate the variation explained. **(B)** The corresponding loadings for the principal components indicate that anaerobic fungi are the major cause of sample separation in PC1, and archaea in PC2.

Impact of DNA extraction methods and fractions on observed bacterial community composition

On average only 26.3% of the annotations for the bacterial taxa included genus level identification. Therefore, mainly the OTU and family level (average of 56.2% annotation) was used in the data analysis. Weighted UniFrac distance based PCoA at the OTU-level showed that the bacterial communities observed in RBB, FDSS, and PQIAmini-derived extracts generally grouped together, whereas the bacterial communities associated with PBB-derived extracts clustered separately (Fig S3A). This was not seen in the unweighted UniFrac distance based PCoA, however, samples appeared to cluster more by rumen fraction instead (Fig. S3A and S3B).

In order to test to what extent different extraction methods and rumen fractions contributed to explaining the observed variation in bacterial community composition, redundancy analysis (RDA) was applied using family-level relative abundance data. This analysis showed that the

PBB (p = 0.012) and FDSS (p = 0.024) DNA extraction methods were separated relative to RBB and PQIAmini on the first canonical axis (Fig. 2). On the second canonical axis samples were separated by fraction (p = 0.012) (Fig. 2). Ruminococcaceae appeared to be positively associated with the PBB method and the fibrous content. The following three families were positively associated with the FDSS method and fibrous content: Fibrobacteraceae, Unclassified Synergistetes and Unclassified Bacteroidales (Fig. 2). The Prevotellaceae were positively associated with the FDSS method and rumen fluid fraction.

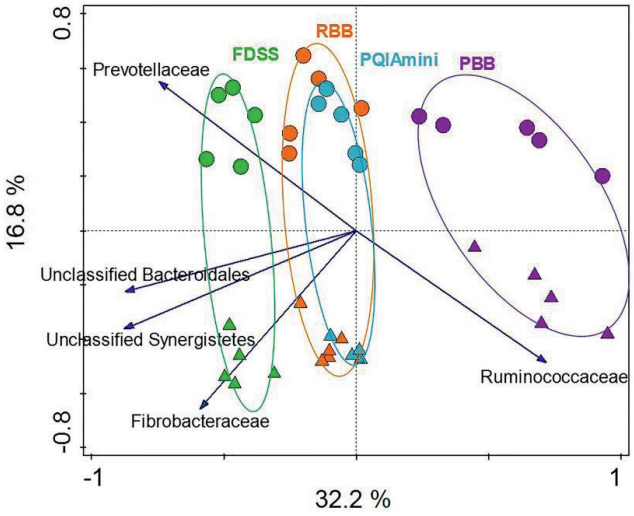

Figure 2. Redundancy analysis triplot (RDA) showing the relationship between the top five family-level phylogenetic groupings, the variation of which is most strongly associated with DNA extraction methods (RBB, PBB, FDSS, PQIAmini) and fractions (RF, ○ and FC, △). The canonical axes are labelled with percentage of total variance explained (%). Arrow length indicates the variance explained by extraction methods and fractions. The GS100 diet has duplicate DNA extracts presented as individual datapoints.

Bacterial community analysis - A more detailed compositional analysis of RF and FC samples showed that the rumen bacterial community consisted of 15 phyla (data not shown), among which on average Firmicutes (46.9 ± 16.1 % RF, 39.7 ± 15.7 % FC) and Bacteroidetes (58.2 ± 15.7 % RF, 26.9 ± 11.2 % FC) were most predominant. The bacterial profiles of RF and FC fractions appeared to be very distinct at the family level (Fig. 3A and 3B). Overall, the relative

abundance of Prevotellaceae was significantly higher in RF samples than in FC samples ($p = 0.001$; Fig. 4A and Table S1A) but was not significantly affected by any of the DNA extraction methods ($p > 0.05$; Fig. 4A and Table S1B). The relative abundance of Fibrobacteraceae was higher in FC compared to RF ($p = 0.020$; Fig. 4B and Table S1A), and was found to be higher ($p = 0.028$) in extracts obtained using the FDSS method as compared to the PBB method (Fig. 4B and Table S1B). The RBB method also resulted in DNA extracts with a higher relative abundance of Fibrobacteraceae in comparison to the PBB method ($p = 0.038$; Fig. 4B and Table S1B). Differences were also observed between RF and FC fractions for Ruminococcaceae. FC samples had significantly higher relative abundances of Ruminococcaceae compared to RF samples ($p = 0.040$; Fig. 4C and Table S1A). The PBB extraction method gave significantly higher relative abundances of Ruminococcaceae compared to the FDSS method ($p = 0.038$; Fig. 4C and Table S1B). Members of the Lachnospiraceae appeared to be predominant in both RF and FC samples, and their relative abundance in FC samples was significantly higher than those in RF samples ($p = 0.006$; Fig. 4D and Table S1A). However, there was no effect of DNA extraction methods on Lachnospiraceae (Fig. 4D and Table S1B). Finally, relative abundances of two other minor (< 1%) families (Anaerolinaceae and Halomonadaceae) were significantly affected by DNA extraction methods (Table S1B) and one minor family (Desulfobulbaceae) was affected by fraction (Table S1A).

At the genus level, *Selenomonas, Succiniclasticum, Ruminococcus, Prevotella, Butyrivibrio, Paraeggerthella, Fibrobacter, Desulfobulbus, Pseudobutyrivibrio, Syntrophococcus* and *Oscillibacter* significantly differed in relative abundance when comparing RF and FC ($p < 0.05$; Table S1A). The genera with higher relative abundance in RF compared to FC fraction were *Desulfobulbus, Succiniclasticum, Paraeggerthella, Prevotella,* and *Selenomonas*, whereas *Syntrophococcus, Pseudobutyrivibrio, Butyrivibrio, Oscillibacter, Ruminococcus* and *Fibrobacter* were significantly higher in their relative abundance in the FC fraction as compared to the RF fraction. In contrast, only the genus *Fibrobacter* was found to be significantly affected by DNA extraction method. The relative abundance of *Fibrobacter* was higher in FDSS extracts compared to those prepared using the PBB method ($p = 0.038$), and higher also in the RBB extracts compared to the PBB ($p = 0.038$) (Table S1B).

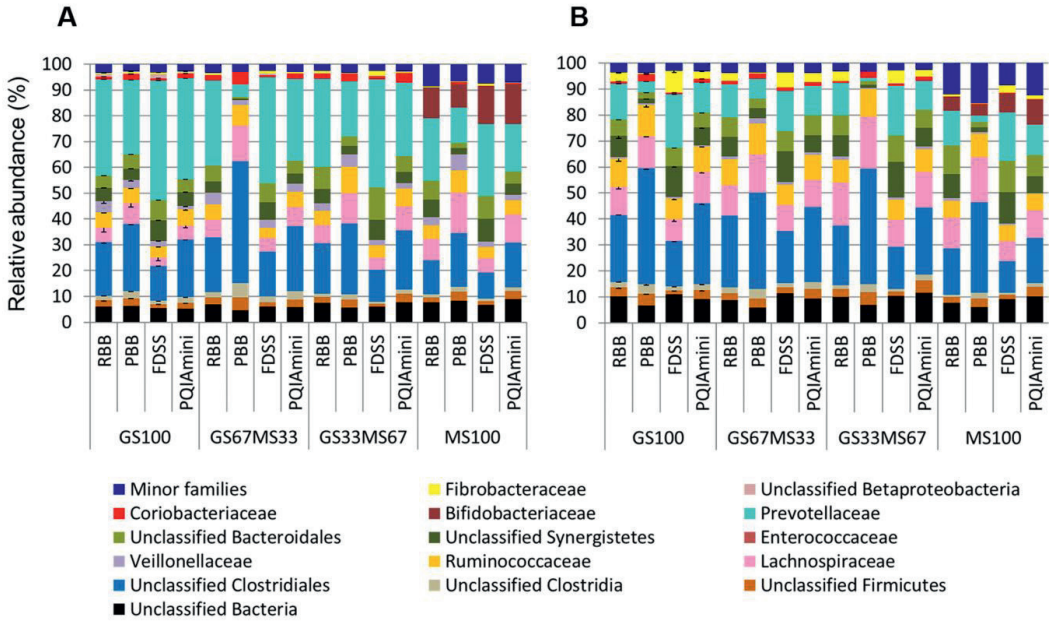

Figure 3. Bacterial family level composition of different DNA extracts obtained from rumen fluid **(A)** and fibrous content **(B)** samples from dairy cows each fed different ratios of grass silage (GS) to maize silage (MS), e.g. GS67MS33 is a diet containing 67 % grass silage and 33 % maize silage. All the stacked bars represent individual sample data except for GS100 which represents the mean of two different DNA extracts (error bars represent the standard deviation). All family level phylogenetic groupings >1 % are shown individually, with those <1 % summed together and presented as minor families.

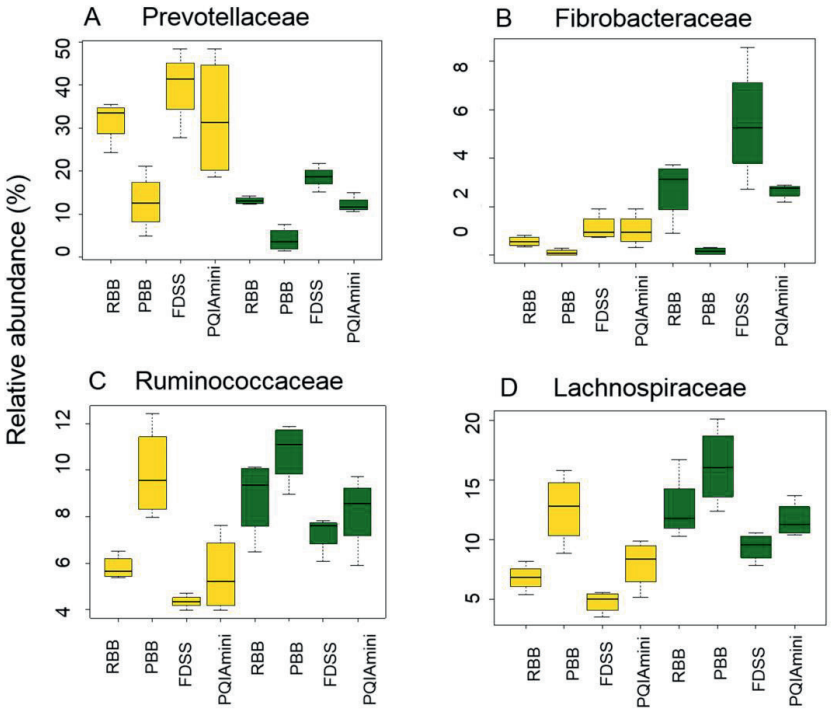

Figure 4. The effect of DNA extraction method (RBB, PDD, FDSS & PQIAmini) on the relative abundance of the bacterial families Prevotellaceae **(A)** Fibrobacteraceae **(B)** Ruminococcaceae **(C)** and Lachnospiraceae **(D)** in rumen fluid (yellow) and fibrous content (green) samples.The boxplots represent the data from 5 observations per rumen fraction, and show the 25th, 50th and 75th percentiles, with whiskers showing the extremes of the data.

Bacterial diversity and richness - Estimates of bacterial sequence richness and diversity were calculated at the OTU level to assess if these parameters were affected by fraction or DNA extraction method. The PBB extracts from RF and FC fractions of GS67MS33 and MS100 fed cows appeared to generally have the lowest bacterial richness (total number of OTUs present in a community) as calculated by the Chao1 index than the corresponding RBB, FDSS and PQIAmini RF and FC extracts (Fig. 5A). A similar trend of the PBB extracts was also seen for diversity (Shannon's index, Fig. 5B). Within the GS100 sample, the Chao1 richness index generally showed higher variability within RF than in FC samples (Fig. 5A). On the other hand, the technical replicates for GS100 appeared to give similar values throughout Shannon's index

analyses (Fig. 5B). The RF samples seemed to have lower Shannon's index values compared to the FC samples, which was not always the case with Chao1 index values.

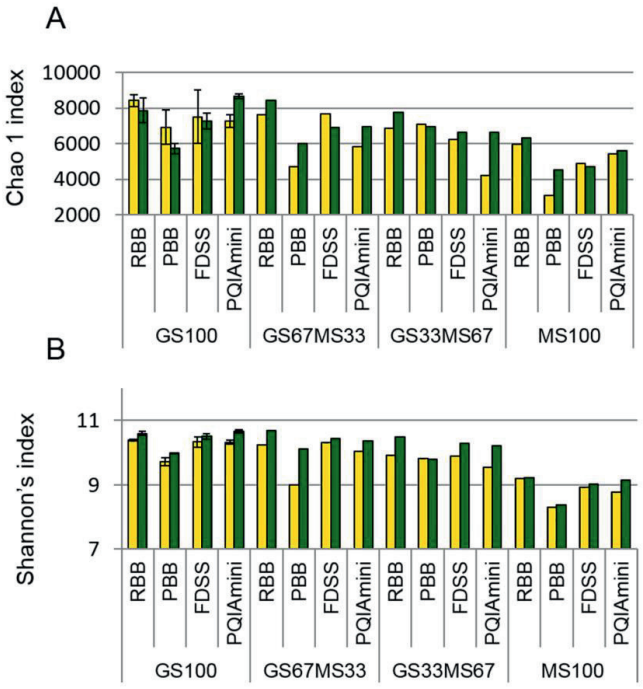

Figure 5. Chao1 richness index **(A)** and Shannon's diversity index **(B)** values for all four DNA extraction methods (RBB, PBB, FDSS & PQIAmini) applied to rumen fluid (yellow) and fibrous content (green) samples from four dairy cows each fed different ratios of grass silage (GS) to maize silage (MS), e.g. GS67MS33 is a diet containing 67% grass silage and 33% maize silage. The GS100 samples represent the mean of two different DNA extracts, and the error bars represent their standard deviation.

Archaeal community analysis

The RF and FC samples were analysed to identify the rumen archaea associated with the different fractions, and how the different DNA extraction methods influenced their detection (Fig. 6A and 6B). Some of the DNA extracts did not yield amplicons for sequencing, despite the fact that all samples were successfully amplified in the archaeal 16S rRNA qPCR. Furthermore,

the PCR failure could also not be directly correlated with either the fraction, sample source (cow/diet) or any of the DNA extraction methods. The FDSS method, however, consistently failed with all the RF samples (Fig. 6A).

Two families belonging to the phylum Euryarchaeota, *i.e.* Methanobacteriaceae and Thermoplasmata-incertae-sedis represented the majority of the sequences. Within the Methanobacteriaceae, two known genera were detected, *Methanobrevibacter* (~83% to 98%) and *Methanosphaera* (~1% to 4%). An unidentified genus within the Methanobacteriaceae was also detected (<1%) (Fig. 6A and 6B). Within Thermoplasmatales-incertae-sedis, only the genus *Thermogymnomonas* (<1%) was identified. Of the samples for which sequence information could be generated, there was no consistent difference in the relative abundances of archaeal genera found relative to the different DNA extraction methods. However, from the two fractions there was generally more *Methanosphaera* seen in the RF as compared to the FC.

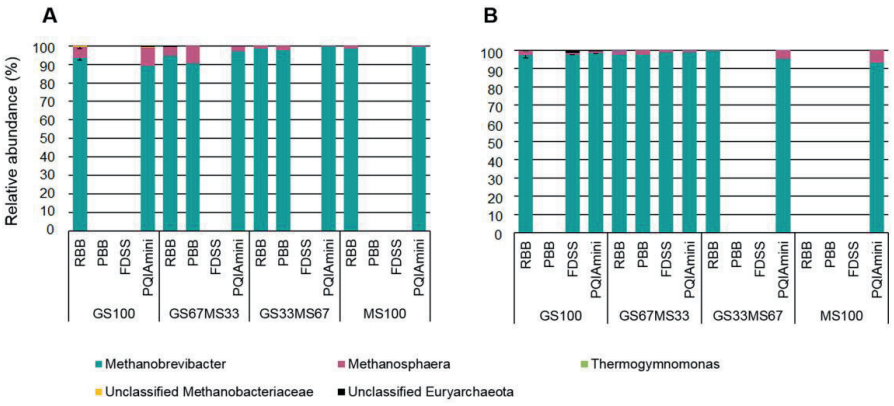

Figure 6 Relative abundance of archaeal taxa at genus level within rumen fluid **(A)** and fibrous content **(B)** samples from dairy cows each fed different ratios of grass silage (GS) to maize silage (MS), e.g. GS67MS33 is a diet containing 67 % grass silage and 33 % maize silage. All samples were subjected to each of the four different DNA extraction methods (RBB, PBB, FDSS and PQIAmini). Missing bars indicate that it was not possible to generate an archaeal PCR product for sequencing. Error bars for the GS100 samples represent the standard deviation associated with two different extracts, except for the PQIAmini extracted GS100 rumen fluid sample (A) where n = 1.

Fungal community analysis

Pyrosequencing analysis of amplified fungal ITS regions revealed the presence of several fungal phyla in both the RF and FC samples, and included aerobic (Ascomycota and Basidiomycota) as well as anaerobic fungi (Neocallimastigomycota). Unidentified fungal taxa (including but not differentiating between unidentified anaerobic and aerobic) and not assigned fungi were dominant (Table 2A and 2B). The identified anaerobic fungi represented less than 1% of the reads in the RF samples (Table 2A). The FC samples on the other hand were characterized by much higher relative abundances of the anaerobic fungi, which were represented by four genera: *Cyllamyces* (0 to 3.2%), *Anaeromyces* (0 to 5.2 %), *Neocallimastix* (0 to 8.1%) and *Piromyces* (0 to 31.5%) (Table 2B). Due to the limited and variable number of anaerobic fungal reads, an in depth analysis of this phylum relative to the DNA extraction method was not possible.

DISCUSSION

DNA quantity, purity and integrity

The different cell wall composition and structure of bacteria, archaea and fungi largely determines their susceptibility to mechanical or enzymatic lysis methods (Fredricks et al., 2005; Henderson et al, 2013). In this study, all the methods employed mechanical disruption of cells by bead beating, albeit with differences in agitation times and type of beads. Several studies have shown that disruption of bacteria with tough cell walls, such as those belonging to the phyla Firmicutes and Actinobacteria, is more efficient with a mechanical approach than by an enzyme-based protocol (Lazarevic et al., 2013). In the present study, all four DNA extraction methods yielded high molecular weight DNA (> 3kb), from both RF and FC fractions based on agarose gel analysis but the mechanical disruption caused shearing of DNA to different extents. The RBB method yielded the most intact genomic DNA compared to the PBB, FDSS and PQIAmini methods. Although the RBB method employs two rounds of bead beating in the presence of high concentrations of SDS, salt and EDTA, the physical damage of DNA is minimized by removing the lysate from the first round of bead beating to a new micro centrifuge tube followed by a second bead beating step to lyse any remaining intact cells. The DNA yields for RBB were lower compared to the other methods assessed. DNA yields previously reported for faecal samples (10-30 µg/g faeces: (Zoetendal, 2006) were slightly higher compared to the range observed for RF, and lower than that observed for the FC.

It has previously been shown that different agitation speeds can affect DNA extraction, as samples subjected to disruption at 4,800 rpm yielded more DNA than those subjected to 2,400 rpm (Fujimoto, 2004). In our study, although RBB and PQIAmini methods both used an agitation speed of 5.5 m/s, the DNA yields for PQIAmini were 3.0 and 3.8 times higher as compared to the RBB method for RF and FC samples, respectively. In this case, either the reagents used during lysis or the different disruption times (3×1 min for RBB and 3×45 s for PQIAmini) might be responsible for the different DNA yields obtained from these two methods for RF and FC.

In this study, the average 260/230 ratios observed for RF (1.4 $\pm$ 0.6) and FC (1.2 $\pm$ 0.5) samples indicated the presence of humic acids or guanidine carried over during the washing steps of the silica columns and the beads. For some extraction methods, the 260/230 ratio seemed particularly low for FC samples as compared to RF samples, presumably due to impurities associated with the lignocellulose components of a plant fibrous material rich in aromatic ring structures similar to humic acids. The 260/230 ratio for samples extracted with the FDSS method were the lowest as compared to the other extraction methods (Table 1). The FDSS protocol has previously been reported to give high DNA yields with soil samples, but still containing contaminants such as humic acid residues (Devi et al., 2015). It is also important to note that phenol-based DNA extraction methods, including the PBB and PQIAmini methods, can give higher 260/280 ratios as any residual phenol absorbs at 280nm. Nevertheless, no PCR inhibition was evident in any of the qPCR analyses performed. For PCR based community analyses, we observed that bacterial and fungal pyrosequencing PCR was successful. However, the pyrosequencing PCR targeting archaeal 16S rRNA genes did not work for all samples. This is presumably due to the lower number of PCR cycles used with this method (25 cycles) compared to that of the bacteria (30 cycles) and fungi (35 cycles). Noteworthy is the observation that only PQIAmini DNA extracts generated archaeal amplicons for both fractions for all the samples.

Pyrosequencing analysis

Bacteria community analysis - The clustering of bacterial communities was distinct for the PBB method and FDSS method as compared to the RBB and PQIAmini methods (Fig. 2). From the PCoA plots (Fig. S3), the latter three methods however, had a gradual shift of the bacterial communities between methods, suggesting that all four DNA extraction methods had an effect on the observed bacterial community structure to some extent. Further analysis of the data

confirmed that DNA extraction method affected the relative abundances of various families and genera.

The predominant phyla detected in this study were Firmicutes and Bacteroidetes, which is in line with other bovine rumen based studies (De Menezes et al., 2011b; Li et al., 2014; Huws et al., 2016; Van Lingen et al., 2017a). The predominant family-level taxa belonging to Firmicutes in the RF fraction were: Ruminococcaceae, Lachnospiraceae and unclassified Clostridiales. Within the Bacteroidetes, Prevotellaceae was the predominant family. These observed families were in line with a previous study (Mao et al., 2015). The FC fraction showed a significantly higher relative abundance of Fibrobacteraceae, Ruminococcaceae and Lachnospiraceae compared to the RF fraction. These three families were also pre-dominant in our study, which is in accordance with another bovine rumen microbiota study (McCann, 2014). Ruminococcaceae were observed at significantly higher relative abundances in extracts prepared with the PBB method as compared to the FDSS method. This suggests that the PBB method was more effective in lysing these cells, or conversely that it was less effective in lysing cells of other microbial groups since the data is based on relative abundance. Lachnospiraceae, on the other hand, was not affected by any extraction method. In a study from Fouts et al. (2012), two members of the Lachnospiraceae, namely *Butyrivibrio* and *Blautia,* were reported to have significantly higher relative abundance in the FC as compared to the RF fraction. Partly in agreement with this, we observed a fraction effect for *Butyrivibrio* but not for *Blautia*. There was a significant decrease of the family Fibrobacteraceae for the PBB method as compared to the RBB and FDSS for the FC fraction samples, indicating that the PBB method was less effective in extracting Fibrobacter DNA compared to other methods. Similarly, for many other genera like *Selenomonas, Succiniclasticum, Ruminococcus, Prevotella, Paraeggerthella, Syntrophococcus, Fibrobacter, Oscillibacter, Desulfobulbus* and *Pseudobutyrivibrio* we observed a fraction effect indicating a distinct separation of microbial communities associated with RF and FC fractions, which is line with the bovine rumen study of (Fouts et al., 2012). This fraction effect might be explained by the different feed components available in the RF and FC fractions (insoluble polymers versus soluble monomers), as well as the difference in ability of cells to adhere to the plant fibres.

Together, these data reinforce the notion that not all bacterial community members and rumen fractions are equally affected by the tested extraction methods, making it difficult to come up with informed decisions as to which extraction method generates DNA that is most

representative of the rumen bacterial community. To this end, synthetic communities of defined bacterial composition could provide additional insight, in analogy to defined mock communities assembled at the DNA level that have been used to assess the influence of different steps during molecular community assessment (Ramiro-Garcia, 2016). One could argue, however, that such synthetic communities would not sufficiently represent *in vivo* rumen conditions, especially for the FC fraction, and thus, particular attention will need to be paid to the design of such analyses.

Archaea community analysis - The DNA extracts obtained from the four extraction methods amplified well for qPCR but when used for 16S rRNA gene-based archaeal community assessment, not all the samples yielded PCR products. One of the possible reasons for this observation, as mentioned above, is the lower number of PCR cycles used for this particular taxon. Furthermore, archaeal diversity was found to be very limited compared to bacteria. A previous study on the comparison of DNA extraction methods on rumen fractions revealed *Methanobrevibacter* spp. as the most dominant methanogen from all extraction methods applied (Henderson et al, 2013). In the Henderson et al. (2013) study one universal primer pair was used to simultaneously amplify the 16S and 18S rRNA genes of bacteria, archaea and ciliate protozoa. This type of approach would avoid the issues encountered in this study with limited amplification of the archaeal 16S rRNA gene in some samples. A universal 16S rRNA sequencing approach, simultaneously amplifying 16S rRNA genes of both the bacteria and archaea, could also be used (Van Lingen et al., 2017). A potential drawback of a universal primer approach could be that if bacteria are more abundant, archaea might not be detected at all. As a consequence, attention should be paid to an appropriate sequencing depth that would safeguard detection and identification of archaeal populations of relative abundances >1%.

The relative distribution of different archaeal populations has previously been shown to be affected by several factors such as diet, host age or species, season and geographical region (Huang et al., 2016). In this study, in the samples for which a PCR product could be generated, the genera *Methanobrevibacter* followed by *Methanosphaera* were the dominant archaeal taxa in all the RF and FC samples. Similar to our results, both *Methanobrevibacter* and *Methanosphaera* were found to be conserved members of the methanogenic population in other bovine studies which focused on physiological interactions within the rumen microbial food web (Janssen and Kirs, 2008; Henderson et al, 2013; De Mulder et al., 2016). *Methanobrevibacter* species can utilize H_2, CO_2 and formate, whereas *Methanosphaera* species can produce CH_4 only via reduction of methanol with H_2 (Carberry et al., 2014b). From a recent study by (Van Lingen,

2016) it was shown that there is no benefit for the methane producers if H_2 or formate are consumed, as there is no energetic limitation due to H_2/formate accumulation in the rumen.

Interestingly in the current study, qPCR showed high numbers of methanogenic archaea in the FC fraction as compared to the RF fraction. This is consistent with the results obtained in a recent study by de Mulder and co-authors (De Mulder et al., 2016) indicating that the methanogenic archaea make up an intrinsic part of the solid fraction in the cow rumen. The presence of archaea in the FC fraction, however, is not surprising considering the close physical and metabolic interactions of methanogens with anaerobic fungi, which extensively colonise and invade rumen FC (Cheng, 2009; Jin et al., 2011). It was noted in this study that *Methanosphaera* seemed to have a lower relative abundance in the FC fraction compared to the RF fraction, however, further work is needed to verify this due to the limited number of biological samples used in this study.

Fungal community analysis - Sequences from anaerobic fungi *(*Neocallimastigomycota phylum) were obtained from five genera: *Piromyces, Anaeromyces, Neocallimastix, Cyllamyces* and *Orpinomyces*. These fungi are involved in the degradation of the lignocellulose fraction of plant material in the rumen (Kittelmann, 2012). In line with this, a larger proportion of anaerobic fungal reads was on average observed in the FC fraction as compared to the RF fraction in the fungal community analysis. This is also consistent with the qPCR analysis, which revealed a higher abundance of anaerobic fungi in FC relative to RF fractions. In our study, the anaerobic fungal community in FC fraction samples was mainly composed of the genera *Cyllamyces* (2 to 3%), *Neocallimastix* (1 to 3%) and *Piromyces* (1 to 2%) with sequences assigned to *Orpinomyces* only detected in the GS33MS67 FC sample subjected to the RBB method (Table 2B). On the other hand in RF samples, no sequences from *Orpinomyces* were detected and a more limited amount of all of the other genera were detected compared to FC samples (Table 2A). The overall higher detection of anaerobic fungal genera in FC, as compared to the RF fraction, is likely to be due to the motile zoospores being only transiently present within rumen fluid for a short time after feeding (Orpin, 1974; 1975; 1976; 1977; Griffith et al., 2009).

Besides the identification of the five genus level groups mentioned above belonging to Neocallimastigomycota*,* we observed a large number of unidentified fungi belonging to both anaerobic and aerobic fungi (Table 2A and 2B) as well as a high proportion of sequences that could not be further assigned to any phylum. As Neocallimastigomycota are considered to be the

key fungal phylum relative to rumen function, community assessment of this specific community using targeted anaerobic fungal primers would provide a better approach, as amplification of aerobic fungi associated with ingested feed and water would be avoided. The larger read depth this would generate would also improve the ability to interpret the impact of different experimental factors on the taxa within the phylum, particularly as there is an increasing evidence of anaerobic fungal niche differentiation within the rumen (Griffith et al., 2009). Furthermore, a custom ITS1 database is also available specifically for the Neocallimastigomycota phylum (Koetschan, 2014).

CONCLUSIONS

DNA extraction methods clearly have an impact on the outcome of downstream rumen microbial community analyses, including relative abundances of specific community members. From this study, this effect was evident with the bacterial community, however, no single extraction method could be concluded as being ineffective. Rather, every extraction method presented its own strengths and weaknesses in observing specific bacterial families. DNA extracted using the PBB method resulted in higher relative abundance of Ruminococcaceae than the FDSS method, whereas relative abundance of Fibrobacteraceae was lower compared to the RBB method. Whilst the effect of DNA extraction method was limited compared to that of rumen fraction, differences due to both DNA extraction method and fraction were observed for certain taxa. Further investigation is needed to determine if this is due to an issue with the physical nature of the different fractions, or merely due to the inherent differences in the microbes present within the fractions. Furthermore, careful selection of the microbial community assessment approach is needed to avoid the issues encountered within this study with respect to archaea and anaerobic fungi. Archaeal 16S rRNA gene barcoded amplicons are best generated in combination with other taxa (bacteria or bacteria and protozoa), whilst anaerobic fungi should be generated with phylum specific primers rather than those designed to cover the entire fungal kingdom. In summary, the comprehensive assessment of observed communities of bacteria, archaea and fungi described here provides insight into a rational basis for selecting an optimal methodology to obtain a representative picture of the rumen microbiome.

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Table 1. Purity and yield of genomic DNA extracted from rumen samples taken from cows fed different diets (GS, grass silage and MS, maize silage) and separated into different fractions (rumen fluid (RF) and fibrous content (FC)). Four different DNA extraction methods were used (RBB, PBB, FDSS and PQIAmini), and the DNA extraction of the 100% grass silage diet (GS100) samples was done in duplicate (I & II).

Diet	DNA extraction methods	DNA yield (RF vs FC)		DNA purity (RF)		DNA purity (FC)	
		RF (µg/ml RF)	FC (µg/g FC)	A260/280	A260/230	A260/280	A260/230
GS100	RBB (I)	6.4	38.7	2.0	2.1	1.7	1.2
	RBB (II)	6.6	28.8	1.9	1.7	1.8	1.3
	PBB (I)	55.9	327.7	1.8	1.6	1.6	1.0
	PBB (II)	50.1	251.5	1.8	1.5	1.6	1.1
	FDSS (I)	20.4	131.4	1.9	0.3	1.9	0.6
	FDSS (II)	23.9	107.4	1.9	0.6	1.9	0.9
	PQIAmini (I)	25.1	83.1	1.8	1.5	1.9	1.8
	PQIAmini (II)	24.4	103.2	1.8	1.7	1.9	1.8
GS67MS33	RBB	6	25.9	2.0	1.8	1.8	1.4
	PBB	57.2	378.2	1.9	1.6	1.7	1.1
	FDSS	21	82.6	1.9	0.5	1.9	0.4
	PQIAmini	20.2	95.2	1.8	1.5	1.9	1.5
GS33MS67	RBB	11.7	30.5	2.1	2.1	1.8	1.2
	PBB	73.7	208.2	1.9	1.5	1.7	1.3
	FDSS	20.9	103.4	1.7	0.2	1.9	0.4
	PQIAmini	27.8	169	1.9	1.7	1.9	1.7
MS100	RBB	7.5	18.1	2.0	1.7	1.9	1.7
	PBB	117.6	187.5	2.0	1.9	1.9	1.9
	FDSS	15.8	96.6	1.9	0.4	1.9	0.5
	PQIAmini	17.7	84.6	1.9	1.5	1.9	1.8

Table 2. Anaerobic fungal composition at genus level for the the rumen fluid **(A)** and fibrous content **(B)** samples from dairy cows each fed different ratios of grass silage (GS) to maize silage (MS), e.g. GS67MS33 is a diet containing 67 % grass silage and 33 % maize silage. The unidentified fungi represent aerobic and anaerobic fungi that could not be classified to the genus level, and the remaining fraction (not indicated in the table) belongs to fungi that could not be classified at the phylum level. DNA extraction technical replicates for the GS100 samples are denoted I & II. nd refers to not detected

A

Diet	DNA extraction methods	Relative abundance (%)					
		Piromyces	Neocallimastix	Cyllamyces	Anaeromyces	Orpinomyces	Unidentified fungi
GS100	RBB I	0.05	nd	0.1	nd	nd	71.41
	RBB II	nd*	nd	0.21	nd	nd	52.22
	PBB I	nd	nd	0.07	nd	nd	80.24
	PBB II	nd	nd	0.19	nd	nd	78.09
	FDSS I	0.28	0.09	0.28	nd	nd	63.88
	FDSS II	nd	0.08	nd	nd	nd	79.97
	PQIAmini I	nd	nd	nd	nd	nd	86.47
	PQIAmini II	nd	nd	nd	nd	nd	88.59
GS67MS33	RBB	nd	nd	nd	nd	nd	55.11
	PBB	nd	nd	nd	nd	nd	31.84
	FDSS	nd	nd	nd	0.03	nd	76.36
	PQIAmini	nd	nd	nd	nd	nd	66.77
GS33MS67	RBB	0.16	0.31	nd	nd	nd	31.42
	PBB	nd	nd	nd	nd	nd	60.51
	FDSS	0.1	nd	0.05	0.05	nd	35.23
	PQIAmini	nd	nd	nd	nd	nd	53.51
MS100	RBB	nd	0.03	nd	nd	nd	66.4
	PBB	nd	0.04	nd	nd	nd	32.77
	FDSS	nd	0.02	nd	nd	nd	75.03
	PQIAmini	nd	nd	nd	nd	nd	75.18

B

Diet	DNA extraction methods	Relative abundance (%)					
		Piromyces	Neocallimastix	Cyllamyces	Anaeromyces	Orpinomyces	Unidentified fungi
GS100	RBB I	nd*	0.99	nd	nd	nd	2.97
	RBB II	nd	nd	nd	nd	nd	19.2
	PBB I	0.06	nd	nd	nd	nd	16.56
	PBB II	2.81	1.25	2.5	nd	nd	14.38
	FDSS I	1.59	0.79	3.17	nd	nd	6.35
	FDSS II	1.23	3.7	1.23	nd	nd	13.58
	PQIAmini I	1.54	3.08	3.08	nd	nd	27.69
	PQIAmini II	1.16	2.33	1.16	1.16	nd	30.23
GS67MS33	RBB	0.86	3.45	0.86	5.17	nd	18.1
	PBB	1.56	nd	nd	1.56	nd	73.44
	FDSS	1.22	nd	2.44	0.61	nd	6.1
	PQIAmini	nd	0.88	0.88	nd	nd	36.84
GS33MS67	RBB	nd	nd	nd	nd	0.6	1.79
	PBB	0.73	1.22	nd	nd	nd	1.46
	FDSS	1.61	8.06	nd	nd	nd	nd
	PQIAmini	0.83	1.65	nd	nd	nd	7.44
MS100	RBB	nd	0.37	nd	nd	nd	42.61
	PBB	31.51	1.37	nd	nd	nd	39.73
	FDSS	nd	nd	nd	nd	nd	17.89
	PQIAmini	nd	0.54	nd	nd	nd	63.39

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SUPPLEMENTARY MATERIAL

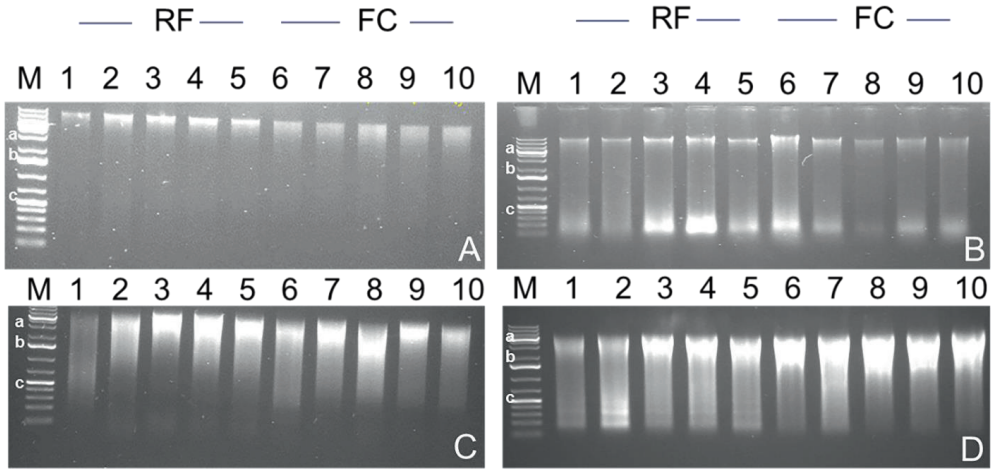

Figure S1 Integrity of genomic DNA extracted with different methods visualized on a 1% agarose gel. DNA obtained from rumen fluid (RF: lanes 1-5) and fibrous content (FC: 6-10) samples using method RBB **(A)**, PBB **(B)**, FDSS **(C)** and PQIAmini **(D)**. Lanes represent cows fed the different diets as follows: MS100 (1 & 6), GS33MS67 (2 & 7), GS100 (3, 4, 8, and 9, as two technical replicates for this diet) and GS67MS33 (5 and 10). Lane M: 1 kb plus DNA size marker was used for all gels, a: 5000bp, b: 1000bp, c: 500bp.

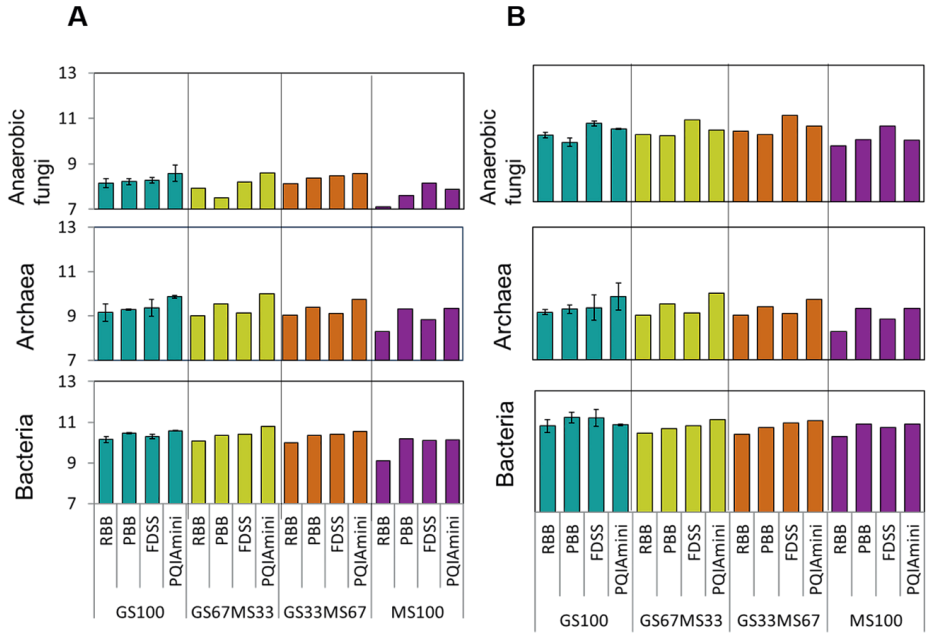

Figure S2. Bacterial and archaeal 16S rRNA and anaerobic fungal 5.8S rRNA gene copy numbers per ml of rumen fluid (RF) or gram fibrous content (FC) samples from dairy cows fed different ratios of grass silage (GS) to maize silage (MS), as measured in DNA extracted using four different methods (RBB, PBB, FDSS and PQIAmini). Bars represent the mean of triplicate qPCR determinations for a single sample DNA extract with the exception of GS100, where they represent the mean of duplicate DNA extracts and the error bars represent their variation. Data is presented as log10 copy numbers per ml RF **(A)** and gram FC **(B)**.

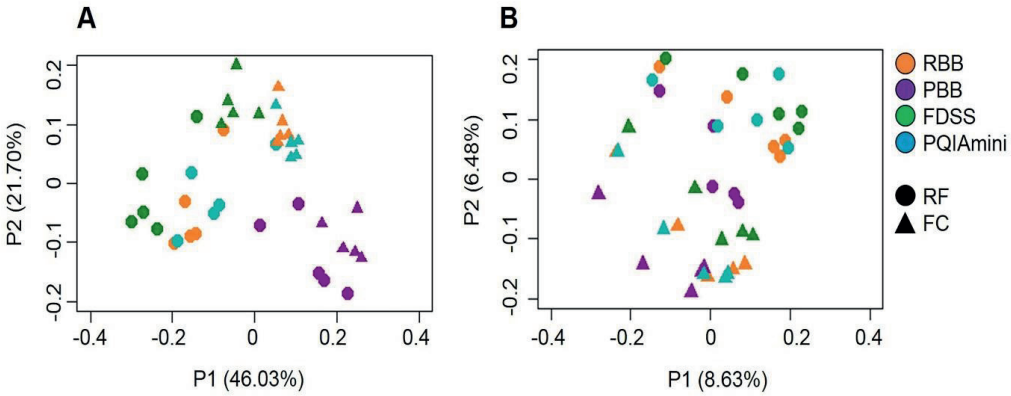

Figure S3. Weighted **(A)** and unweighted **(B)** UniFrac principal coordinate analysis (PCoA) of the rumen bacterial community at the OTU level. Datapoints are coded in terms of DNA extraction method (RBB, PBB, FDSS and PQIAmini) and fraction (rumen fluid (RF) and fibrous content (FC)). The GS100 diet has duplicate DNA extracts presented as individual datapoints. The percentages given at P1 and P2 indicate the amount of variation represented by the co-ordinate axes.

Table S1. Bacterial families and genera that significantly differ ($p<0.05$) in relative abundance in rumen fluid (RF) versus fibrous content (FC) fraction are shown in **(A)**, and different DNA extraction method (RBB, PBB, FDSS and PQIAmini) comparisons are shown in **(B)**. Relative abundance (%) values presented are the mean ± standard deviation.

A

Taxon	RF	FC	p value
Family			
Desulfobulbaceae	0.1 ± 0.1	0.0 ± 0.0	0.030
Fibrobacteraceae	0.6 ± 0.5	2.7 ± 2.3	0.020
Lachnospiraceae	8.0 ± 3.5	12.5 ± 3.3	0.006
Prevotellaceae	29.4 ± 11.1	11.6 ± 3.1	0.001
Ruminococcaceae	6.1 ± 1.7	9.0 ± 1.9	0.040
Genus			
Butyrivibrio	0.8 ± 0.3	1.7 ± 0.8	0.0001
Desulfobulbus	0.1 ± 0.0	0.0 ± 0.0	0.036
Fibrobacter	0.5 ± 0.4	2.8 ± 2.3	0.021
Oscillibacter	0.0 ± 0.0	0.1 ± 0.1	0.027
Paraeggerthella	0.1± 0.0	0.0 ± 0.0	0.0002
Prevotella	18.4 ± 8.1	4.7 ± 2.9	<0.0001
Pseudobutyrivibrio	0.2 ± 0.1	0.5 ± 0.2	0.010
Ruminococcus	0.3 ± 0.2	1.0 ± 0.5	<0.0001
Selenomonas	0.2 ± 0.1	0.0 ± 0.0	<0.0001
Succiniclasticum	2.1 ± 1.1	0.5 ± 0.3	<0.0001
Syntrophococcus	0.2 ± 0.1	0.3 ± 0.1	0.009

B

Taxon	DNA extraction method	p value
Family		
Anaerolineaceae	PQIAmini (0.1 ± 0.1) vs PBB (0.3 ± 0.1)	0.027
Anaerolineaceae	PBB (0.3 ± 0.1) vs RBB (0.1 ± 0.0)	0.038
Fibrobacteraceae	FDSS (3.2 ± 1.4) vs PBB (0.2 ± 0.1)	0.028
Fibrobacteraceae	PBB (0.2 ± 0.1) vs RBB (1.6 ± 0.7)	0.038
Halomonadaceae	FDSS (0.02 ± 0.0) vs PQIAmini (0.01 ± 0.01)	0.008
Halomonadaceae	PBB (0.2 ± 0.1) vs FDSS (0.02 ± 0.0)	0.028
Ruminococcaceae	FDSS (5.8 ± 0.6) vs PBB (9.6 ± 1.8)	0.038
Genus		
Fibrobacter	RBB (1.6 ± 1.4) vs PBB (0.1 ± 0.1)	0.038
Fibrobacter	PBB (0.1 ± 0.1) vs FDSS (3.5 ± 3.0)	0.038

Chapter 3

Characterization of dairy cow rumen bacterial and archaeal communities associated with grass silage and maize silage based diets

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ABSTRACT

Replacing grass silage (GS) with maize-silage (MS) in the diets of dairy cattle offers an effective strategy to decrease enteric methane (CH_4) production without negatively affecting dairy cow performance. The present study aimed to characterise the rumen bacterial and archaeal communities in dairy cows fed different ratios of MS and GS, and places the findings in the context of ruminal fermentation as well as previously reported CH_4 emissions. Rumen fluid samples from 12 rumen cannulated dairy cows were collected after 10 and 17 days of feeding one of four diets. All diets had a roughage: concentrate ratio of 80 : 20 based on dry matter (DM). Roughage consisted of either 1000 g/kg DM GS (GS100), 1000 g/kg DM MS (GS0), or a mixture of both silages [667 g/kg DM GS and 333 g/kg DM MS (GS67); 333 g/kg DM GS and 677 g/kg DM MS (GS33)]. In terms of ruminal VFA, no significant diet effects were found but the molar proportions of isovalerate were affected by time, being lower on day 17 than day 10. Bacterial and archaeal concentrations were not affected by diet, but increased from day 10 to day 17. Bacterial community composition was affected by diet, time and diet × time, whereas archaeal community composition was only affected by diet. Several bacterial and archaeal genera could be associated with diet, but not with time. In the case of archaea, relative abundance of *Methanobrevibacter* was positively associated with the GS0 diet. Bacterial genera belonging to the Succinivibrionaceae and Ruminococcaceae were among those associated with maize silage diets, suggesting a role in the low methane emissions observed in a previous study. The observed time effects indicate that the rumen microbiome was not stable after 10 days of feeding the diets.

KEYWORDS

Dairy cow; rumen fluid; grass silage; maize silage; rumen bacterial community; rumen archaeal community; fermentation products

INTRODUCTION

Dietary composition, geographical location, cow breed and the health of the host animal are known factors that influence the rumen microbial community structure (De Menezes et al., 2011a; Henderson et al., 2015b). Of all these factors, diet is considered to be the largest driver of change in ruminal fermentation, as well as of changes in the associated microbiota and enteric methane (CH_4) production (van Lingen et al., 2016; van Lingen et al., 2017b). Enteric CH_4 is produced by ruminal methanogenic archaea and not only contributes to 16% of the total anthropogenic global greenhouse gas emissions but also represents 2-12% energy loss for the host (Johnson and Johnson, 1995; IPCC, 2006). Enteric fermentation in ruminants is considered the largest source of CH_4 emissions in agriculture (IPCC, 2014). Decreasing CH_4 emissions has, therefore, become a major concern in ruminant livestock production, and has been a prime research focus in recent years. One of the most effective ways of reducing CH_4 emissions from dairy cattle is through dietary strategies.

Grass silage (GS) and maize silage (MS) represent the major components in dairy cow diets. Generally, GS has a higher fibre content (i.e., neutral detergent fibre and acid detergent fibre), whereas MS has a higher starch content. Fermentation of starch favours the ruminal production of propionate and decreases ruminal pH, which reduces hydrogen availability and activity of rumen methanogens and consequently enteric CH_4 production (van Kessel, 1996; Hook et al., 2010; Brask et al., 2013). Recently, van Gastelen et al. (2015) demonstrated that replacing GS with MS in dairy cow diets has an impact on ruminal fermentation and CH_4 emission. Replacing GS with MS led to a decrease in CH_4 emissions and an increase in the molar proportions of butyrate, whereas the total volatile fatty acid (VFA) concentration and the molar proportions of acetate and propionate were unaffected (van Gastelen et al., 2015).

It is unclear how the changes reported by van Gastelen et al. (2015) in CH_4 emissions and fermentation characteristics are related to changes in the rumen microbiota. Additionally, the majority of rumen microbial studies to date have primarily focussed on starch in the context of cereal grains (Pitta et al., 2010; De Menezes et al., 2011a; Jiang et al., 2015) rather than different types of roughages. Hence, the objectives of the present study were (1) to investigate the effect of replacing fibre-rich GS with starch-rich MS on the rumen bacterial and archaeal diversity and concentrations using samples collected 10 and 17 days after the

introduction of the experimental diets, and (2) to place the findings in the context of ruminal fermentation as well as previously reported data on CH_4 emission (van Gastelen et al., 2015).

MATERIALS AND METHODS

Samples and ethics statement

The rumen fluid (RF) samples used in the present study were collected from 12 rumen cannulated dairy cows. These animals represented a subset of the 32 dairy cows used in a previously published study (van Gastelen et al., 2015) that was conducted at the animal research facilities of Wageningen University & Research (Wageningen, the Netherlands). As described by van Gastelen et al. (2015), all the experimental procedures were in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University.

Study design and diets

The study design is similar to van Gastelen et al. (2015). In short, 12 rumen cannulated dairy cows, were allocated into groups of four cows according to lactation stage, parity, and milk production. Within each group cows were randomly assigned to one of four dietary treatments. All dietary treatments had a roughage-to-concentrate ratio of 80:20 based on dry matter (DM) content. The composition of the concentrate was similar for all four treatments, whereas the roughage was GS, MS, or a mixture of both (ingredient as percentage of the total amount of roughage in the diet; DM basis): 100% GS (**GS100**), 67% GS and 33% MS (**GS67**), 33% GS and 67% MS (**GS33**), and 100% MS (**GS0**). The treatment period lasted 17 days consisting of a dietary adaptation period of 12 days followed by a 5 day period in climate respiration chambers to determine CH_4 emission. The cows were fed ad libitum during the first 7 days of the adaptation period. From day 8 to 17, feed intake was restricted to 95% of the ad libitum dry matter intake (DMI) of the cow within a group consuming the lowest amount of feed during day 5 to 8. During the study one of the cows was diagnosed with mastitis and treated locally (i.e., in the udder directly) with antibiotics and intravenously with a painkiller. However, the cow was retained in the study as it was otherwise healthy and eating well.

Rumen sampling

The RF was sampled on days 10 and 17, i.e., before and directly after the climate respiration chamber phase, respectively. The RF was collected 4 hours after morning feeding according to the method described by van Zijderveld et al. (2010) and collected in three equal volumes from the front and middle of the ventral sac and from the cranial sac of the rumen. The RF sampled from the three regions was subsequently pooled, aliquoted in ~50 mL portions, immediately frozen on dry ice, and within 2 hours of collection transported to the lab where it was stored at -80 ^{0} C until DNA extraction and VFA analysis. The sampling tube was rinsed with warm water between sampling from different cows.

Determination of rumen VFA concentrations

RF aliquots (1 mL) from the 12 cows at the two time points (i.e., day 10 and day 17) were centrifuged at 10,000 g for 10 min, after which the metabolites dissolved in the supernatant were separated by a Spectrasystem HPLC (Thermo Scientific, Breda) equipped with a Metacarb 67H column (Agilent, 300 × 65 mm) and quantified with a Refractive Index detector. Column temperature was 45ºC, and 5 mM sulphuric acid was used as eluent. Flow rate was set at 0.9 mL/min. HPLC data analysis was performed in Chromeleon 7 software. The internal standard used was DMSO (10 mM in 0.1N H_2SO_4).

DNA extraction

Total genomic DNA was extracted from 24 RF samples (i.e., two time points for each of the 12 animals). Prior to DNA extraction, RF aliquots (1 mL) were centrifuged at 15,000 g for 10 min at 4 ºC, and the cell pellets were used for DNA extraction as previously described (van Lingen et al., 2017b). Briefly, cells were lysed using repeated bead beating, and the lysate was further processed in a customized MaxWell® 16 Tissue LEV Total RNA Purification kit cartridge (XAS $_{1200}$) (Promega Biotech AB, Stockholm, Sweden) (van Lingen et al., 2017b). The quantity and purity of the DNA in the obtained extracts were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA).

qPCR analysis

Quantitative PCR (qPCR) assays targeting bacterial and archaeal 16S ribosomal RNA (rRNA) genes were performed using a BioRad CFX96 system (Bio-Rad Laboratories). The qPCR

reactions were carried out in triplicate as previously described by van Lingen et al. (2017b), except that the reaction volumes were 25 µl.

For standard curve preparation, a bacterial and an archaeal 16S rRNA gene PCR product was prepared as previously described (van Lingen et al., 2017b). DNA concentration and amplicon size were used to calculate the number of amplicon copies, and 10-fold serial dilutions were prepared in water from 10^8 to 10^0 amplicon copies/µl.

Barcoded 16S rRNA gene amplicon sequencing

For the analysis of bacterial and archaeal community composition, barcoded amplicons of the 16S rRNA genes were generated using a 2-step PCR strategy (Tian et al., 2016). For bacterial composition profiling, the forward primer 27F-DegS: 5′- GTTYGATYMTGGCTCAG -3′ (van den Bogert et al., 2011), and the reverse primer mix of 338R–I: GCWGCCTCCCGTAGGAGT (Daims et al., 1999a) and 338R–II: GCWGCCACCCGTAGGTGT (van den Bogert et al., 2013) were used with attached UniTag1 (forward primer: GAGCCGTAGCCAGTCTGC) and UniTag2 (reverse primer mix: GCCGTGACCGTGACATCG) linkers, respectively (Tian et al., 2016). For archaea composition profiling, 518F (5′ - CAGCMGCCGCGGTAA -3′) (Wang and Qian, 2009) was used as the forward primer with UniTag1, and the reverse primer 905R (5′ - CCCGCCAATTCCTTTAAGTTC – 3′) (Kvist et al., 2007) with UniTag2. The first PCR step was performed in a total volume of 50 µL containing 10 µL 1× HF buffer (Finnzymes, Vantaa, Finland), 1 µL dNTP Mix (10 mM; Promega), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (2 U/µL) (Finnzymes), 500 nM each of the primers UniTag1-27f Deg S and UniTag2-338R-I + II (for bacteria) or UniTag1-518f and UniTag2-905r (for archaea) and 20 ng of sample DNA. The cycling conditions for the first step consisted of an initial denaturation at 98 °C for 30 s; 25 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C (for bacteria) or 60 °C (for archaea) for 20 s, and elongation at 72 °C for 20 s; with a final extension at 72 °C for 10 min.

The second PCR step was then employed to add an 8-nucleotide sample specific barcode to the UniTag primer target sequences obtained from the first amplicon, as previously described (Hamady et al., 2008). The second step PCR was performed in a total volume of 100 µL containing: 1× HF buffer, 2 µL of dNTP Mix, 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (2 U/µL) and 500 nM of a forward and reverse primer targeting the

UniTag1 and UniTag2 sequences, respectively, that were each appended with an 8 nt sample specific barcode at the 5' end of the respective primer. The cycling conditions of the second step for both bacteria and archaea consisted of an initial denaturation at 98 °C for 30 s followed by 5 cycles of: 98 °C for 10 s, 52 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 10 min. Incorporation of the sample specific barcodes, yielding a PCR product of ~350 bp and ~385 bp for bacteria and archaea, respectively, was confirmed by agarose gel electrophoresis. Control PCR reactions were performed alongside each separate amplification with no addition of template, and consistently yielded no product. PCR products were then purified using HighPrep^{TM} (MagBio Europe Ltd, Kent, United Kingdom) and quantified using a Qubit fluorometer in combination with the dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). Purified PCR products were mixed in equimolar amounts into pools together with defined synthetic mock communities that allowed assessment of potential technical biases (Ramiro-Garcia et al., 2016). Pools then underwent adaptor ligation followed by sequencing on the Illumina MiSeq platform (GATC-Biotech, Konstanz, Germany).

Sequence data quality control and processing

The 16S rRNA gene sequencing data was analyzed using NG-Tax Galaxy version 1.0, an in-house pipeline (Afgan et al., 2016; Ramiro-Garcia et al., 2016). Paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and those barcodes were used to demultiplex reads by sample. Operational taxonomic units (OTUs) were defined at 100% identity using an open reference approach, and taxonomy was assigned to those OTUs using the SILVA version 128 16S rRNA gene reference database (Quast et al., 2013).

Data analysis and visualization

Parameters related to ruminal fermentation and microbial concentrations were analyzed using the MIXED procedure in SAS (edition 9.3, SAS Institute Inc., Cary, NC, USA). The parameters were subjected to repeated measures ANOVA to take repeated sampling from the same animal into account. The model included dietary treatment, time, and dietary treatment × time as fixed effects, and group and cow as random effects. Post-hoc analyses were carried out using the Tukey-Kramer test for pairwise comparisons. Significance of treatment effects was declared at $P \leq 0.05$, and trends at $0.05 < P < 0.10$.

Alpha diversity of the 16S rRNA gene data was estimated with the phylogenetic diversity (PD) index. Normality of the bacterial and archaeal PD dataset was assessed using

the Shapira-Wilk's normality method, with a P-value > 0.05 confirming normal distribution. Consequently, effects of diet, and diet × time were assessed using Kruskal-Wallis test on the PD dataset while the time effect on the PD was assessed by a paired T-test. To assess the beta diversity in bacterial and archaeal communities in the cows across all four experimental diets, unweighted and weighted UniFrac distances were used to perform principal co-ordinate analysis (PCoA). Adonis was used to test for significance of sample groupings in the PCoA with respect to diet, time and diet × time (Anderson, 2001). The R packages used to perform and visualise the community based analysis in RStudio were: ape, vegan, microbiome, phyloseq, picante and ggplot2 (Philip, 2003; Paradis et al., 2004; Kembel et al., 2010; Lahti and Shetty, 2017).

Constrained partial redundancy analysis (RDA) of bacterial and archaeal 16S rRNA gene sequence data was performed to assess the relationship between genus-level phylogenetic groupings and explanatory variables diet with covariance time, time with covariance diet and diet × time, using Canoco 5 (Smilauer, 2014). Significance of explanatory variables was tested using a Monte Carlo permutation test with a total of 999 permutations.

All P-values for the alpha and beta-diversity statistical analysis were corrected for multiple testing using False discovery rate (FDR) correction, and significant effects were declared at $P \leq 0.05$, and trends at $0.05 < P < 0.10$.

Data availability

The 16S rRNA amplicon sequencing data for the bacterial and archaeal composition analysis has been deposited as one study in European Nucleotide Archive (ENA) under accession number PRJEB24373.

RESULTS

Ruminal fermentation characteristics

Ruminal fermentation products are presented in Table 1. The molar proportions of butyrate tended to be affected by diet (P = 0.065). Total VFA tended to be affected by time (P = 0.076) and the molar proportions of isovalerate were affected by time (P = 0.002), being lower on day 17 than on day 10.

Quantification of bacteria and archaea

The rumen bacterial and archaeal concentrations are presented in Table 1. The bacterial concentrations were affected by diet (P = 0.026) and time (P = 0.008). The bacterial concentrations were lower for the GS0 diet compared with the other three diets (GS100, GS67, and GS33), and the bacterial concentrations were higher on day 17 compared with day 10. The archaeal concentrations were only affected by time (P = 0.022) with the archaeal concentration being higher on day 17 compared with day 10.

Changes in rumen bacterial community composition

Overall, the genus-level taxon *Prevotella* 1 (58.1% $\pm$ 0.06) dominated in all the samples. The next most predominant genus level taxon was the NK4A214 group belonging to Ruminococcaeae (5.5% $\pm$ 0.01), with all other genus level groupings being < 1% on average (Fig.1). Whilst these two predominant taxa did not vary greatly in relative abundance, other genus level groups did, such as *Prevotella* UCG-003 (as evidenced by the large standard deviations in Fig. 1), indicating inter animal variation.

With respect to alpha diversity, the PD values were confirmed to have a normal sample distribution (P = 0.196). There was no significant effect of diet (P = 0.500), time (P = 0.761) or diet × time (P = 0.697) on bacterial PD (Fig. 2A and 2B).

PCoA analysis at the OTU-level, using unweighted UniFrac distances (Fig. 2C), showed significant clustering of the bacterial RF samples with diet (P = 0.006) and time (P = 0.005), with no diet × time interaction (P = 0.763). With respect to diet, profiles of GS100 diet fed animals generally clustered at the top of PCoA axis 2 and the GS0 diet generally clustered at the bottom of the PCoA axis 2, with the mixed proportion diets (GS67 and GS33) generally situated between the two extreme diets (Fig. 2C). With respect to time, samples taken at day 17 and day 10 were generally separated along PCoA axis 1 (Fig. 2C). In the corresponding weighted UniFrac analysis, there was a tendency for an effect of diet (P = 0.062), whereas no time effect (P = 0.139) or diet × time interaction (P = 0.911) were seen (Fig. S1).

In order to assess the contribution of diet, time and diet × time interaction to the observed variation in the bacterial community composition at the genus level, constrained RDA analyses were performed. Diet significantly contributed to explaining the observed variation in bacterial community composition (P = 0.002) and was associated with 24.5% of

the total variation in the bacterial community. In the RDA triplot (Fig. 3A), samples of animals fed the different experimental diets separated along the first canonical axis according to the decreasing proportion of GS (i.e., from left to right). Several of the genera had highest relative abundance with one of the extreme diets (i.e., either GS100 or GS0). The following genera were positively associated with the GS0 diet: the UCG-002 group belonging to the Succinivibrionaceae, a genus containing *Eubacterium coprostanoligenes, Moryella,* the UCG-014 group belonging to the Ruminococcaceae, *Lactobacillus, Succinivibrio,* the YAB-2003 group belonging to the Prevotellaceae and a genus that could only be reliably annotated to the phylum Saccharibacteria. In contrast, the genera *Prevotella* 2*, Leuconostoc,* and *Candidatus* 'Saccharimonas' were positively associated with the GS100 diet, along with several genus level groupings that could only be annotated to the family (Piscirickettsiaceae, vadin BE-97) or phylum level (SR1(Absconditabacteria)).

Partial RDA analysis with diet as covariate that time had an effect (P = 0.015) on the bacterial composition and explanatory variables accounted for 10.56 % of the residual variation in the bacterial community (Fig. 3B). The full RDA analysis showed that there was a diet × time interaction (P = 0.011) which accounted for 39.04% of the total variation in the bacterial community (Fig. S2). Generally, the diets separated along the first canonical axis (GS decreasing from left to right) and time on the second canonical axis (day 10 to the top and day 17 to the bottom). The extent of the differences between days 10 and 17 varied with diet, with the differences being smallest for GS67 and largest for GS33.

Table 1. Ruminal fermentation products and rumen bacterial and archaeal concentrations measured using rumen fluid (RF) samples from dairy cows after 10 or 17 days of feeding different proportions of grass and maize silage (e.g. GS100 indicates 100% grass silage). Numbers represent the mean concentrations for total volatile fatty acid (VFA), bacteria and archaea, while individual VFA products (acetate, propionate, butyrate and isovalerate) are shown as mean molar proportions. SEM refers to standard error of the mean. An ANOVA test was used to assess the effect of diet, time and their interaction, with a post-hoc Tukey-Kramer multiple comparison test performed (van Gastelen et al., 2015). P values lower than 0.05 are considered to indicate significance and those higher than 0.05 but lower than 0.1 are considered to indicate a tendency to be significant. Values with a different superscript (^{a,b}) indicate a significant difference in diet while superscripts x or y indicate a significant difference in time.

Index	Diet (D)				Time (T)		SEM	ANOVA (p-value)		
	GS100	GS67	GS33	GS0	10	17		D	T	D x T
Total VFA (mM)	105.4	97.3	99.1	103.5	96.8	105.9	6.88	0.571	0.076	0.877
VFA (% of total VFA)										
Acetate	64.9	65.1	65.2	62.9	64.6	64.5	1.20	0.320	0.859	0.939
Propionate	20.0	19.3	18.8	17.7	19.2	18.8	0.76	0.101	0.326	0.850
Butyrate	13.8	14.1	14.5	17.6	14.1	16.5	1.09	0.065	0.168	0.917
Isovalerate	1.3	1.5	1.4	1.8	1.7^{x}	1.3^{y}	0.22	0.414	0.002	0.384
Microbial concentrations (log10 (16S rRNA gene copies/ml RF))										
Bacteria	10.9^{a}	10.8^{a}	10.8^{a}	10.5^{b}	10.6^{x}	10.9^{y}	0.10	0.026	0.008	0.762
Archaea	8.2	8.2	8.2	8.0	8.1^{x}	8.2^{y}	0.20	0.174	0.022	0.492

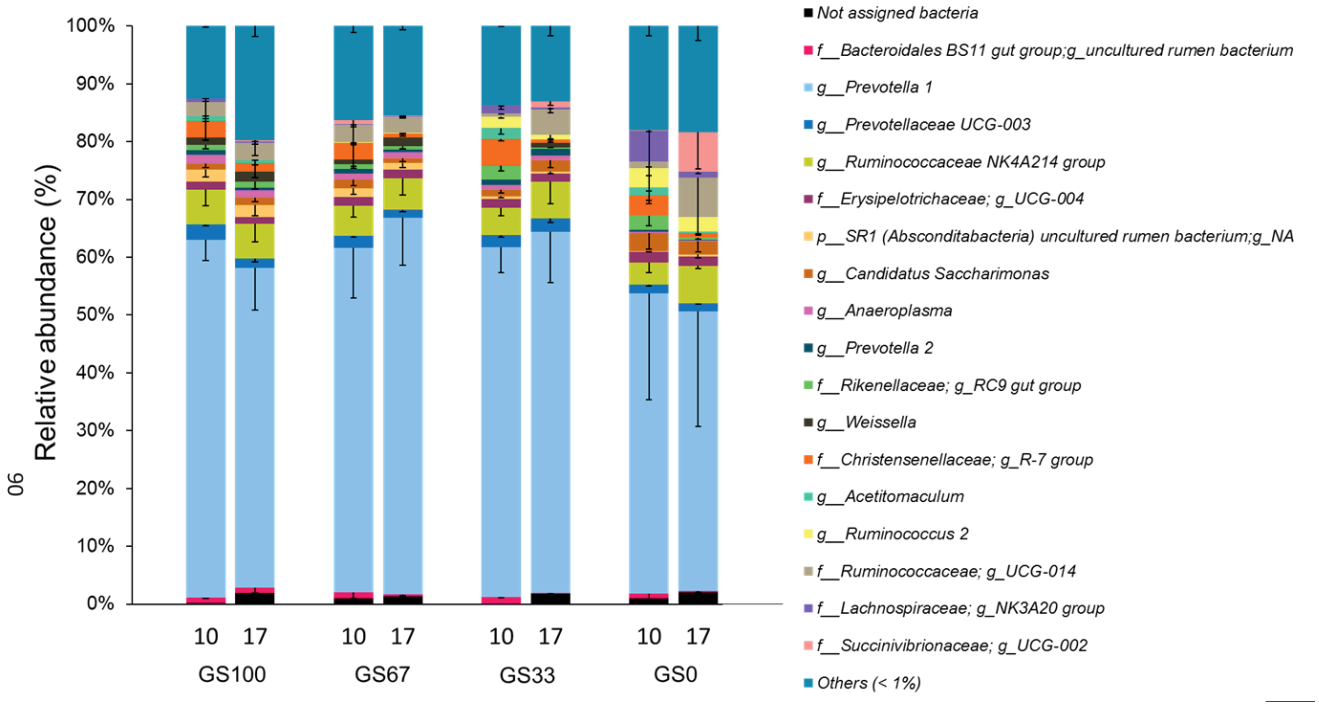

Figure 1. Relative abundance of major (>1 %) bacterial genus level groups in rumen fluid samples from dairy cows with genera <1% (in at least one sample) summed as 'Others'. Sample codes indicate the number of days that the diet had been fed (10 or 17) and the different grass and maize silage proportions in the diet, for example GS100 (100% grass silage). Bars represent means of n=3. Error bars represent the standard deviation. g_NA means that genus could not be annotated.

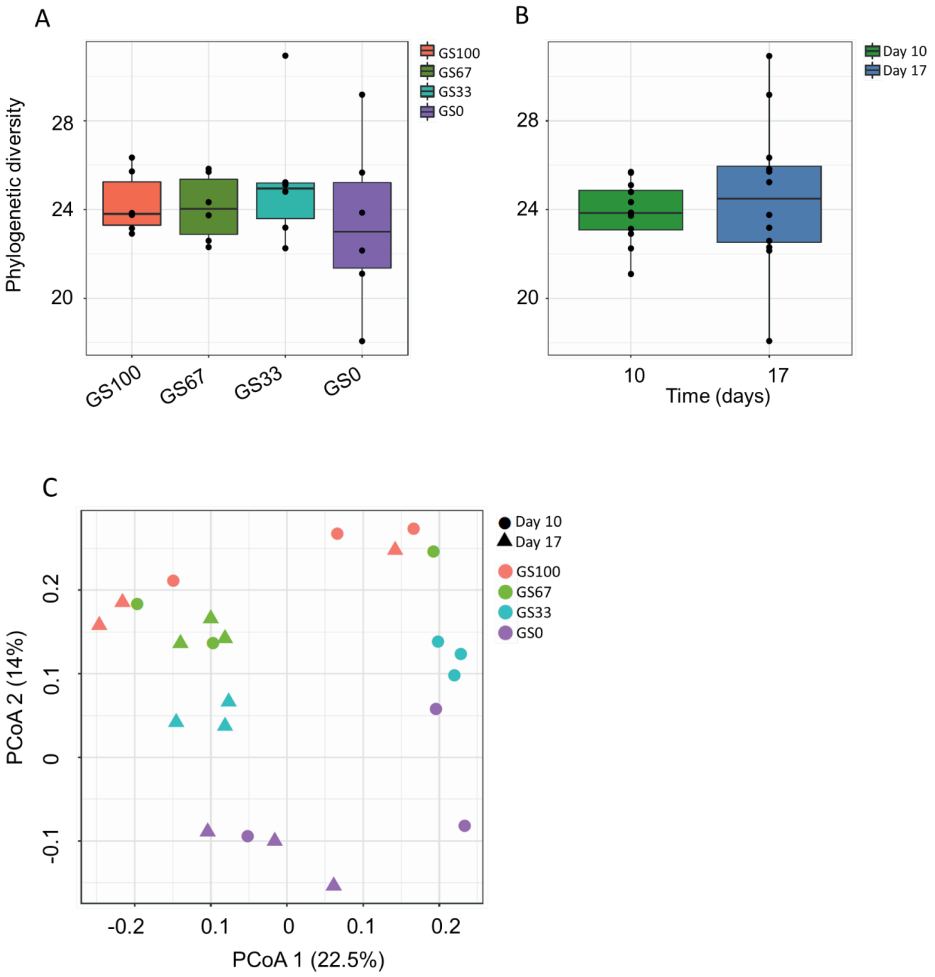

Figure 2. Phylogenetic diversity (PD) of bacterial communities associated to different ratios of grass and maize silage **(A)** and different time points **(B)**. Sample codes indicate the number of days that the diet had been fed (10 or 17) and the different grass and maize silage proportions in the diet, for example GS100 (100% grass silage). Principal co-ordinate analysis **(**PCoA) analysis of bacterial community composition from rumen fluid samples (n = 24) using the unweighted UniFrac distance metric **(C)**. The percentage of variation explained is indicated on the respective axes.

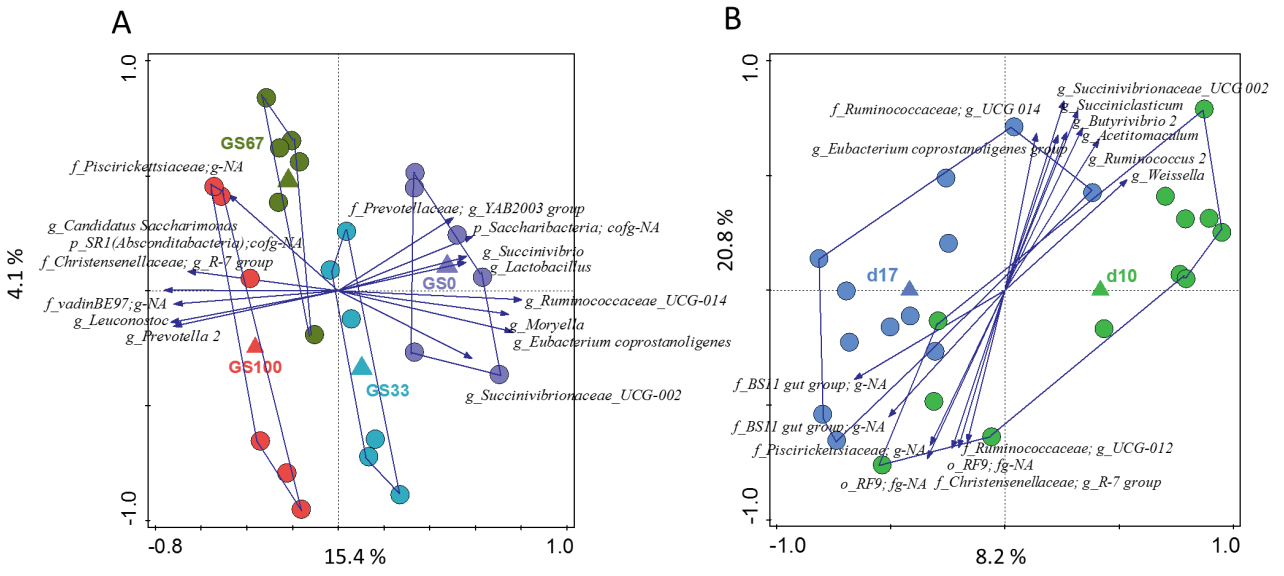

Figure 3 Redundancy analysis triplot of partial RDA showing the relationship between the top 15 genus level phylogenetic groupings of the OTU explaining the variance of diet with covariate time **(A)** and time with covariate diet **(B)**. The axes are labelled with the amount of variation they explain relative to the explanatory variable diet **(A)** or time **(B)**. Please note that for Figure 3B, only the first axis is constrained, and thus the explained variance is higher for the second (i.e. the first unconstrained) axis. Arrow labels indicate the taxonomic identification of genus-level phylogenetic groupings, with the level [i.e., phylum (p), class (c), order (o), family (f), or genus (g)). Abbreviation NA indicate the levels that could not be annotated. For example "f_BS11 gut group; g-NA" was reliably assigned to the family BS11 gut group but the genus could not be annotated. Sample codes for the means indicate either different grass and maize silage proportions in the diet (i.e. GS100 (100% grass silage)) **(A)** or the number days that the diet had been fed (i.e. d10 is 10 days of feeding) **(B)**.

Changes in rumen archaea community composition

Annotation of the 347 detected OTUs showed that *Methanobrevibacter* was the predominant archaeal genus followed by *Methanosphaera* (Fig. 4). Archaeal PD values were confirmed to have a normal distribution (P = 0.189). There was no effect of diet (P = 0.300), time (P = 0.525) or diet × time (P = 0.272) on the archaeal PD (Fig. 5A and 5B). With unweighted UniFrac PCoA of the archaeal community, a tendency for an effect of diet was noted (P = 0.075), whereas no effect of time (P = 0.505) or diet × time (P = 0.582) was observed (Fig. 5C). In the weighted UniFrac analysis there was no effect of diet (P = 0.862), time (P = 0.430) or diet × time (P = 0.534) (Fig. S3).

The contribution of diet, time and diet × time interaction to variation in the archaeal community composition at the genus level was assessed using partial RDA analyses. The effect of diet was significant (P = 0.032), and explanatory variables accounted for 28.34% of the residual variation in the dataset when time was used as covariate (Fig. 6). Almost all of this variance was represented by the first canonical axis, where both GS100 and GS67 separated from GS33 and GS0 (which did not differ from each other). *Methanobrevibacter* showed a positive association with the GS0 and GS33 diets. The GS100 diet appeared to be most positively associated with *Methanosphaera*. The GS67 diet was most positively associated with a non-annotated genus level group that belonged to the family Methanobacteriaceae. RDA analysis further showed that time did not affect the archaeal community composition (P = 0.284), and only accounted for 1.24 % of the residual variation with diet being used as covariate (Fig. 6B). No diet × time interaction was observed (P = 0.312) (data not shown).

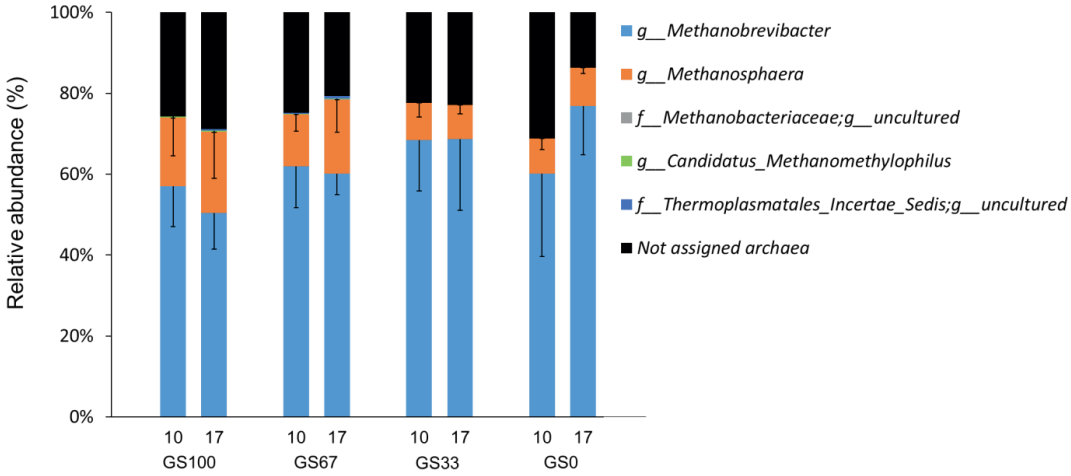

Figure 4. Relative abundance of all archaeal taxa within rumen fluid samples from dairy cows fed different proportions of grass silage (GS) to maize silage (MS). Sample codes indicate different grass and maize rations in the diet, for example GS100 (100% grass silage) and the number of days that the diet was fed (i.e. 10 or 17). Bars represent sample means from cows fed the same diet (n=3), and error bars represent their standard deviation.

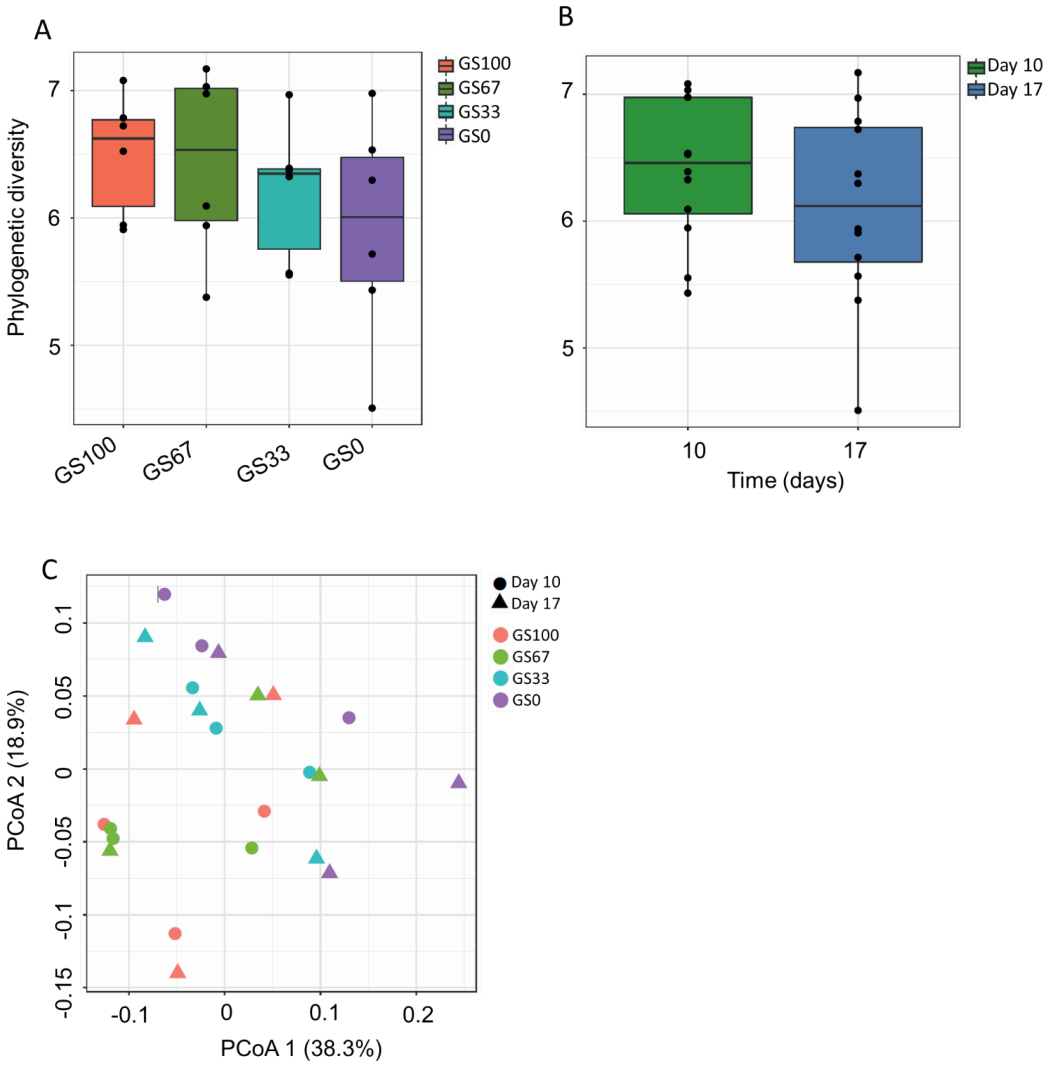

Figure 5. Phylogenetic diversity (PD) comparisons of archaeal communities associated to different ratios of grass and maize silage **(A)** and those associated to the different number of days the diet had been fed **(B)**. Diet sample codes indicate different grass and maize silage proportions in the diet, for example GS100 (100% grass silage). Principal co-ordinate analysis **(**PCoA) analysis of archaeal community composition from rumen fluid samples (n = 24) using the unweighted UniFrac distance metric **(C)**. The percentage of variation explained is indicated on the respective axes.

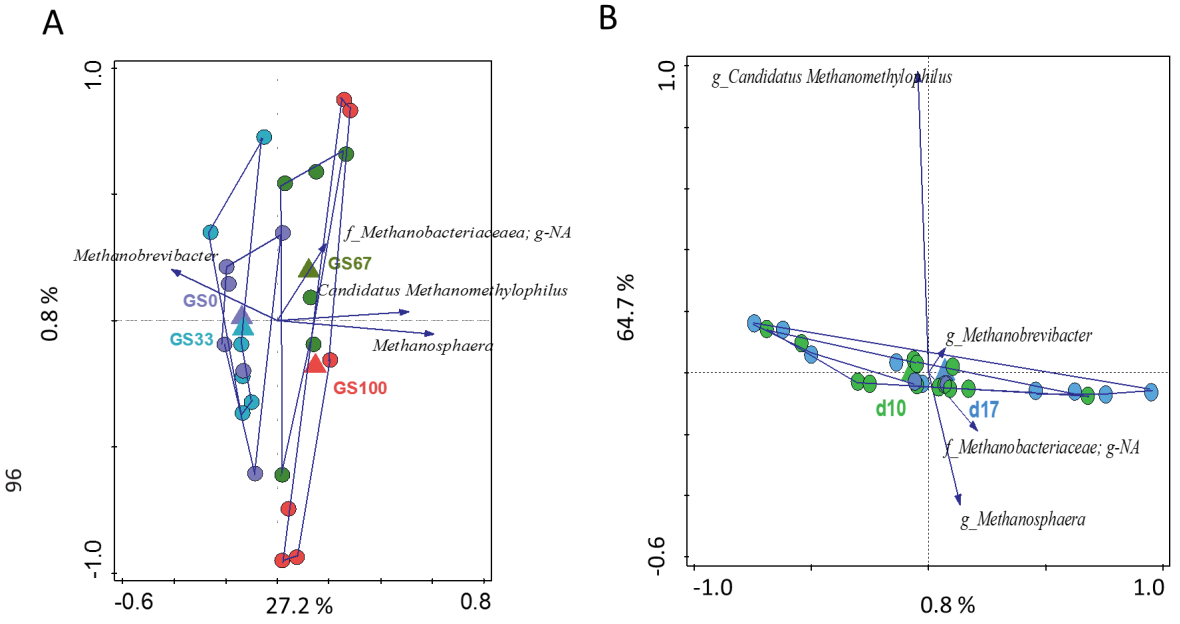

Figure 6. Redundancy triplot of partial RDA showing the relationship between the genus level phylogenetic groupings of the archaeal OTUs explaining the variance of diet with covariate time **(A)** and time with covariate diet **(B)**. The explanation of the plots and the labels for the taxa and samples are as previously described in Figure 3.

DISCUSSION

The potential of reducing CH_4 emissions from lactating dairy cows has been shown in a previous study (van Gastelen et al., 2015) where, in comparison with the GS100 diet, CH_4 yield (g/kg DMI) was 11% reduced for the GS0 diet. The objective of the current study was to explore whether the rumen bacteria and archaea were also affected by the different ratios of grass and maize silage used, as well as by the number of days the diet had been fed. These aforementioned analyses will aid in understanding how observed changes in VFA and CH_4 relate to underlying changes in the rumen microbiome.

Effect of diet and time on VFA production

The results of the rumen fermentation products (i.e., total VFA and molar proportions of individual VFA) in this study are largely in agreement with the results reported previously by van Gastelen et al. (2015). Both the present study and van Gastelen et al. (2015) report no effect of diet on total VFA and the molar proportions of acetate and propionate. In the present study, however, the molar proportions of butyrate tended to be affected by diet, whereas van Gastelen et al. (2015) reported a linear increase in molar proportions of butyrate upon increasing the level of MS at the expense of GS. Additionally, van Gastelen et al. (2015) reported that isovalerate tended to be affected by diet, whereas in the current study no diet effect on isovalerate was found. These differences between the two studies are likely to be associated with the differences in frequency and timing of sampling.

In the present study, the RF samples were collected on day 10 (before the climate respiration chamber phase) and day 17 (after the climate respiration chamber phase), but only 4 h after morning feeding. In the study of van Gastelen et al. (2015) the RF samples were collected 1 h before and 1, 2, 4, 6 and 8 h after morning feeding on days 10 and 11 (i.e. before the climate respiration chamber phase). It is well documented that there are significant temporal variations in the molar proportions of individual VFA after feeding (van Lingen et al., 2017b). Furthermore, differences in VFA sample preparation and analysis between the two studies may have affected the results. Hence, direct comparison between the VFA results of the present study with those reported by van Gastelen et al. (2015) is not feasible.

Isovalerate molar proportions were affected by time in the present study, with a decrease of isovalerate from day 10 to day 17. Usually, isovalerate is associated with microbial protein synthesis and fermentation of plant cell walls (Fenner et al., 1970). The decrease in isovalerate

molar proportions might suggest a reduction of the cellulolytic bacterial populations for which isovalerate is an essential growth factor, as has been previously demonstrated in pigs (Liu et al., 2016). However, this seems unlikely, as no bacterial genera could be clearly associated with the effect of time on the bacterial community composition (Fig. 3B).

Effect of diet and time on rumen microbial concentrations

Diet affected bacterial concentrations, which were lower for the GS0 diet compared with the other three diets. This is despite the expectation of higher bacterial concentrations in the rumen liquid of animals fed the GS0 diet that contains starch, as starch is a more easily fermented substrate for bacteria than the fibre in the GS100 diet (Deckardt et al., 2013). This decrease in bacterial concentrations in the absence of grass silage in the diet is interesting. This finding suggests that the bacterial concentrations might not be directly MS related, as we did not observe a linear decrease in bacterial concentration upon increasing levels of MS. our findings are in contrast to the study of Lettat et al. (2013), who used a 3 × 3 latin square design where all nine dairy cows received all dietary treatments. Lettat et al. (2013) reported increased bacterial concentrations upon replacing fibre-rich alfalfa completely with starch-rich maize silage, and in this study samples were collected 4 hours after morning feeding. Starch in maize silages is generally readily available for the ruminant due to the moisture and softness of the kernel (Indugu et al., 2017). However, this availability of starch also depends upon maturity and processing of the maize kernel at harvest (Indugu et al., 2017). This might contribute to the observed decrease of bacterial concentrations with increasing amounts of maize silage in the present study, although we are not aware of any peculiarities with respect to harvest of the maize used in this study. There was no diet effect on archaeal concentrations which is in line with the study of Lettat et al. (2013), who also did not observe any effect of diet on the methanogenic archaea.

The observed increase in bacterial concentrations from day 10 to day 17 is suggestive of an increase in fermentation. However, total VFA only tended to be affected by time indicating the increase in bacterial concentration was not biologically significant. Also archaeal concentrations were found to increase from day 10 to day 17. The reason for this increase is presumably linked to the similar change in the bacterial concentrations, as no significant change in archaeal community composition occurred with time (Fig. 6B). The biological significance of this change in archaeal concentrations is not known, as CH_4 emissions were only measured from days 12 to 17 in the study of van Gastelen et al. (2015).

Effect of diet and time on rumen bacterial composition

Bacterial alpha diversity was not affected by diet but bacterial community composition was (Fig. 2). This effect was observed in the unweighted UniFrac PCoA analysis (Fig. 2C) but not the corresponding weighted analysis (Fig. S1). This suggests that the diet effect was mainly due to differences in the presence or absence of low abundant bacterial taxa, rather than differences in the relative abundance of predominant bacterial taxa. In both the unweighted PCoA and the RDA, the bacterial community composition showed a transition that was consistent with the decreasing amount of GS. The RDA analysis showed that several genus-level groupings were positively associated with the GS0 diet: the UCG-002 group belonging to the Succinivibrionaceae, a genus represented by *Eubacterium coprostanoligenes, Moryella*, the UCG-014 group belonging to the Ruminococcaceae*, Lactobacillus, Succinivibrio*, an unidentified genus (cofg-NA) within the phylum Saccharibacteria, and the YAB-2003 group belonging to the Prevotellaceae.

The higher amount of starch present in MS has been associated with increased relative abundances of members of the families Ruminococcaceae and Succinivibrionaceae (Kozakai et al., 2007; Deusch et al., 2017), which is consistent with the positive association of UCG-002, *Succinivibrio* and UCG-014 with the GS0 fed cows in the present study. Low CH_4 producing cows have been positively associated with lactate and succinate producing bacteria (Danielsson et al., 2017). This is also consistent with the positive association of *Lactobacillus* (a lactate producer) and *Succinivibrio* (a succinate producer) in the GS0 diet, as this diet was also associated with the lowest CH_4 emission by van Gastelen et al. (2015). A metagenomics study has linked increased abundance of Succinivibrionaceae with reduced CH_4 emission in dairy cows (Wallace et al., 2015). This can be explained by the fact that Succinivibrionaceae compete with hydrogenotropic methanogenic archaea for H_2 and produce succinate, which is then converted to propionate (McCabe et al., 2015). *Moryella*, which was positively associated with the GS0 diet in our study, has also been reported as one of the dominant groups in a maize starch fed diet (Li et al., 2012).

The relevance of the positive association of a genus-level group that includes *Eubacterium coprostanoligenes* with the GS0 diet in the present study is not clear, as the characterized species *E. coprostanoligenes* does not hydrolyse starch (Freier et al., 1994). Also, only a few other *Eubacterium* spp from the rumen (i.e. *Eubacterium uniforme* and *Eubacterium xylanophilus*) have been previously associated with maize silage derived feeds (Deusch et al., 2017). However, *E. coprostanoligenes* is interesting as until now it has been associated with hydrolysis of cholesterol

esters to produce coprostanol (Madden et al., 1999). Species in the *Eubacterium* genus are saccharolytic and ammonia producing, playing a role in amino acid fermentation. As ammonia production in the rumen consumes H_2 gas, *Eubacterium* spp. might play a role in decreasing CH_4 emissions by depriving methanogens of H_2 (van Zijderveld et al., 2010; Cunha et al., 2018). The relation between the unclassified group belonging to the Saccharibacteria and the YAB-2003 group belonging to the Prevotellaceae and the decreasing amount of GS is also unclear due to the limited knowledge with respect to the physiology of these organisms.

Several genera were positively associated with the GS100 diet. The positive association of *Prevotella* 2 with the GS100 diet (Fig. 3A; which also suggests a negative association with GS0) suggests that *Prevotella* 2 plays a role in fibre degradation. The majority of the characterized species within this genus have been isolated from the human oral cavity (for example *Prevotella marshii, P. shahii)* (Sakamoto et al., 2004; Downes et al., 2005) while others have been isolated from human faeces (for example *P. stercorea*) (Hayashi et al., 2007). Among these species, *P. shahii* and *P.stercorea* were abundant in deers fed oak leaves in the study of Li et al. (2013). The positive association of *Leuconostoc* to GS100 observed in this study is harder to explain in the context of fermentation. *Leuconostoc* spp. are lactic acid bacteria that are typical inhabitants of silage, and fermentation of maize silage is generally faster than that of grass silage. Members of Christensenellaceae have been associated with degradation of fibre (Mao et al., 2015), in line with the positive association of this group with the GS100 diet.

The alpha diversity of the bacterial communities was affected by time, suggesting that the community was not completely stable following 10 days of diet adaptation (Fig. 2B). Indeed, this is also reflected in the finding that bacterial concentrations were found to be higher on day 17 compared to day 10. Whilst separation of day 10 and day 17 samples occurred in the RDA, no taxa were strongly associated with either of the days (Fig. 3). Although all animals were on the same dietary treatment on day 10 and day 17, these treatments were restricted to 95% from day 8 as reported in van Gastelen et al. (2015) which might at least contribute to the observed shift from day 10 to day 17.

Effect of diet and time on archaea

Our analysis did not show any significant differences in the archaeal alpha diversity in response to diet, time or diet × time (Fig. 5A and 5B). Changes in feed fermentation products can induce changes in the methanogenic community structure, however, as only limited changes occurred in

this study it is perhaps not surprising that the weighted and unweighted PCoA showed no significant diet effect in archaeal beta diversity (Fig. 5). However, significant diet effects were observed in the RDA analysis, which was based on relative abundance distributions, whereas PCoA analyses were based on the phylogenetically weighted Unifrac distances. The relative abundance of *Methanobrevibacter* was positively associated with the GS0 diet, whereas *Methanosphaera* and *Candidatus* Methanomethylophilus were negatively associated with the GS0 diet. *Methanobrevibacter* and *Methanosphaera* are two pre-dominant archaea found in all dietary treatments. *Methanobrevibacter* spp. are usually formate, H_2 and CO_2 dependent hydrogenotrophs while *Methanosphaera* spp. are H_2 dependent methylotrophs (Carberry et al., 2014a). The weak associations with diet may indicate competition for H_2 between *Methanobrevibacter* and *Methanosphaera* within the rumen (Carberry et al., 2014a), suggesting that the concentrations and partial pressures of H_2 in the rumen are key factors affecting methanogenesis (van Lingen et al., 2016).

CONCLUSIONS

In this study, we assessed changes in the rumen microbiota in response to dietary treatments differing in the roughage composition after 10 and 17 days of feeding. These changes were used to help understand corresponding changes in ruminal VFA and previously observed CH_4 measurements. In conclusion, diet had a significant effect in shaping the bacterial and archaeal communities in the rumen of dairy cows. The bacterial families Succinivibrionaceae and Ruminococcaceae were among the bacterial taxa were associated with the maize silage diets, indicating their role in the low methane observed from the previous study. *Methanobrevibacter* was positively associated with maize silage diet. Furthermore, time had a significant effect on both bacterial and archaeal concentrations, but only on bacterial community composition. These findings indicate that the rumen microbiome had not stabilized after for 10 days of feeding the experimental diets, which might at least in part be due to the fact that feeding was restricted to 95% of the DMI of the cow eating the least within each group from day 8.

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SUPPLEMENTARY MATERIAL

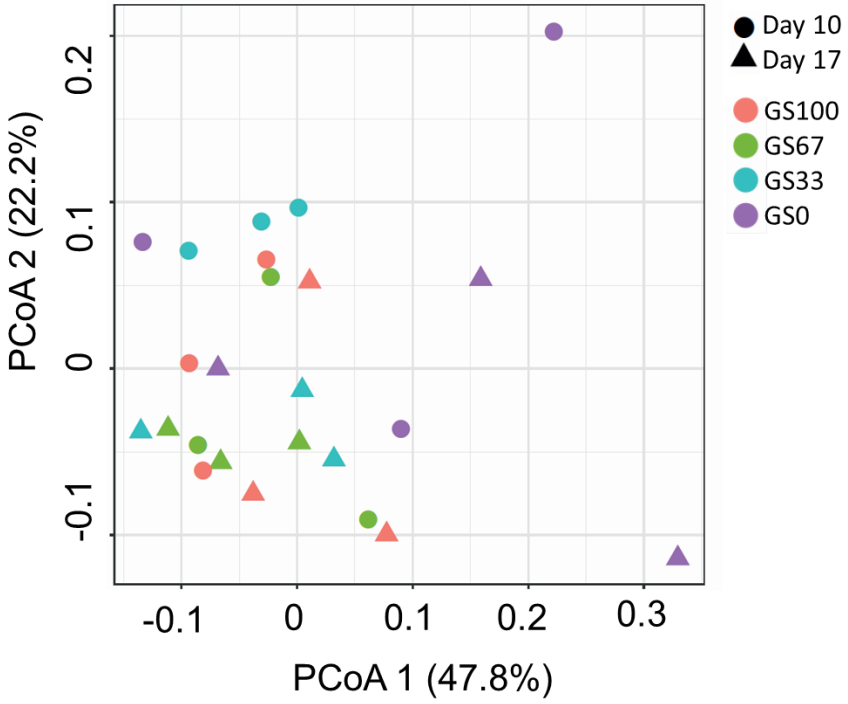
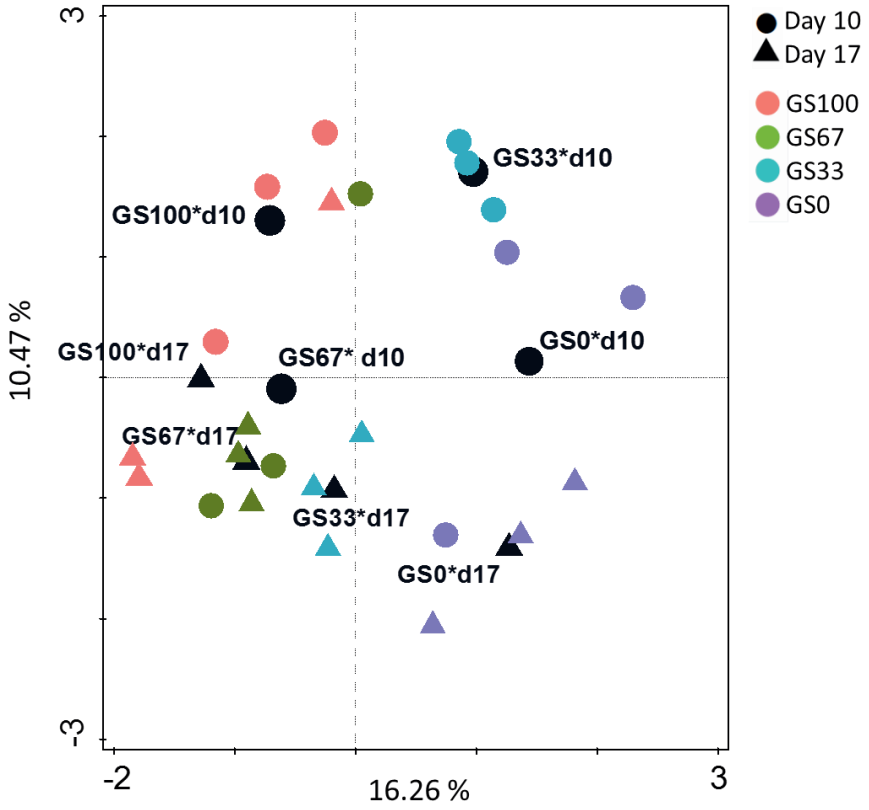


Figure S1. Principal co-ordinate analysis (PCoA) analysis of rumen fluid bacterial community composition (n=24) using weighted UniFrac distance metrics. Colours indicate the different grass and maize silage proportions in the diet (i.e. GS67 (67% grass silage and 33% maize silage)), and the symbol shapes indicate the number of days that the diet had been fed (10 or 17).The percentage of variation explained is indicated on the respective axes.



Supplementary Figure S2. Redundancy analysis triplot explaining the variance associated with the diet × time. The canonical axis are labelled with the amount of variation they explain relative to the explanatory variable diet × time. Sample codes for the means indicate different grass and maize silage proportions in the diet (i.e. GS67 (67% grass silage and 33% maize silage)) and the number of days that the diet had been fed (d10 or d17).

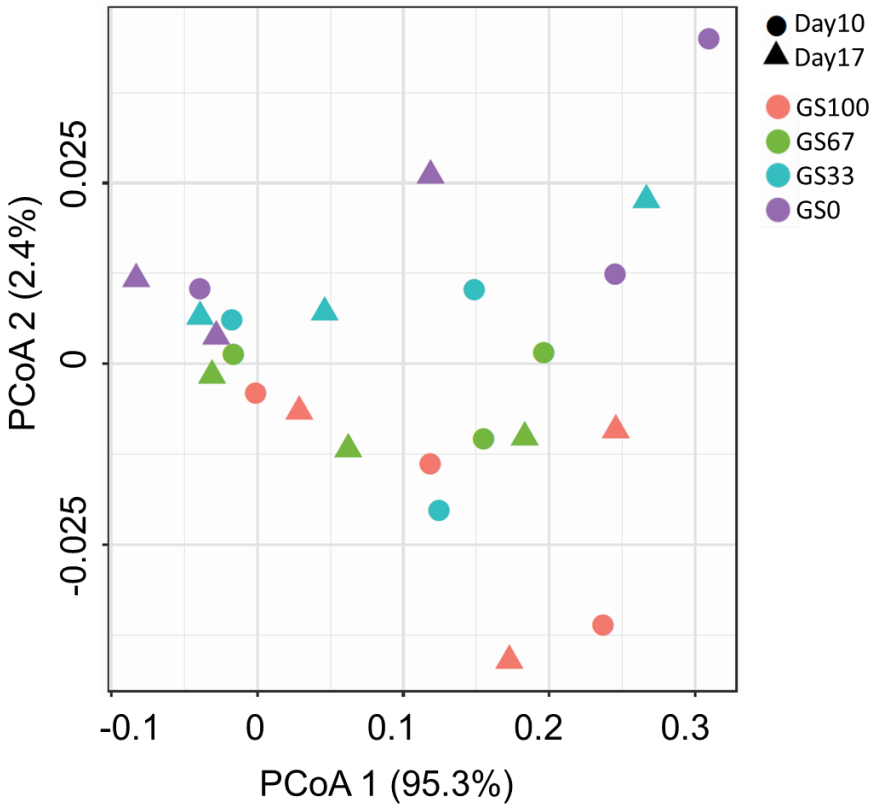


Figure S3. Principal co-ordinate analysis (PCoA) analysis of archaeal community composition from rumen fluid samples (n=24) using the weighted UniFrac distance metric. Colours indicate the different grass and maize silage proportions in the diet (i.e. GS67 (67% grass silage and 33% maize silage)), and the symbol shapes indicate the number of days that the diet had been fed (10 or 17). The percentage of variation explained is indicated on the respective axes.

Chapter 4

Enrichment of rumen fibre associated bacteria derived from ruminal fibrous content in dairy cows fed grass or maize silage

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ABSTRACT

Ruminant animals gain most of their energy from plant fibres, which are degraded by complex microbial communities in the rumen. In the past, rumen microbiologists focused on elucidating the attachment of bacteria to feed particles and their role in initial fibre degradation. Nonetheless, many fibre associated microbes remain uncultured and unidentified. Therefore, in this study rumen fibre associated microbes were enriched from grass silage (GS) or maize silage (MS) fibres recovered from the bovine rumen. Enrichments used pre-autoclaved ruminal silage fibres (GS or MS, respectively, for each isolation source) as an attachment matrix in bottles containing different fibre related components (cellulose, amylopectin or xylan) in duplicate. Furthermore, bottles without any additional C source other than the pre-autoclaved silage fibres were included as well. All these bottles, termed the primary enrichments (PE), were incubated at 39°C for 14 days during which samples were obtained (day 0, 3, 5, 7, and 14) for analysis of metabolite production and substrate degradation. The fibres from the PE bottles were transferred as the inoculum for the sub-enrichment 1 (SE1) bottles, which were incubated again for 14 days under the corresponding conditions with intermittent cold storage between transfers. A similar approach was taken to further enrich four more times (SE2-SE5). Genus *Ruminofilibacter* dominated all the MS SE5 bottles, whereas all GS SE5 bottles contained a more diverse community (*Prevotella 1* and *Bacteroides*). The in vitro experimental approach of serial two week incubation, seems likely to be important to enrich for fibre associated bacteria, i.e. *Ruminofilibacter* that are not favoured by shorter incubation times.

KEYWORDS

Rumen fibre, enrichments, grass silage, maize silage, fermentation

INTRODUCTION

Rumen microbial fermentation of feed

The cow rumen microbiota is a complex community of bacteria, methanogenic archaea, protozoa, anaerobic fungi and viruses. Of these, the bacteria are the most diverse. The ruminant is dependent on rumen microbes for digestion of complex plant lignocellulosic compounds provided as feed. The conversion of complex plant polysaccharides to simple compounds initially involves microbial attachment to the plant fibres, which is a key limiting step for ruminal feed degradation (McAllister et al., 1994). The microbial attachment is then followed by fibre degradation which involves hydrolysis and fermentation of the degradation products into volatile fatty acids (VFAs) namely acetate, propionate and butyrate, and some minor branched-chain VFAs (iso-butyrate, iso-valerate and valerate) in addition to products like hydrogen (H_2), carbon dioxide (CO_2), formate and methyl-containing compounds. Acetate, formate (formed in the production of acetate), H_2, CO_2, and methyl-containing compounds then subsequently serve as substrates for methanogenesis. VFA's are not commonly used as substrates in the rumen since these are absorbed by the animal as energy substrates (Hook et al., 2010). Fibrous feed like grass silage fermentation is associated with higher methane formation as compared to corn or maize silage based diets in dairy cows (van Gastelen et al., 2015). Investigation of fibre associated rumen microbes that are involved in the breakdown of the fibrous components of feed can contribute to improved understanding of the link between fibre attachment, degradation and methane formation.

Plant material contains fibres that have two main constituents: cellulose and hemicellulose. Cellulose is composed of β-1, 4-linked glucose residues, whereas hemicellulose mainly consists of xylan assembled from β-1, 4-linked xylose residues and is substituted with acetyl, arabinosyl and glucuronyl residues. Rumen microbes require hydrolytic enzymes to utilize these fibre components. Bacteria and anaerobic fungi produce a wide range of highly active plant cell wall degrading enzymes, contributing to 80% of the total fibre degrading activity in rumen (Dijkstra, 1995). Due to the slower generation times of anaerobic fungi (6-9 hrs) in comparison to bacteria (0.5-3.5 hrs) (Varga and Kolver, 1997) bacteria are considered to be the pre-dominant players in the degradation of dietary fibre (Petri et al., 2012).

The variation in rumen microbiota composition depends on the diet of the host, with diet causing the most profound changes in bacterial communities compared to the archaea (Pitta et al., 2010; De Menezes et al., 2011b). Bacteria inhabiting the rumen are classified into 1) free-living

bacteria associated with the rumen liquid 2) bacteria loosely bound to the fibrous content 3) bacteria tightly attached to the fibrous content (FC), and 4) bacteria associated/attached to the epithelial wall of the rumen (Cheng and Costerton, 1980; McAllister et al., 1994). Bacteria loosely bound and/or attached (i.e. associated) to the fibrous content represent 70-80 % of the total bacterial microbiota (Craig et al., 1987) whereas the free-floating bacteria represent ~30 % (Petri et al., 2012). Bacteria associated to epithelial cells constitute a very small fraction (~1 %) of the total ruminal bacterial microbiota and have no significant contribution to ruminal digestion (Petri et al., 2012). Bacteria associated with feed particles are responsible for 88% to 91% of ruminal endoglucanase and xylanase activity, 70% of the amylase activity, and 75% of the protease activity in the rumen (Cheng and Costerton, 1980; Miron et al., 2001). This information indicates that fibre-associated bacteria are pivotal for ruminal fibre degradation.

The first characterised rumen cellulolytic micro-organism was *Fibrobacter succinogenes* isolated by Robert Hungate (Hungate, 1950). *F. succinogenes* was identified as an anaerobic, Gram negative micro-organism, which produced succinic acid and acetic acid as major fermentation products from carbohydrates. Later, *Ruminococcus albus* and *Ruminococcus flaveflaciens* were also identified as cellulose degraders (Hungate, 1957). Since then, these three bacteria have been considered the key-stone species associated with ruminal fibre degradation. Moreover, these three species have been tested *in vivo* previously for their attachment and degradation capability, confirming their role in ruminal fibre utilization (Miron et al., 2001).

Degradation of complex polymers requires hydrolytic enzymes. In 2009, the genes encoding hydrolytic enzymes were identified in *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Koike and Kobayashi, 2009; Morrison et al., 2009). Additionally, genome sequencing also revealed genes encoding multiple glycosyl hydrolases in all three species (Krause et al., 2003). In both *R. flavefaciens* and *R. albus,* a cellulosome structure has been identified where the cellulosomal protein containing is involved in protein anchoring to the Gram-positive bacterial cell envelope (Rincon et al., 2005; Jindou et al., 2008).

Non-fibrolytic bacteria, that are able to degrade one or more plant components, can stimulate the activity of fibrolytic bacteria through interactions, particularly cross-feeding. For example, cellulolytic species *(F. succinogenes, R.albus or R.flavefaciens)*, when co-cultured with *Treponema bryantii, Prevotella ruminicola* and *Selenomonas ruminantium,* show improved fibre digestion compared to the corresponding monocultures (Wolin et al., 1997). The non-fibrolytic species are

motile and move inside the plant cells facilitating the entry of non-motile fibrolytic species (Sawanon et al., 2011). These findings indicate that there is a close association between specific bacteria when degrading ruminal plant fibrous content. Other known non-cellulolytic rumen bacterial species involved in fibre degradation include *Clostridium longisporum, Clostridium lochheadii, Butyrivibrio fibrosolvens, Prevotella ruminicola, Eubacterium ruminantium* and *Eubacterium cellulosolvens* (Stewart et al., 1997).

Evaluation of ruminal microbial communities through next generation sequencing techniques using 16S ribosomal RNA (rRNA) gene based PCR approaches has expanded our knowledge of the composition of rumen microbial communities associated with rumen fibrous content and liquid fraction across different diets, ruminant species and geographical locations (De Menezes et al., 2011b; Fouts et al., 2012; Pitta et al., 2014; Henderson et al., 2015b; Noel et al., 2017). The microbial communities adhering to the fibrous content are distinct from those in the liquid fraction (Fouts et al., 2012). The liquid fraction of the rumen is characterized by high relative abundances of bacteria belonging to the genera *Zhangella*, *Tannerella* (both members of the order Bacteroidales) and *Prevotella*, whereas the genera *Blautia* and *Butyrivibrio* (both members of the order Clostridiales) are generally more abundant in the fibrous content (Fouts et al., 2012).

Among the known members of fibre associated bacterial communities, only 2-31% have more than 97% similarity with cultivated species (Koike et al., 2003a; Kobayashi, 2006). This highlights the need to perform enrichment, isolation and characterization of bacteria that are associated with the fibrous content in order to better understand the metabolism and role of microorganisms involved in fibre-attachment and degradation. Therefore, the objective of this study was to enrich fibre associated bacteria using a range of different fibre based substrates from both ruminal maize silage and grass silage fibres and characterise their metabolism and bacterial community composition.

MATERIALS AND METHODS

Ethics statement

The ruminal fibrous content (FC) samples used in this study represented a subset of those collected as part of a larger study, the details of which have been published elsewhere (van Gastelen et al., 2015). The current study was conducted in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research.

Fibres for inoculum & cultivation medium composition

The ruminal FC was sampled from two Holstein Friesian dairy cows fed different diets. Both diets had a roughage-to-concentrate ratio of 80:20 based on dry matter, and for one cow the roughage consisted of 100% grass silage (GS) and for the other cow 100% maize silage (MS). The solid (fibrous) fraction was collected via the rumen cannula after 4 hours of feeding, and then firmly squeezed by hand. All samples were collected within a time span of 30 min, after which they were transported to the laboratory. The ruminal GS and MS fibres were immediately stored in anoxic 25% glycerol - phosphate buffered saline (PBS) stock at - 80ºC until use as an inoculum for the primary enrichments (PE).

In addition, approximately 7.5 g wet weight (WW) of GS and MS fibres were collected and placed in a dry ice box before being transferred to the laboratory where the fibres were transferred into falcon tubes and stored at -80 ºC. These fibres were used in all the enrichment bottles as a matrix for attachment and substrate, with the type of fibre used (i.e. GS or MS) matched to the type of inoculum fibre.

Experimental design

An overview of the study design is provided in Fig 1. For both the MS and GS fibre enrichments, serum bottles were prepared in duplicate containing an anaerobic, bicarbonate buffered mineral salt medium (BM, see next section for details) supplemented with the following: one representative plant substrate ((i.e. 1g/L of either cellulose (Avicel), xylan from birchwood or amylopectin (Sigma-Aldrich, Steinheim, Germany)), 0.1 g/L yeast extract and the corresponding type of fibre (0.1 g/bottle) (as an attachment matrix/substrate). Additionally, there were duplicate bottles supplemented without any representative plant substrate and containing only the respective type of fibre (0.1 g) (as an attachment matrix/substrate). The inoculated serum bottles for the primary enrichment (PE) were incubated for 14 days at 39ºC and then stored at 4^0C for ~20 days. To further enrich the bacteria attached to the fibrous matrix, sub-enrichments (SE) were made by transferring the fibres (GS or MS) from the PE serum bottles to the sub-enrichment 1 (SE1) serum bottles (Fig. 1). These bottles were prepared in the same manner as for the PE, and were incubated for 14 days at 39 ºC followed by transferring the fibres from SE1 to SE2 (14 days enrichment period at 39ºC) and similarly until SE5 (Fig. 1). There was a cold storage of three to four weeks in between each transfer of fibres from primary enrichment to sub-enrichment and further. All inoculations and transfers were performed aseptically and in an anaerobic glove box.

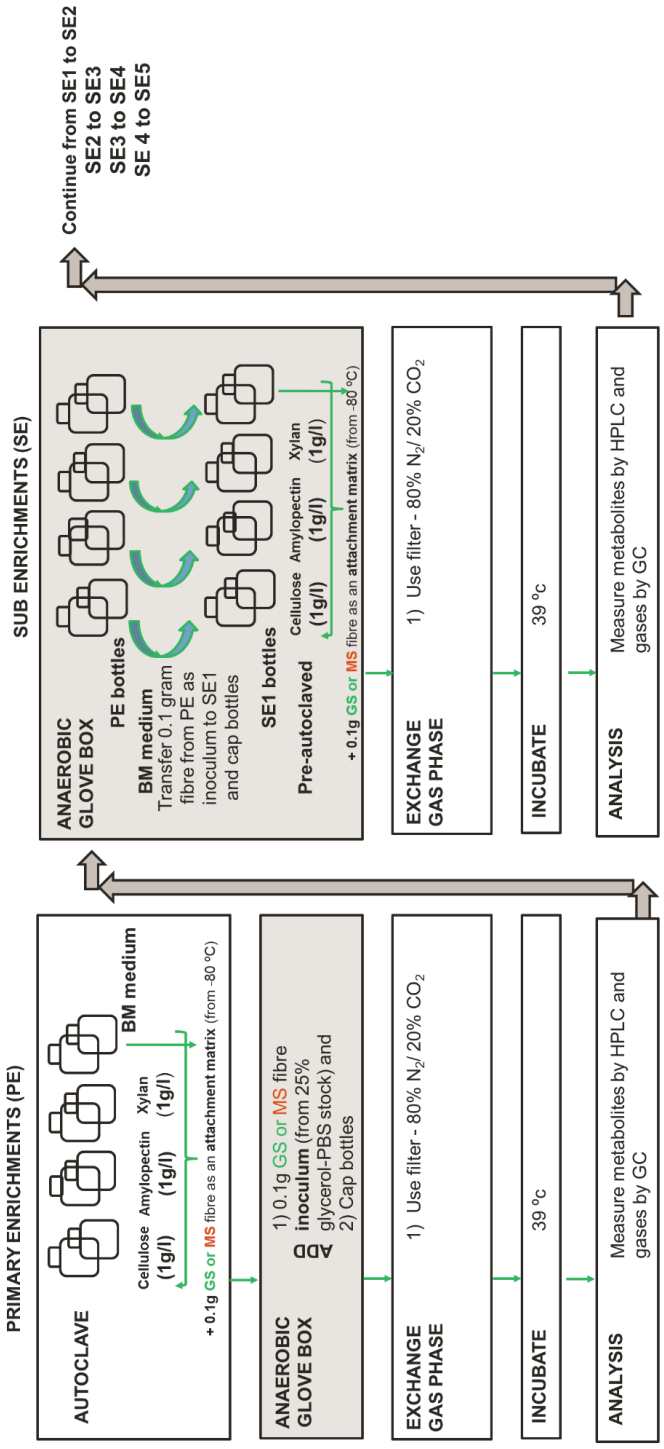


Figure 1. Schematic representation of the experimental design.

Media composition & preparation

The BM was used as previously described (Plugge, 2005). Prior to adding 48 ml of BM to the 117 ml serum bottles, all serum bottles were supplemented with yeast extract (0.1g/L final concentration) in addition to either ruminal grass (GS) or maize (MS) fibres (0.1g WW per bottle) which would serve as an attachment matrix. Either cellulose, xylan or amylopectin was also added before dispensing the BM medium. These representative plant components were weighed as a powder (1 g/ L final concentration). All serum bottles were sealed with rubber septa and aluminium crimp caps after addition of BM, the headspace was flushed with a mixture of N_2-CO_2 (80:20, v/v; 1.7 atm) and autoclaved at 121°C for 20 min. After sterilization, the medium was supplemented with an anoxic filter-sterilized calcium/vitamin stock solution (i.e. 10 ml $CaCl_2$ (11 g/L) + 1 ml vitamin solution per L of medium) (Stams et al., 1993) and reduced with filter-sterilized a Cysteine-HCl-Na_2S-$NaHCO_3$ solution (i.e. 50 ml $NaHCO_3$ solution (80 g/L) + 1 ml $Na_2S.9\ H_2O$ solution (240 g/L) + 0.5 g cysteine·HCl per L of medium (Stams et al., 1993). Furthermore, final concentrations of hemin (5 mg/L) and vitamin K1 (50 mg/L) were filter-sterilized and added after the autoclaved the medium was reduced. The final pH of the medium was 7.0.

Inoculating the primary enrichments from glycerol stock fibres

GS and MS fibres from the 25% glycerol stock (stored at -80ºC) (inoculum) were washed with anoxic sterile phosphate buffered saline (PBS) twice to remove excess glycerol attached to the fibres, as well as any loosely associated microbes. The washed fibres were then inoculated (0.4% w/v) into the serum bottles as outlined above (see experimental design section). All manipulations were done in an anaerobic glove box (tent). The serum bottles were then tightly capped with the rubber stopper and aluminium cap inside the glove box, brought outside the glove box, and the headspace of each bottle was flushed with a mixture of N_2-CO_2 (80:20, v/v; 1.7 atm) through a sterile 0.2 µm filter. All bottles were incubated at 39^0C, in the dark.

Sampling for metabolite and microbial analyses

From each serum bottle liquid samples (1 ml) were collected aseptically into 1.5 ml microcentrifuge tubes using 1 ml syringes (BD Plastipak™, Spain) and 0.5 mm x 0.16 mm needles (BD Microlance™ 3, Ireland) at the following times after inoculation: 0, 3, 5, 7, 14

days. These liquid samples were centrifuged at 14,000 g for 5 min, and the supernatant then used for VFA analysis using HPLC (as described below). Headspace gas samples (0.8 mL) were also collected at the same time points as the liquid phase samples using a 1 ml gas-tight syringe connected to a needle. Gas samples were directly injected to the GC for analysis (as described below).

Fibres from the original inoculum (OI) along with fibres from sub enrichment 5 (SE5) were collected after 14 days of incubation for microbial composition analysis with bacterial 16S rRNA gene amplicon barcoded sequencing. Fibres were sampled by opening the bottles inside the anaerobic glove box and using sterile forceps to transfer the fibres (approx. 0.15 g WW) to a 1.5 ml eppendorf tube. Sampled fibres were then stored at -80ºC until DNA extraction. .

Analysis of VFA and gaseous metabolites

The VFA in the culture supernatants were separated by a Spectra system HPLC (Thermo Scientific, Breda) equipped with a Metacarb 67H column (Agilent, 300 × 65 mm). Column temperature was 45°C. A 5 mM sulphuric acid solution was used as an eluent. Flow rate was set at 0.8 mL/min. Metabolites were quantified with a refractive index detector.

The concentration of methane and hydrogen in the headspace samples was analyzed using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) with a packed column (Molsieve 12X column 60/80 mesh, 2m x 3 mm, Varian, Middleburg, The Netherlands) and a thermal conductivity detector (TCD) with a current of 70 mA. The column temperature and injection temperature was 100 °C. Argon was used as carrier gas (flow rate 30 mL min-1). After measurement, the concentration of the gases was calculated based on the headspace volumes in each bottle.

DNA extraction

Genomic DNA was extracted from fibre samples using the Maxwell system as previously described (van Lingen et al., 2017b). The quantity and purity of the resulting DNA extracts was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA).

MiSeq sequencing of 16S rRNA gene amplicons

For bacterial composition profiling, barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated using a 2-step PCR strategy. The forward primer (UniTag1-27F-DegS) included UniTag1 (5′-GAGCCGTAGCCAGTCTGC-3′) at the 5′-end, and the reverse primer mix (UniTag2-338R-I + II) had UniTag2 (5′-GCCGTGACCGTGACATCG-3′) at the 5′-end (Tian et al., 2016). The first PCR step was performed in a total volume of 50 µL containing 10 µL 1× HF buffer (Finnzymes, Vantaa, Finland), 1 µL dNTP Mix (10 mM; Promega), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (2 U/µL) (Finnzymes, Vantaa, Finland), 500 nM each of the primers and 20 ng of sample DNA. The cycling conditions for the first step consisted of an initial denaturation at 98 °C for 30 s; 25 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 20 s, and elongation at 72 °C for 20 s; and a final extension at 72 °C for 10 min. The presence and size of the amplification products were determined by agarose gel electrophoresis containing 1X SYBR Safe® (Invitrogen).

The second PCR step was then employed to add an 8 nucleotide sample specific barcode to the 5'- and 3' -end of the PCR products. The PCR was performed in a total volume of 100 µL containing 1× HF buffer, 2 µL of dNTP Mix, 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (2 U/µL), 500 nM of a forward and reverse primer equivalent to the Unitag1 and UniTag2 sequences, respectively, that were each appended with an 8 nt sample specific barcode and 5 µl of the PCR product from the first step PCR. The cycling conditions of the second step consisted of an initial denaturation at 98 °C for 30 s; 5 cycles of 98 °C for 10 s, 52 °C for 20 s and 72 °C for 20 s; and a final extension at 72 °C for 10 min. Incorporation of the sample specific barcodes, yielding a PCR product of ~350 bp, was confirmed by agarose gel electrophoresis. Control PCR reactions were performed alongside each separate amplification with no addition of template, and consistently yielded no PCR product. PCR products were then purified using HighPrep^{TM} (MagBio Europe Ltd, Kent, United Kingdom) and quantified using a Qubit dsDNA BR Assay Kit (Invitrogen). Purified PCR products were mixed in equimolar amounts into pools together with defined synthetic mock communities that allow controlling for potential technical biases (Ramiro-Garcia et al., 2016). Pools then underwent adaptor ligation followed by sequencing on the MiSeq platform (GATC-Biotech, Konstanz, Germany).

RESULTS AND DISCUSSION

In the present study, two enrichment series from ruminal grass silage (GS) and maize silage (MS) fibres were obtained through serial transfers into mineral medium supplemented with sterile fibres and representative plant substrates, starting from PE to SE5 (Fig. 2 and 3). All enrichments were incubated at 39ºC for a 14 day period, with storage at 4ºC for 3-4 weeks between transfers. Although overall activity in all the enrichments was low (Fig. 2 and 3), fibre-degrading bacteria were considered to be enriched as visual inspection of PE bottles from both the MS and GS enrichment series showed initial fragmentation or breaking up of the fibre structure. Cultures also produced metabolites indicative of substrate utilization during PE for both the GS (Fig. 2) and MS (Fig. 3).

Grass silage enrichments

The GS enrichments produced acetate as the major metabolite from all enrichments ranging from PE to SE5 (Fig. 2A). Propionate and butyrate were also formed in lower concentrations, although not in all the later enrichments (Fig. 2B and 2C). Generally, after SE2 only acetate was formed at concentrations >1 mM, indicating that the cold storage in between the sub enrichments might have resulted in the loss of populations associated with propionate and butyrate production.

In GS PE bottles supplemented with cellulose, 7 mM acetate was produced after 14 days incubation whereas the SE1 bottle supplemented with cellulose produced less acetate (5 mM) indicating lower concentrations and/or activity of cellulose degrading bacteria over the sub-enrichments (Fig. 2A). In SE2, acetate production was further reduced to 2 mM, but increased again to 8 mM in SE3 cellulose enrichments. From SE3, acetate production by day 14 decreased to around 1 mM for SE5 bottles on day 14 (Fig. 2A). Since the amount of acetate produced by the GS SE5 cellulose enrichment was lower than that formed by the corresponding enrichment without any additional polysaccharide added (GS SE5 fibre) on day 14 (Fig. S1), it can be concluded that the cellulose was no longer utilized by this point.

In GS bottles supplemented with xylan, high acetate production was observed on day 14 for SE2 (12 mM), with lower concentrations being observed in subsequent sub-enrichments (i.e. 4 mM in SE4 and SE5 bottles). Although xylan has been suggested to be more easily degraded compared to cellulose (Horn et al., 2012), our results show that by SE3 the

concentration of acetate produced by the GS xylan enrichments was lower than that observed for the corresponding GS cellulose enrichments (Fig. 2A).

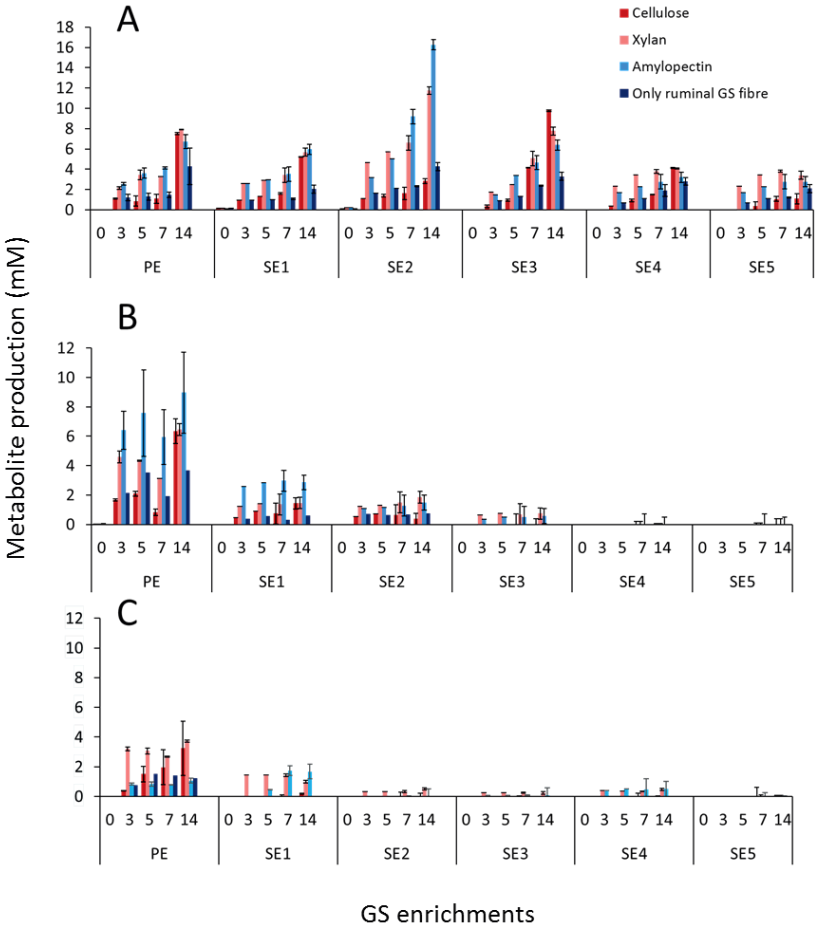


Figure 2. Acetate **(A)**, propionate **(B)** and butyrate **(C)** production from primary (PE) and sub enrichments (SE1 to SE5) in the presence of grass silage (GS) fibres and different polysaccharides during incubation for 14 days, with samples taken at 0, 3, 5, 7 and 14 days of each incubation. The error bars represent variation in the duplicate bottles.

In GS bottles supplemented with amylopectin, acetate production was the highest in SE2 suggesting that the additional substrate was readily utilized by the microbial consortia (Fig. 2A). However, acetate production in SE4 and SE5 on day 14 did not differ from that

observed for the GS-fibre only bottles, suggesting that the amylopectin degradation in the latter enrichments was minimal. It is speculated that this low activity might have been due to the cold storage periods. Moreover, as the sub-enrichments were proceeding, the fibres were losing integrity and therefore during transfers of inoculum (fibres from one sub-enrichment to another) there might have been less bacteria (attached onto fibres) transferred.

Propionate production was seen in PE bottles with all four substrates (cellulose, xylan, amylopectin and only GS fibres) at a final concentration of 6.2, 5.9, 8.4 and 3.1 mM respectively. During further sub-enrichments, however, propionate production declined steadily to almost undetectable values in SE4 and SE5 (Fig. 2B). Butyrate was only produced in low concentrations (<4 mM) from cellulose and xylan enrichments during the PE (Fig. 2C). The rest of the substrates (amylopectin and only GS fibres) showed limited butyrate production (~1 mM) in all bottles.

Maize silage enrichments

The MS enrichments also showed acetate as the major metabolite produced, irrespective of the presence of an additional plant substrate (Fig. 3). In all cases, the amount of acetate produced was higher than that observed in enrichments that only contained the MS-fibre, suggesting that the additional substrates (cellulose, amylopectin and xylan) were always utilized to some extent (Fig. 3A).The cold storage period in between the transfers did not seem to affect acetate production by the MS fibre enrichments (Fig. 3A).

The MS enrichments produced propionate in PE and SE1 enrichments with or without any additional substrates (Fig. 3B). The propionate production in SE1 ranged from 4 to 6 mM. In further subcultures, lower concentrations of propionate (< 1 mM) were detected.

The MS enrichment bottles produced butyrate from all substrates from PE to SE5, except for cellulose, amylopectin and MS-fibre only enrichment bottles during SE1 and SE2. During PE, butyrate could be measured from day 7 in all bottles.

Gaseous metabolites

Overall, CH_4 production was low (<0.2 mM) in all the PE for the GS and MS enrichments. In SE2, xylan (~2 mM) and amylopectin (~5 mM) had more CH_4 detected than cellulose and only fibres GS and MS enrichments (Fig. S1). In later transfers (SE5), CH_4 was mainly

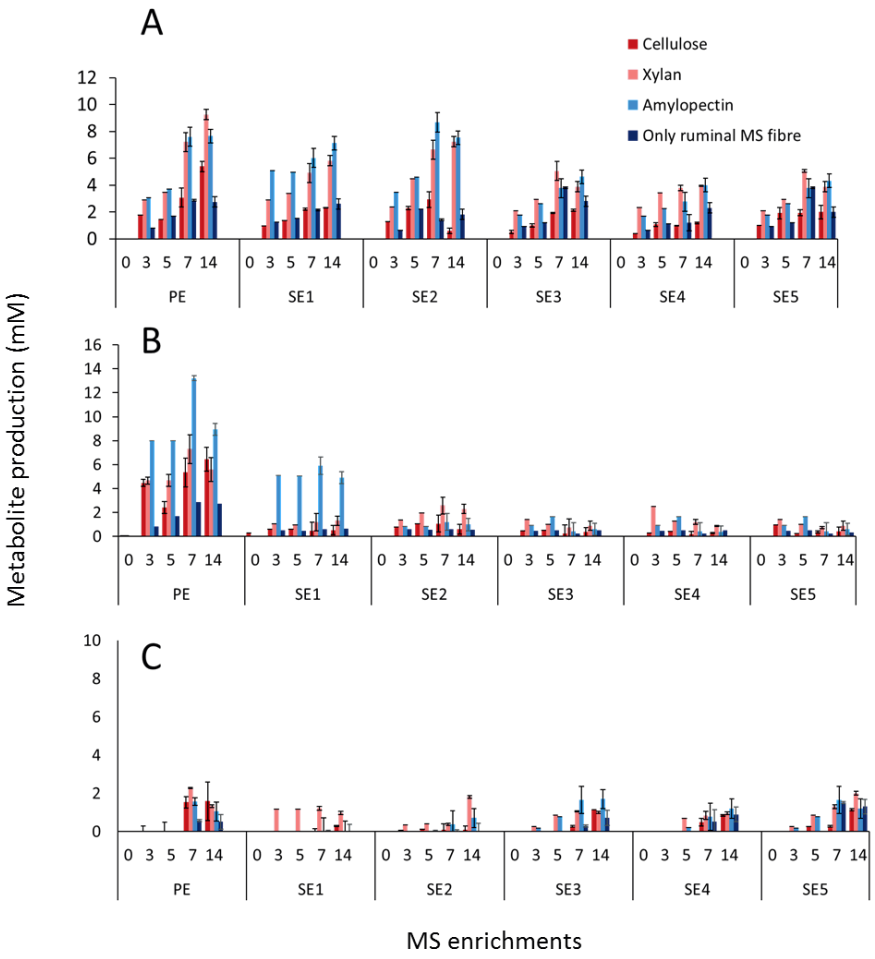


Figure 3. (A) Acetate, **(B)** propionate and **(C)** butyrate production (mM) from primary (PE) and sub enrichments (SE) in maize silage (MS) and different polysaccharides during incubation for 14 days, with samples taken at 0, 3, 5, 7 and 14 days of each incubation. The error bars represent variation in the duplicate bottles.

observed from amylopectin in GS and MS enrichments (Fig. S1). H_2 was not detected in any of the enrichments.

Bacterial community analysis using MiSeq

Bacterial community analysis was performed on the original inoculum (OI) (Fig. 4A) and the last sub-enrichment (SE5) using bacterial 16S rRNA gene barcoded amplicon sequencing

(Fig. 4B). A number of bacterial genera present in SE5 differed in their relative abundances compared to the original inoculum, for both the GS and MS fibre enrichments. These differences could be due to low abundant bacteria becoming further enriched following sub culturing and/or more abundant bacteria dying off, although the cold storage between subcultures may also have influenced the bacterial community composition.

Compared to the bacterial communities in the original inocula, those observed in SE5 were characterized by a lower alpha diversity and were dominated by specific genera (Fig. 4). The predominant genus-level taxon in both the GS and MS inocula was *Prevotella* 1, accounting for 37.8 % and 41.3% of the fibre-associated communities, respectively. The same group was consistently predominant in all the GS SE5 enrichments, ranging from 18.8 % – 51.9 %. In contrast, this genus was no longer observed in any of the MS sub-enrichments. Instead, the MS SE5 enrichments were dominated by *Ruminofilibacter* (range 66.4 to 85.0 %) (Fig. 4).

Compared to the OI, the GS SE5 bottles with fibre as the sole carbon source were also enriched in *Ruminoclostridium* 1 (23.7%), *Bacteroides* (18.7%), *Prevotella* 1 (18.8 %) and *Aminobacterium* (14.6 %). In the GS SE5 bottles with cellulose, *Prevotella* 1 (51.9 %) became the dominant member that was enriched, indicating their preference for grass silage fibres. In contrast, *Proteiniphylum* (45.1 %) and *Prevotella* 1 (31.3%) were both highly enriched with xylan. The GS SE5 bottles with amylopectin was enriched mainly with *Aminobacterium* (31.3 %) and *Bacteroides* (14.9 %) (Fig. 4). Members of *Prevotella* 1 (example *Prevotella ruminicola*) are usually correlated with propionate production and are potentially involved in hemi-cellulose degradation, along with their importance in utilization of proteins and peptides (Emerson, 2017). Our in vitro study not only shows their enrichment in the presence of hemicellulolytic components, like xylan, but also suggests their capability to utilize other complex insoluble substrates such as cellulose and amylopectin in the GS enrichments. *Bacteroides* spp. have been reported as being cellulose degraders capable of producing acetate, propionate or succinate (Chassard et al., 2010). However, succinate is not accumulated in the rumen but gets rapidly de-carboxylated to propionate. In our study, although *Bacteroides* was observed in all GS sub-enrichment bottles, there was neither succinate nor propionate production seen in SE5 indicating there must be co-fermentation of cellulose by *Bacteroides* and other fibre associated microbes, resulting in acetate production (Scheifinger, 1973). Finally, the genus *Gracilibacter* was detected in GS SE5 bottles with no

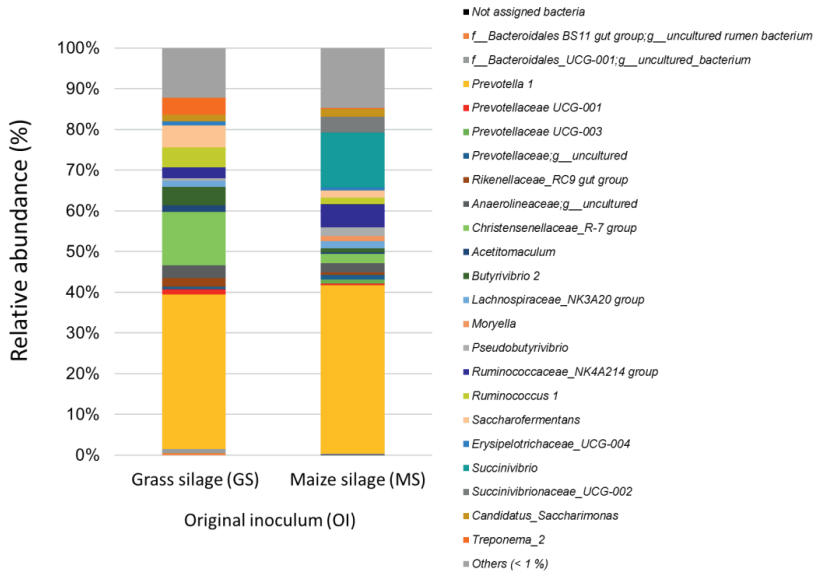
additional carbon source. Members of this genus are known to ferment glucose to produce mainly lactate, acetate, and ethanol (Lee et al., 2006).

In all maize silage (MS) enrichment bottles, *Ruminofilibacter* was highly enriched, suggesting this genus to be associated with the degradation of maize fibre. In contrast, this genus was only present at 0.1 % and 0.2 % relative abundance in the GS and MS inocula, respectively. This genus has been reported to be a rumen bacterium capable of digesting xylan (Krober et al., 2009). It has also been detected in a biogas plant fed with maize silage, green rye and liquid manure (Krober et al., 2009), as well as in birch and conifer pulp fermentations (Nissila et al., 2012). More recently, *Ruminofilibacter*, a genus from the family of Marilinabiaceae, has also been detected in anaerobic biogas plants supplemented with maize silage as the main substrate (Wojcieszak et al., 2017). It is interesting to note that this genus appeared only after 42 days of fermentation in an in vitro system (Nissila et al., 2012), suggesting that this genus was a minor member of the bacterial community and required a long enrichment period. This also reflects the outcome of our study, although as only the OI and SE5 were sequenced it is not clear how long it took before this genus dominated the MS enrichments.

In cellulose and xylan MS enrichments, *Aminobacterium* was enriched to 11.9 % and 11.7 % relative abundance. Moreover, in the cellulose MS enrichment bottles the *R-7 group* (1.9 %) belonging to Christensenallaceae, and *Desulfovibrio* (2.6 %) were detected which were high relative to the OI. The MS enrichment bottles with amylopectin as an additional carbon source were enriched for *Pyramidobacter* (12.7 %) and *Proteiniphylum* (9.5 %) compared to the MS OI (Fig. 4B). Members of the genus *Pyramidobacter* have been reported previously as anaerobic bacteria that are asaccharolytic that produce mainly acetate and minor amounts of phenylacetic, isobutyric, propionic and succinic acid mainly from the proteolytic activity (Downes, 2009). Proteolytic activity in rumen is known to occur immediately after feeding (Wallace et al., 2000). The products observed from the enrichment bottles might be associated with the proteins from the maize kernels. Members of the genus *Proteiniphilum* are known to be strictly anaerobic and proteolytic, and they are also able to use pyruvate, peptone, yeast extract, glycine, L-arginine as carbon sources. However, they are not able to degrade carbohydrates, alcohols, and fatty acids with the exception of pyruvate (Chen and Dong, 2005). This suggests that members of this non-fibrolytic genus may play a role in the

rumen by possibly supporting other fibrolytic bacteria by cross feeding, and should be addressed in future studies.

A.



B.

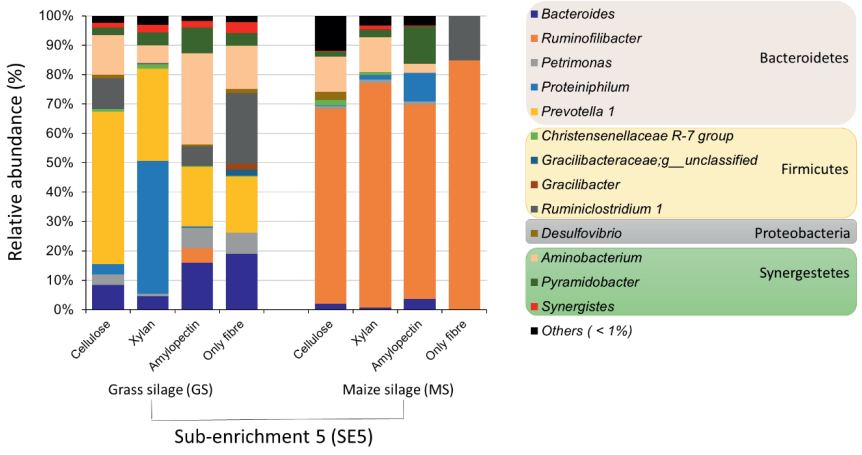


Figure 4. Genus level composition of the original inoculum **(A)** from silage derived rumen fibres, and the subsequent SE5 bottles **(B)**. All genera found at average relative abundances of less than 1 % are regarded as "Others".

In conclusion, this study gives insight into rumen fibre-associated communities that are capable of utilising different plant fibre components. The enrichment of microorganisms from ruminal GS and MS fibres resulted in primarily acetate production. *Ruminofilibacter* dominated all the MS enrichments, whereas a more diverse range of bacteria (including *Prevotella* 1 and *Bacteroides*) were associated with GS derived ruminal fibres. Enrichments of bacteria associated with ruminal GS and MS fibres had limited activity in sub-enrichments, presumably as the biomass was cold stored for long periods (2-3 weeks) in between transfers. The in vitro experimental approach of serial two week incubations seems likely to be important in enriching fibre associated bacterial groups that are otherwise not favoured by shorter incubation times, i.e. *Ruminofilibacter*.

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SUPPLEMENTARY MATERIAL

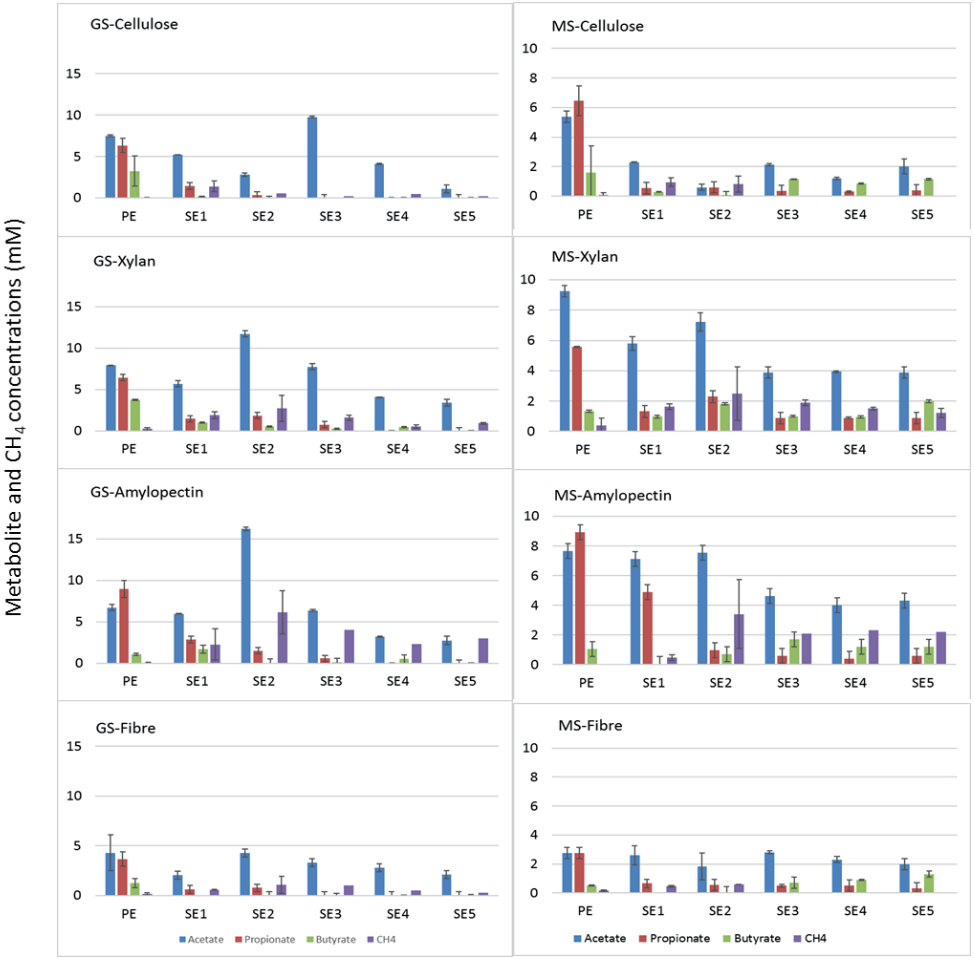

Figure S1. Volatile fatty acids (acetate, propionate and butyrate) and methane (CH_4) concentrations on day 14 obtained from primary enrichments (PE) and sub-enrichments (SE) 1-5 using grass and maize silage rumen derived fibres. The bottles with GS-fibre or MS-fibre indicate grass or maize derived pre-autoclaved rumen fibres as the sole carbon source while bottles with GS/MS-Cellulose, GS/MS-Xylan, GS/MS-Amylopectin have cellulose, xylan, and amylopectin as an additional carbon source, respectively.

Chapter 5

Characterization of *Propionibacterium ruminifibrarum* sp. nov., isolated from cow rumen fibrous content

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Submitted

ABSTRACT

A novel propionate producing bacterium, strain JV5^T, was isolated from the rumen fibrous content of a Holstein Friesian dairy cow. Strain JV5^T tested Gram-positive, is non-motile and oxygen tolerant. Growth occurred between 35 °C and 45 °C, with an optimum at 39 °C. The pH range for growth is 6.5-8, with an optimum at pH 7. The 16S rRNA gene sequence of strain JV5^T is 98.4 % and 96.5 % identical to that of *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T), respectively. Genome wide average nucleotide identity and digital DNA-DNA hybridization were 88.3 % and 35.5 %, respectively, against *P. australiense* (DSM 15818^T). The G + C % of JV5^T was 66 %. JV5^T did not produce urea and was able to metabolize glutamate but not aspartate and glycine. JV5^T was able to ferment a range of substrates including certain simple and complex carbohydrates, sugar alcohols and amino acids. Chemotaxonomic analysis of JV5^T revealed the presence of meso – diamino pimelic acid isomers similar to what has been found in *P. australiense* but different from *P. acidifaciens.* The observed major (>10%) cellular fatty acids in JV5^T ($C_{18:1}$ w9c, anteiso $C_{15:1}$ A, $C_{16:0}$ and $C_{17:0}$ and alcohol $C_{16:0}$ N) were also different from those observed in *P. australiense* and *P. acidifaciens*. Based on these findings, a novel species is proposed within the genus *Propionibacterium*, *Propionibacterium ruminifibrarum* sp. nov. (type strain JV5^T = DSM 106771^T).

KEYWORDS

Rumen fibres, *Propionibacterium*, 16S rRNA, methylcellulose, dairy cow, propionate

Ruminants, such as dairy cows, have evolved to effectively utilise fibrous plant material due to the presence of a specially adapted forestomach. The forestomach is comprised of three compartments, the largest of which is the rumen. The anaerobic microorganisms present in the rumen degrade the fibrous material in a step-wise process: i) hydrolysis of complex plant structural carbohydrates into monomers; ii) fermentation of the monomers into short chain fatty acids (SCFA's), lactate, succinate, (m)ethanol, H_2 and CO_2; iii) methanogenesis from H_2 and CO_2, formate and methanol. Knowledge on the range of microorganisms involved in initial fibre attachment and breakdown, and their physiology, however, is still limited.

In 1909, the genus *Propionibacterium* was first described by Orla-Jensen and is comprised of species that produce propionate as the main fermentation end-product (Orla-Jensen, 1909; Goodfellow and Williams, 1983). Propionate is a valuable energy source to the ruminant when it is absorbed by the ruminal epithelial cells, as it is the only major volatile fatty acid that directly contributes to gluconeogenesis. Members of the genus *Propionibacterium* are Gram-positive and have a versatile physiology, from anaerobic to aero tolerant, and are non-spore forming. Traditionally, species within this genus were grouped as classical or cutaneous based on phenotypic traits, 16S rRNA gene analysis and source of isolation such as mature cheese, cattle rumen, human skin, fermented vegetables and silage (Merry and Davies, 1999; Lucena-Padros et al., 2014).

Recently, the taxonomy of the whole family of Propionibacteriaceae was re-evaluated (Scholz and Kilian, 2016). The re-evaluation was based on decreasing the genus *Propionibacterium* to one branch containing the classic *Propionibacterium*, and creating a new genus for the other branches. Members of the redefined genus *Propionibacterium* possess meso-2,6-diaminopimelic acid as the diagnostic amino acid in their peptidoglycan (Scholz and Kilian, 2016), which separates them from the other genera (*Acidopropionibacterium, Cutibacterium* and *Pseudopropionibacterium*) that are characterized by LL-diaminopimelic acid (LL-2 A_2PM) (Goodfellow et al., 2012).

Propionibacterium spp. have the ability to perform bio-hydrogenation of poly-unsaturated fatty acids (PUFA) to generate health-promoting fatty acids like conjugated linoleic acid (Raeth-Knight et al., 2007; Hennessy et al., 2012). Moreover, *Propionibacterium* spp. are dominant in silage and consequently are ingested with the silage, or are used as direct-fed microbials to increase ruminal propionate (Elghandour et al., 2015). Propionate has

been shown to improve energetic efficiency, reduce ketosis (Wolin, 1960) and decrease methane (CH_4) emissions from dairy cows (Dean et al., 2018a). In this study, we describe the properties of strain JV5^T, a novel propionate producing bacterium isolated from enrichments derived from rumen fibrous content.

The rumen fibrous content was collected from a rumen fistulated Holstein Friesian cow fed grass silage and concentrate (80:20), which was part of a larger study conducted in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University (van Gastelen et al., 2015). The fibrous content was sampled manually through the rumen fistula, squeezed to remove excess liquid and immediately stored in a glycerol/phosphate buffered saline (1:3) solution at - 80 °C until cultivation. Cultivation was routinely carried out in 120 ml serum bottles filled with 50 ml of bicarbonate-buffered anaerobic medium (BM) supplemented with a substrate. BM medium composition was as follows (per litre): 0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NH_4Cl; 0.3 g NaCl; 0.1 g $MgCl_2.6H_2O$; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 1 ml vitamin solution; 0.5 mg resazurin; 4 g $NaHCO_3$; 0.25 g Na_2S.7-9 H_2O; 0.5 g cysteine; 0.1 g yeast extract; 10 ml of haemin (5 mg in 1 ml 1N NaOH dissolved in 100 ml distilled H_20); 0.2 ml of vitamin K1 (0.1 ml vitamin K1 dissolved in 10 ml 95% ethanol). The trace element and vitamin solutions were prepared as described previously (Stams et al., 1993). All compounds were sterilised by autoclaving except for the vitamin solution, haemin and vitamin K1, which were all added as filter sterilised solutions to the medium prior to inoculation. The final pH of the medium was 7.0, and all experiments were carried out at 39 °C without shaking unless stated otherwise.

Preparation of the inoculum for the enrichments involved detachment of the bacteria from the rumen fibres using a previously described methylcellulose method (Alterskjær and Svein, 1998). Briefly, fibres were transferred to BM medium containing 0.1% (v/v) methylcellulose and incubated for 5 minutes. The bacteria that detached from the fibres into the liquid phase were then serially diluted 10-fold into 50 ml bottles containing BM medium supplemented with 1 % (w/v) insoluble cellulose (Avicel) as the carbon source. Microbial activity was monitored by measurement of metabolites using HPLC. After 2 weeks of incubation, the highest dilution (10^{-2}) where metabolites were produced was used to prepare another dilution series. A liquid sample from the 10^{-2} dilution was plated on BM medium supplemented with 0.8% (w/v) agar and 0.5g/L cellulose (Avicel), and then anaerobically

incubated in an anaerobic box for 3 weeks at 39ºC. After this, streak plating was performed from individual colonies. After 3 to 4 weeks, small round white colonies appeared on the BM cellulose plates. Single colonies were then transferred into reinforced clostridium medium (RCM) for rich/fast growth, and 10-fold serial dilutions were prepared in 35 ml serum bottles, which were then incubated at 39ºC. Cell morphology and purity of strains was examined routinely during this process with light microscopy, and a pure culture was obtained that was termed strain JV5^T.

Culture (1ml) was sampled aseptically using a needle and syringe from JV5^T grown on RCM medium and the cell pellet (13,000 g for 7 min) used for genomic DNA extraction using the repeated bead beating (RBB) protocol (Vaidya et al., 2018). The 16S rRNA gene was then amplified using universal bacterial primers 27F and 1492R (Lane et al., 1991), and the product purified using a PCR purification kit (Qiagen, Germany) before being Sanger sequenced (GATC-Biotech, Konstanz, Germany). Sequences were manually checked for errors using Chromas software programme, and the subsequent alignment and contigs were prepared using DNA Baser (version 4.20.0). Based on BLASTN search of the NCBI database, the 16S rRNA sequence (1430 bp) of strain JV5^T was most closely related to that of *Propionibacterium australiense* (DSM 15818^T) (98%) and *Propionibacterium acidifaciens* (DSM 21887^T) (96%). Pairwise sequence similarities calculated for the 16S rRNA sequences via the GGDC web server (Meier-Kolthoff et al., 2013) confirmed that the 16S rRNA sequence of strain JV5^T was 98.4% similar to *P. australiense* (DSM 15818^T) and 96.5% similar to *P. acidifaciens* (DSM 21887^T). The phylogenetic relationship of strain JV5^T and its closest related species (*P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T)) along with other distantly related *Propionibacterium* species was assessed using the 16S rRNA gene. Sequences were aligned using Clustal X2 and a neighbour joining tree was constructed in MEGA version 6.0 (Tamura et al., 2013) from distance matrices generated using a maximum composite likelihood method (Fig. 1). Clustering revealed strain JV5^T to be closest to *P. australiense* and *P. acidifaciens*, in addition to known *Propionibacterium* spp. that were of dairy origin. The GenBank/EMBL/DBBJ accession number for the 16S rRNA gene sequence of strain JV5^T (DSM = 106771^T) is MG783038.

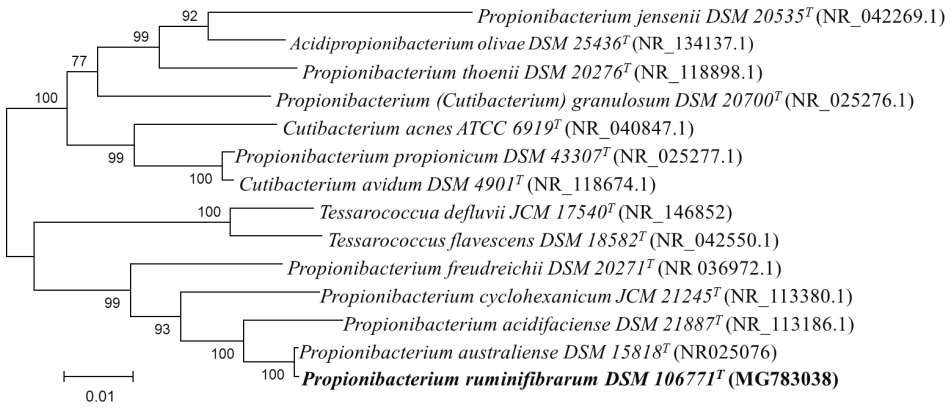

Figure 1. 16S rRNA gene based phylogenetic tree showing the relationship of strain JV5^T (in bold) with related species within the genus *Propionibacterium*. Bar represents 1% sequence divergence. Numbers on the tree next to the branch nodes indicate percentage bootstrap values determined

As the identity of the 16S rRNA gene sequences of JV5^T and *P. australiense* is on the borderline with the most recently described threshold (98.7%) to delineate a new species (Rossello-Mora and Amann, 2015), further taxonomic analyses were performed with strains of *P. australiense* (DSM 15818^T) and *P. acidifaciense* (DSM 21887^T) obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. Genome-based comparison tools (Rosselló-Móra and Amann, 2015) were applied to confirm the difference between the strain JV5^T and *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T). Genomic DNA was extracted from cells of JV5^T and *P. australiense* (DSM 15818^T), pre-grown in BHI medium incubated at 39ºC for 72 hours, using the MasterPure DNA kit, and the genome was sequenced at GATC on an Illumina MiSeq sequencer. The draft genome sequence of strain JV5^T has been uploaded to the EBI database under accession number ERS1670027 in project PRJEB19533, with contigs OMOH01000001-OMOH01000036. Subsequently, the draft genome sequences of strain JV5^T and *P. australiense* (DSM 15818^T) were compared along with the genome of *P.acidifaciens*, which was obtained from NCBI (DSM 21887^T). Genome wide average nucleotide identity (gANI) values were calculated with JSpecies version 1.2.1 (Richter and Rossello-Mora, 2009), while the digital DNA-DNA hybridization (dDDH) was calculated using the genome BLAST distance phylogeny version 2.1 web browser from DSMZ (Meier-Kolthoff et al., 2013).

gANI for strain JV5^T was 88.3% compared to *P. australiense* (DSM 15818^T), and 84.2% compared to *P. acidifaciens* (DSM 21887^T). Both of these values are lower than the species cut-off value of 96.5% (Varghese et al., 2015). dDDH between strain JV5^T and *P. australiense* (DSM 15818^T) was 35.5%, and between strain JV5^T and *P. acidifaciens* (DSM 21887^T) 12.9%. Again, both values were significantly lower than the 70% threshold value (Wayne et al., 1987). Both the gANI and dDDH results can, therefore, be used confidently for species delineation (Goris et al., 2007; Rossello-Mora and Amann, 2015). The genomic DNA G+C content of strain JV5^T was calculated using an in house custom python script and found to be 66% (Table 1). This value lies within the DNA G+C content range (57-70%) described for many other species of the genus *Propionibacterium* (Lucena-Padros et al., 2014; Aubin et al., 2016).

Fatty acid composition, analysis of diaminopimelic acid (DAP) and respiratory quinones was performed at DSMZ for strain JV5^T, *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T). Major cellular fatty acids (>10%) observed for strain JV5^T were the unsaturated fatty acid $C_{18:1}$ w9c, the branched unsaturated anteiso $C_{15:1}$ A, the two saturated fatty acids $C_{16:0}$ and $C_{17:0}$ and the alcohol $C_{16:0}$ N (Table S1). The cellular fatty acid profile of strain JV5^T differed from that of *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T). From the above mentioned major cellular fatty acids, anteiso $C_{15:1.}$ A was not detected in *P. australiense* (DSM 15818^T). Conversely, $C_{15:0}$ was not detected in strain JV5^T but was detected as a major cellular fatty acid (>10% in total) in *P. australiense* (DSM 15818^T). Differences between strain JV5^T and *P. acidifaciens* (DSM 21887^T) were more in terms of minor (<10%) cellular fatty acids (Table S1). Whole cell hydrolysates were used for DAP analysis, and were examined using thin layer chromatography on cellulose plates (Rhuland et al., 1955). Strain JV5^T contained meso-DAP isomers which were similar to those observed for *P. australiense* (DSM 15818^T), whilst both were different from *P. acidifaciens* (DSM 21887^T) (Table 1).There were no respiratory quinones detected in strain JV5^T, *P. australiense* or *P. acidifaciens*.

Cell wall morphology of strain JV5^T was determined using a standard method (Plugge et al., 2000), with cells pre-grown in brain heart fusion (BHI) medium at 39 ºC. Cell morphology was examined with a light microscope (Leica, Heerbrugg, Switzerland). For electron microscopy, strain JV5^T was grown for 24 hours in pre-autoclaved BM medium supplemented with plant fibres retrieved from the rumen (10 fibre pieces of ~1.5 cm length).

The fibres grown with strain JV5^T were then fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at room temperature for 2 hours. Subsequently the fibres were rinsed in 0.1 M phosphate buffer (pH 7.2), and stained with 1% osmium tetroxide in 0.1 M phosphate buffer. After washing in 0.1 M phosphate buffer, the samples were dehydrated in graded ethanol solutions 30%, 50%, 70% , 80%, 90%, 96%, 2 x 100% (for 10 min each). The samples were subsequently critical point dried (CPD) with carbon dioxide in a Leica EM CPD300 before being mounted on an SEM stub by carbon adhesive tabs (EMS Washington USA) and coated with 12 nm Tungsten (Leica MED 020). Samples were analysed at 2 KV, 6 pA, in a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands). Strain JV5^T stained gram positive, and is a non-motile, short curved rod, present as single cells (0.5-0.8 µm wide by 1.0-1.2 µm long) or in small clusters. Cells were also observed to from micro-colonies on the surface of silage derived plant fibres (Fig. 2).

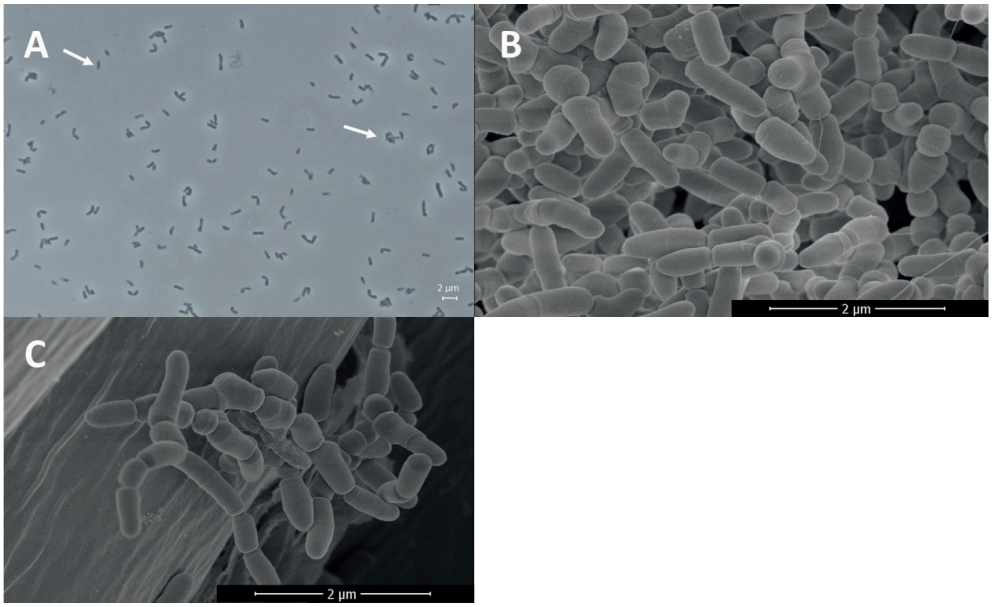

Figure 2. Strain JV5^T visualised by light microscopy **(A)** and scanning electron microscopy **(B and C)**. Cells of Strain JV5^T are present as single cells or in clusters of more than two (indicated with arrows) **(A)**. Cells associate to silage derived plant fibres where they form micro-colonies **(C).**

For biochemical characterization, strain JV5^T, *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) were pre-grown in peptone-yeast-glucose (PYG) medium (pH 7.2) at 39°C unless stated otherwise. Carbohydrate assimilation was assessed using API 50 CHB and API 20A kits (bioMérieux, France), whilst enzyme profiling was performed using API ZYM kits (bioMérieux, France). Differentiating characteristics found by the kits were as follows. From the API 50 CHB kit, strain JV5^T produced acid from D-turanose and erythritol while the closest relatives *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) did not (Table 1, S2 and S3). API 20A profiling showed that strain JV5^T is urea, gelatin, indole and catalase negative whilst being esculin positive (Table 1 and Table S4). These properties differed between one or more of the closely related strains, particularly in terms of urea hydrolysis activity, which both *P. australiense* and *P. acidifaciens* possessed unlike strain JV5^T. API ZYM profiling showed strain JV5^T was positive for valine aminopeptidase, cysteine aminopeptidase and beta – glucosidase, whilst its close relatives were negative (Table 1 and Table S5).

Fermentation of selected substrates and electron donors (20mM) and concomitant metabolite production by strain JV5^T was tested in bottles (BM medium with 0.1g/l yeast extract, in duplicate) for 14 days (Table S2). D-adonitol, galactose, glucose, inositol, DL-lactate, mannose, meso-erythritol, ribose and sorbitol were converted to mainly propionate and acetate, and sometimes succinate and/or formate. Pyruvate and aspartate were fermented to only acetate. D-arabinose, L-arabinose, cellobiose, glutamate, glycerol, glycine, L-sorbose, rhamnose and xylose were not utilized (Table S2).

Fermentation products and the carbon balance of strain JV5^T was determined in BM medium with 30 mM glucose. A liquid sample (1 ml) was centrifuged for 7 min at 13,000 rpm, and the metabolites dissolved in the supernatant were measured with HPLC while gas formation from the bottle was measured by gas chromatography (GC) (van Lingen et al., 2017b). After 14 days, strain JV5^T produced 23.9 mM propionate, 14.7 mM acetate, 18.4 mM formate and 2.4 mM succinate from 30 mM glucose. The redox balance was used to calculate the CO_2.The calculated carbon recovery was 74.4 % (without accounting for biomass).
The glucose fermentation stoichiometry for strain JV5^T was:
1 glucose + 0.8 CO_2 --------> 0.9 Propionate + 0.5 Acetate + 0.7 Formate + 0.1 Succinate
No hydrogen (H_2) was detected.

The ability of strain JV5^T to utilize representative plant polymeric components was tested with xylan from oat spelt (2 g /L), xylan from birch wood (2 g /L), pectin (2 g /L), cellulose (2 g /L) and soluble starch (2 g /L) in BM medium supplemented with 0.5 g/L yeast extract. Uninoculated bottles of BM medium with polymeric substances served as negative controls. Strain JV5^T was able to produce trace amounts of acetate from xylan (oat spelt: 2 mM; birchwood: 3 mM) as well as succinate and propionate in very low concentrations (< 1 mM). Cellulose degradation resulted in only succinate production (1.2 mM), while soluble starch was not utilized at all. These results suggest that strain JV5^T could not directly use plant polymeric components, but probably can associate with fibres to use the compounds that are released from them.

The optimal temperature for growth of strain JV5^T was assessed in duplicate at a range of 10 to 60 ºC (with intervals of 5 ºC) in bottles with 10 ml PYG medium. The test was also performed using a narrower temperature range (39-47 ºC) with intervals of 1 ºC. Bottles were incubated for two weeks. Growth was assessed using optical density (OD_{660}) measurements. Strain JV5^T was able to grow at temperatures ranging from 35 to 45 ºC with an optimum at 39 ºC, which is different for *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) (Table 1). The pH range and optimum for strain JV5^T was determined from 4.0 to 9.0 (with intervals of 0.5) in 10 ml PYG medium in duplicates using 20 mM Tris-Borate buffer (pH 8.0 to 9.0) or 20mM $NaHCO_3$ for (pH <8.0). The pH of the bottles was adjusted using 1M NaOH or HCl. Bottles were incubated for two weeks at 39 ºC. Growth was assessed using optical density (OD_{660}) measurements. Strain JV5^T was able to grow in a pH range from 6.5 – 8.0 with an optimum at pH 7.0 (Table 1). To define salt tolerance, strain JV5^T, *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) were grown in BM medium in duplicate with 0.5 %-6 % NaCl (w/v; at intervals of 0.5%) and incubated for four weeks. Strain JV5^T tolerated up to 5% NaCl, which was higher than what has been observed for *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) (Table 1).Oxygen tolerance was tested by growing strain JV5^T, *P.australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) in BM medium without any reducing agent with 0.5%, 1%, 5% or 21 % O_2 in the headspace. Growth was monitored using optical density (OD_{660}) measurements. All three strains were able to tolerate oxygen up to 5 % but did not grow at atmospheric (21%) oxygen levels, indicating they are micro-aero tolerant.

Table 1: Differential characteristics of strain JV5^T and closely related reference strains. 1 – Strain JV5^T (DSM 106771^T), 2 – *P. australiense* (DSM 15818^T), 3 – *P. acidifaciens* (DSM 21887^T). The temperature, pH, and colony colour for **2** and **3** are taken from (Bernard et al., 2002) and (Downes and Wade, 2009), respectively. NR indicates not reported.

Characteristics	**1**	**2**	**3**
Cell morphology	Short curved rods	Short rods	Short curved rods
Cell size (µm)	0.5-0.8 × 1.0-2	0.6-0.7 × 1.2	0.7-0.8 × 1.2-4
Colony colour	White	Cream	White to pale cream
Isolation source	Cow rumen fibres	Bovine lesions	Human mouth
pH range/ optimum	6.5-8.0/7.0	NR/7	6.0-6.72/NR
Temperature range/ optimum (°C)	35-45/39	NR/35-37	NR/37
Salt (NaCl) tolerance range	0.5 to 5%	0.5 to 3%	0.5 to 3%
DAP (Di-amino pimelic acid) isomer	meso-DAP	meso-DAP	LL-DAP
G+C content (%)	66	68.3	70
Hydrolysis of			
Esculin	+	+	-
Gelatin	-	-	+
Urea	-	+	+
Glucose fermentation products	Propionate, Acetate, Formate, Succinate	Propionate, Acetate, Succinate	Propionate, Acetate, Succinate
API 50 CH: Acid formation from			
Arbutin	-	+	-
D-Adonitol	+	+	-
D-Mannitol	-	+	-
D-Maltose	+	+	-
D-Raffinose	-	+	-
D-Ribose	+	+	-
D-Sorbitol	+	+	-
D-Trehalose	+	+	-
D-Turanose	+	-	-
Dulcitol	-	+	-
D-Xylose	-	+	-
Erythritol	+	-	-
Inositol	-	+	-
Inulin	-	-	+
Salicin	-	+	-
API ZYM			
Valine aminopeptidase	+	-	-
Cysteine aminopeptidase	+	-	+
Beta-glucosidase	+	-	+

DESCRIPTION OF *PROPIONIBACTERIUM RUMINIFIBRARUM* SP. NOV.

Propionibacterium ruminifibrarum

ruminifibrarum (ru.mi.ni.fi.bra'rum. L. neut. n. *rumen* gullet, rumen; L. fem. n. *fibra* a fibre; N.L. gen. pl. n. *ruminifibrarum* of rumen fibres).

Cells are Gram-positive, non-motile, small short curved rod-shape, 0.5-0.8 × 1.0-2 µm and are present as single cells or in clusters. Cell growth is observed at temperatures ranging from 35 to 45 ºC with an optimal temperature of 39 ºC. The pH for growth is 6.5 to 8.5 with an optimal pH of 7.0. Can tolerate salt concentrations up to 5%. Micro-aero tolerant. Major cellular fatty acids (>10% in total) observed were $C_{18:1}$ w9c, $C_{15:1}$ A, $C_{16:0}$ and $C_{17:0}$ and alcohol $C_{16:0}$ N (Table S1). Characterized by meso-diaminopimelic isomers. No respiratory quinones detected. Using API 50 CHB, JV5^T produced acid from D-turanose and erythritol while the closest relatives *P. australiense* (DSM 15818^T) and *P. acidifaciens* (DSM 21887^T) did not (Table S3). Urea, gelatin, indole and catalase negative while esculin positive from API 20A (Table S4). From a bottle test, D-adonitol, galactose, glucose, inositol, DL-lactate, mannose, meso-erythritol, ribose and sorbitol were converted to mainly propionate and acetate, and succinate and/or formate in some cases (Table S2). Degradation of pyruvate only generated acetate. Able to metabolize the amino acid aspartate producing acetate, but not glutamate and glycine (Table S2). Using the API ZYM kit, positive for valine aminopeptidase, cysteine aminopeptidase, beta-glucosidase phosphatase alkaline, esterase, esterase lipase, lipase, leucine aminopeptidase, phosphatase acid, phosphoamidase and beta-glucorinidase (Table S5). Although originating from rumen derived plant fibres, Strain JV5^T could not use any of the tested major plant components directly. The type strain, JV5^T (= DSM 106771^T), was isolated from ruminal fibrous content obtained from Holstein Friesian dairy cow in the Netherlands. The DNA G+C (mol %) content of the type strain is 66 mol %.

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SUPPLEMENTARY MATERIAL

Table S1 Cellular fatty acid profiles (% of totals) of strain JV5^T and closely related reference strains. 1 – Strain JV5^T (DSM 106771^T), 2 – *P. australiense* (DSM 15818^T), 3 – *P. acidifaciens* (DSM 21887^T), ND – not detected. Bold numbers indicate large differences in cellular fatty acid profiles between strains 1, 2 and 3.

Cellular fatty acids	**1**	**2**	**3**
Saturated			
$C_{13:0}$	0.56	0.59	0.20
$C_{14:0}$	2.61	1.36	0.58
$C_{15:0}$	ND	**39.37**	ND
$C_{16:0}$	**10.07**	5.43	2.69
$C_{17:0}$	**15.27**	8.70	6.16
$C_{18:0}$	ND	0.8	0.46
Unsaturated			
$C_{18:1}$ w6c (6,9,12)	ND	0.94	ND
$C_{18:1}$ w7c	ND	ND	0.49
$C_{18:1}$ w9c	10.11	10.16	8.42
Branched saturated			
iso $C_{14:0}$	1.04	ND	0.52
iso $C_{15:0}$	5.19	3.10	**15.75**
iso $C_{16:0}$	1.52	ND	1.08
iso $C_{17:0}$	ND	ND	1.15
iso $C_{18:1}$	**4.78**	ND	ND
anteiso $C_{17:0}$	ND	ND	3.30
anteiso $C_{15:0}$	3.27	1.97	**25.25**
Branched unsaturated			
iso-$C_{18:1}$ H	**ND**	3.74	2.26
iso $C_{17:1}$ I and/or anteiso B	ND	ND	1.21
anteiso $C_{15:1}$ A	13.83	**ND**	8.92
alcohol $C_{16:0}$ N	**8.65**	2.59	0.94
Hydroxy			
$C_{18:1}$ 2 OH	ND	0.62	ND

Table S2 Metabolites produced from substrate fermentation by strain JV5^T. All incubations were performed in BM medium (with 0.1 g/L yeast extract and Cysteine-Na_2S as the reducing agent) with 20 mM of substrate in 120 ml serum bottles incubated at 39ºC for a period of 14 days. A BM bottle with only yeast extract (0.1g) was also tested for metabolite production. Metabolites were measured on day 14 by HPLC and the symbols in the table indicate which metabolites were produced (+) or not (-). All analyses were carried out in duplicate.

Substrate	**Propionate**	**Acetate**	**Formate**	**Succinate**
BM with yeast	-	-	-	-
Adonitol	+	+	+	+
D-arabinose	-	-	-	-
L-arabinose	-	-	-	-
Aspartate	-	+	-	-
Cellobiose	-	-	-	-
Galactose	+	+	+	+
Glucose	+	+	+	+
Glutamate	-	-	-	-
Glycerol	-	-	-	-
Glycine	-	-	-	-
Inositol	+	+	-	+
DL-Lactate	+	+	+	-
Mannose	+	+	+	-
Meso-erythritol	+	+	-	-
Pyruvate	-	+	-	-
Rhamnose	-	-	-	-
Ribose	+	+	+	+
Sorbitol	+	+	+	+
L-Sorbose	-	-	-	-
Xylose	-	-	-	-

Table S3. Carbohydrate metabolism of 1 - strain JV5^T, 2 - *P. australiense* (DSM 15818^T) and 3 - *P. acidifaciens* (DSM 21887^T) as derived from the **API 50CH** test performed using duplicates. Symbols indicate acid formation (+) or no acid production (-) in the test.

Carbohydrate	**1**	**2**	**3**
Amygdalin	-	-	-
Arbutin	-	+	-
D-Adonitol	+	+	-
D-Arabinose	-	-	-
D-Arabitol	-	-	-
D-Cellobiose	-	-	-
D-Fructose	+	+	+
D-Fucose	-	-	-
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Lactose	+	+	+
D-Lyxose	-	-	-
D-Mannose	+	+	+
D-Mannitol	+	+	-
D-Maltose	+	+	-
D-Melibiose	-	-	-
D-Melezitose	-	-	-
D-Raffinose	-	+	-
D-Ribose	+	+	-
D-Saccharose	+	+	+
D-Sorbitol	+	+	-
D-Trehalose	+	+	-
D-Turanose	+	-	-
D-Tagatose	-	-	-
Dulcitol	-	+	-
D-Xylose	-	+	-
Erythritol	+	-	-
Esculin ferric citrate	-	-	-
Gentiobiose	-	-	-
Glycerol	-	-	-
Glycogen	-	-	-
L-Arabinose	-	-	-
L-Arabitol	-	-	-
L-Fucose	-	-	-
L-Rhamnose	-	-	-
L-Sorbose	-	-	-
L-Xylose	-	-	-
Methyl-αD-mannopyranoside	-	-	-
Methyl-αD-glucopyranoside	-	-	-
Methyl-βD-xylopyranoside	-	-	-
Inositol	-	+	-
Inulin	-	-	+
N-Acetylglucosamine	-	-	-
Potassium gluconate	-	-	-
Potassium-2-ketogluconate	-	-	-
Potassium-4-ketogluconate	-	-	-
Salicin	-	+	-
Xylitol	-	-	-

Table S4. Characteristics of 1 - strain JV5^T, 2 - *P. australiense* (DSM 15818^T) and 3 - *P. acidifaciens* (DSM 21887^T) from **API 20A** test performed using duplicates. Symbols indicate positive reaction (+) or negative reaction (-) in the test.

API 20A	**1**	**2**	**3**
Arabinose	-	-	-
Catalase	-	-	-
Cellobiose	-	-	-
Esculin	+	+	-
Gelatin	-	-	+
Glucose	+	+	+
Glycerol	-	-	+
Indole	-	-	-
Lactose	+	-	+
Mannose	+	-	+
Maltose	+	-	+
Melizitose	-	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Saccharose	+	-	+
Salicin	+	-	-
Sorbitol	+	-	-
Trehalose	+	-	-
Urease	-	+	+
Xylose	-	-	-

Table S5. Characteristics of 1 - strain JV5^T, 2 - *P. australiense* (DSM 15818^T) and 3 - *P. acidifaciens* (DSM 21887^T) from **API ZYM** test performed using duplicates. Symbols indicate positive reaction (+) or negative reaction (-) in the test.

APIzym	**1**	**2**	**3**
Phosphatase alkaline	+	+	-
Esterase (C4)	+	+	-
Esterase lipase (C8)	+	+	-
Lipase (C 14)	+	+	-
Leucine aminopeptidase	+	+	+
Valine aminopeptidase	+	-	-
Cystine aminopeptidase	+	-	+
Trypsin	-	-	-
Chymotrypsin	-	-	-
Phosphatase acid	+	+	+
Phosphoamidase	+	+	+
α- galactosidase	-	-	+
β- galactosidase	-	-	+
β- glucorinidase	+	+	+
α- glucosidase	-	-	+
β- glucosidase	+	-	+
β- glucosiaminidase	-	-	-
α- mannosidase	-	-	-
α- fucosidase	-	-	-

Chapter 6

General discussion

Ruminal fermentation is performed by the diverse rumen microbiota consisting of bacteria, archaea, protozoa and anaerobic fungi (Mackie et al., 2000). The rumen is the largest digestive organ of ruminants and provides the animal with energy in the form of proteins from microbial cell protein and volatile fatty acids (VFAs) (Hungate, 1966a). VFAs are taken up in the blood stream of the host via diffusion across the rumen epithelium. Breakdown products of microbial proteins are absorbed after digestion in the small intestine as amino acids, di-peptides and tri-peptides (Webb, 1990). In turn, another end product of feed degradation, methane, is non-desirable as it represents a loss of energy to the animal and also contributes to the greenhouse gas effect. Therefore the development of strategies for the mitigation of methane emissions from dairy cows has received ample attention. Most of the rumen methane mitigation studies to date have focused on manipulating the rumen by changing feed composition, adding chemical agents, enzymes as feed additives or microbial agents (e.g. probiotics) (Haque, 2018). However, characterizing ruminal microbiota with respect to its composition and activity remains vital to any rumen manipulation studies. Furthermore, the rumen microbial communities associated with the rumen fluid and fibrous content are different (Jewell et al., 2015). A prerequisite to understanding rumen microbial composition and function is to study the physiological and metabolic capabilities of rumen microorganisms with respect to fibre degradation. The focus on rumen fibre degradation stems from the fact that it is an important key step to overall ruminal feed degradation, and thus directly linked to ruminal methane emissions. There still is, however, a knowledge gap on the microbes that are involved in metabolizing the fibrous fraction.

In this thesis, **Chapter 1** introduced the current knowledge of the animal associated and experimental factors influencing rumen microbiota composition. This chapter particularly addressed the need for understanding rumen microbes using culture independent and culture dependent approaches to be able to perform rumen manipulations with an aim to reduce levels of rumen methane emissions. The central objective of **Chapter 2** was to determine the effect of different DNA extraction methods on the observed rumen microbial composition of liquid as well as fibrous rumen fractions of dairy cows. Our results showed that DNA extraction methods affected the apparent microbial community composition, and more specifically that of the bacteria. **Chapter 3** described the effects of different diets on rumen microbial composition and related it to the previously reported methane emissions, which were found reduced in animals fed a maize silage based diet. These analyses revealed that diet, i.e. the ratio of grass and maize silage in the roughage, and time after the introduction of the

experimental diets, significantly affected the bacterial composition but not the archaeal composition. Several genus-level taxa were positively associated with maize silage, with some of these taxa being members of the family Succinibrionaceae, which have been linked to low CH_4 emissions in other studies.

Attachment of microbes to feed fibres is important in ruminal feed degradation (McAllister et al., 1994). Microorganisms in the rumen have a variety of surfaces to which they may attach and distinct communities adhere to each surface available (Huws et al., 2014). From an ecological point of view, microorganisms and typically bacteria that attach on feed particles have an advantage over the free floating bacteria that are passing faster through the rumen. Moreover, fibre-attached microorganisms have first access to the nutrients. Three bacterial species (*Fibrobacter succinogenes, Ruminococcus albus* and *R. flavefaciens*) have been established and well characterized as primary degraders of forage cell walls and are known to adhere to cellulose fibres (Bera-Maillet et al., 2004). Furthermore, primary cellulolytic populations have complex interactions with a range of non-cellulolytic fibre degraders, including e.g. *Prevotella ruminicola, Ruminobacter amylophilus, Selenomonas ruminatium, Streptococcus bovis, Succinimonas amylolytica* and *Succinivibrio dextrinosolvens* (Weimer, 1996a). Most of the non-cellulolytic bacteria utilize carbohydrates as growth substrates and/or ferment various di- and monosaccharides. In the research described in this thesis, enrichments of fibre associated microbial communities were made, and bacteria possibly involved in fibre degradation could be identified **(Chapter 4).** Some of the enriched rumen fibre associated bacteria were similar to genera known to be associated with fibre degradation. However, we were also able to enrich several novel rumen bacterial genera including *Ruminofilibacter*. These were possibly enriched due to the cold shock in between the sub-enrichments (SE), as this enrichment approach that was initially implemented due to experimental constraints in this study, is unconventional and has not been systematically used before. In **Chapter 5** a novel fibre associated bacterium was isolated. The results of this chapter describe a novel *Propionibacterium* species in terms of its physiological, biochemical and chemotaxonomic characteristics.

Overall, the work in this thesis complements existing knowledge by assessing rumen microbial composition with respect to rumen feed degradation and characterizing novel bacterial taxa. An overview of cultivation dependent and molecular approaches used in the different studies included in this thesis is presented in Table 1.

Table 1. Overview of rumen microbiota analysis in this thesis

Study	Samples	Number of cows	Cow Diet	Groups	Sampling time (day)	Method
Chapter 2	Rumen fluid and fibrous content	4	GS100,GS67, GS33,GS0	4	17	Pyrosequencing qPCR
Chapter 3	Rumen fluid	12*	GS100,GS67, GS33,GS0	3,4,7	10,17	MiSeq qPCR
Chapter 4	Fibrous content	1	GS100	4	17	Enrichment
Chapter 5	Fibrous content	1	GS100	4	17	Isolation

*The cows were clustered in groups of four, with each of the four cows fed one of four different diets differing in the ratio of grass (GS) and maize (MS) silage.

ASSESSMENT OF RUMEN MICROBIAL DIVERSITY

The rumen is a fermentation chamber which allows input of feed, removal of microbial biomass and end products whilst enabling microbial growth. Many factors influence the rumen microbial community and can be broadly grouped into animal associated and experimental factors, including, e.g., breed, diet, housing and age. Furthermore, methodological aspects, such as sampling, sample storage and DNA extraction methods, influence the way we see these microbial communities **(Chapter 1 and Chapter 2)**. Many studies have evaluated a variety of technical biases when analysing rumen microbial community composition. For example DNA extraction methods, sampling and storage conditions have impact on the observed rumen microbial community composition (Henderson et al., 2013; Fliegerova et al., 2014; Paz et al., 2016; Granja-Salcedo et al., 2017). The method of DNA extraction has been identified as the main factor determining observed microbial composition changes, rather than the sampling method (cheesecloth squeezed, centrifuges, filtered), storage temperature (-80 ºC, room temperature) or cryoprotectants (PBS-Glycerol, ethanol) (Fliegerova et al., 2014; Paz et al., 2016). Commercial DNA extraction kits specifically designed for rumen contents are to the best of our knowledge unavailable, and kits that are designed for other sample types (soil or biofilm or faeces) are often used.

Chapter 2 involved testing of four routinely used DNA extraction methods (Repeated bead beating: RBB; Phenol dependent bead beating: PBB; Fast DNA SPIN Soil Kit: FDSS; and PQIAmini) on rumen fluid (RF) and fibrous content (FC). The methods were evaluated based on DNA yield, quality and molecular microbial analyis (i.e. qPCR and barcoded amplicon sequencing). The results showed that the DNA extraction method impacts the outcome of the bacterial community analysis more than the picture obtained for archaeal and fungal communities. Moreover, every DNA extraction method had its own strengths and weaknesses. In our study, high molecular weight DNA was always recovered, although all methods employed a bead beating step, with differences in agitation times and types of beads. However, there was some shearing of the DNA mainly due to the mechanical force employed by our methods, albeit not to the same extent with the different methods. DNA yields differed between extraction methods tested **(Chapter 2)**, which was also noted in the study of Henderson et al. (2013). At the phylum level, Bacteroidetes and Firmicutes dominated all samples. However, more prominent differences between rumen fractions and DNA extraction methods were observed at the genus level within these and other phyla, such as the Fibrobacteres **(Chapter 2)**.

INFLUENCE OF DIET ON RUMEN MICROBIOTA COMPOSITION

The rumen microbiome is complex and can be influenced by many factors. The study described in **Chapter 3** aimed to characterise the rumen bacterial and archaeal communities in dairy cows fed different ratios of maize silage (MS) and grass silage (GS) wherein the composition of the concentrate was similar for all four treatments, whereas the roughage was GS, MS, or a mixture of both (ingredient as percentage of the total amount of roughage in the diet; DM basis): 100% GS (**GS100**), 67% GS and 33% MS (**GS67**), 33% GS and 67% MS (**GS33**), and 100% MS (**GS0**). We placed the findings in context of ruminal fermentation as well as previously reported CH_4 emissions (van Gastelen et al., 2015). Overall, no effect of diet on total VFA and the molar proportions of acetate and propionate was observed (**Chapter 3** and van Gastelen et al. (2015). Both studies differed with respect to the molar proportions of butyrate and isovalerate. Additionally, Van Gastelen et al. (2015) reported that isovalerate tended to be affected by diet, whereas in the current study no diet effect on isovalerate was found. These differences between the two studies are likely associated with the frequency and timing of sampling, which differed between both studies. More specifically, samples analyzed in this study were taken on days 10 and 17, 4h after morning feeding ,whereas van Gastelen

and coworkers (2015) analysed samples taken one hour before and 1, 2, 4, 6 and 8 h after morning feeding on days 10 and 11, before animals entered a respiration chamber from day 12-17. The observed differences could also be partly attributed to mild stress of the cows. After day 17 (last milking/feeding), cows were moved from the respiration chamber to the free barn and tied. During this process animals may have experienced some mild stress and a small period of time that they would not be able to eat or lie/ruminate.

Due to different ratios of GS and MS in their diet **(Chapter 3)**, ruminants can harbour distinct rumen microbial communities. Bacterial communities in our study were most affected by dietary interventions **(Chapter 3)**. In the study of Zhu et al. (2017), alfa alfa hay feed increased the proportions of *Prevotella* and *Selenomonas* compared to a maize stalk diet. The differences in the microbial composition can affect the ruminant's feeding efficiency through production of VFAs as the microbes are involved in various fermentation pathways and have associations with each other (Malmuthuge and Guan, 2017). One of the strongest associations was between succinate producing, succinate utilizing and amino acid fermenting bacteria and methanogens (namely Methanomassillicoccaceae and *Methanosphaera*). In our study, bacterial families Succinivibrionaceae and Ruminococcaceae were associated with the MS diets, indicating a possible role in the lower methane emissions observed by van Gastelen et al. (2015). *Methanobrevibacter* was also positively associated with MS in the diet. Future studies should focus on mechanistic aspects of such bacteria: archaea interactions, and how this knowledge can be applied towards methane mitigation strategies.

In this thesis, maize (which is mainly starch) silage fed cows showed a neutral pH in their rumen content (Van Gastelen, 2015) (**Chapter 3**). Amylolytic bacteria are important in the rumen of animals fed concentrate diets and starch. Lactic acid is a typical product of their metabolism, however, this does not accumaulate as it is rapidly utilized by propionate producers. Propionate is a hydrogen sink and contributes to reduced methane emission. However, there are different consequences to increasing the amount of starch in the diet. It can increase the passage rate, which subsequently lead to a shift of methanogenesis to the hind gut, abolishing the effect in reducing CH_4 emissions. Also, rapid degradation of easily fermentable substrates can increase the rate of VFA production beyond the absorptive capacity, resulting in pH drop and disruption of rumen microbiota or acidosis (Hook et al., 2011). Also, limited rumination can reduce the inflow of acid neutralizing saliva, and decrease rumen motility resulting in decreased passage rate. Therefore associations of the

rumen microbiota with the rumen size, physiological changes, and passage rates as well as role of host genetics should be considered for defining strategies to rumen manipulation.

FIBRE ASSOCIATED BACTERIA

Ruminal plant fibre digestion relies on colonization and breakdown of the fibre by complex microbial communites (Weimer, 1996b). Fibre-attached bacteria capable of degrading cellulose and/or hemicellulose are pivotal to the maintenance of rumen function, and hydrolysis of plant polysaccharides into monomers which are then utilized by other rumen microbes. Only 2-31% of fibre associated bacterial communities in the rumen, however, are represented by cultivated species (Koike et al., 2003b; Creevey et al., 2014), highlighting the need to isolate these uncultured bacteria to increase our knowledge of their involvement in ruminal fibre degradation and the underlying mechanisms. In this thesis, we identified clear differences in the rumen bacterial communities associated with the rumen fibrous content and the liquid fraction **(Chapter 2),** and enriched in vitro fibre associated consortia **(Chapter 4).**

Fibrolytic bacteria include cellulose, pectin and xylan degraders while the non-fibrolytic bacteria ferment simple carbohydrates including various monosaccharides and disaccharides (Hungate, 1966a). Feeding grain or maize diets has shown to change the ruminal microbial community from fibrolytic to amylolytic (Fernando et al., 2010). This is possibly why starch or soluble carbohydrates utilizing bacteria, a.o. *Proteiniphilum* or *Bacteroides* were enriched in our study when maize fibres were used **(Chapter 4)**. Some previously uncultured rumen bacterial genera (a.o. *Pyramidobacter*, *Ruminofilibacter)* were enriched through serial transfers, with cold shock in between the sub-enrichments (SE) to avoid further fibre fragmentation that might have lead to no inoculum to transfer in the next sub-enrichment and time issues in setting up the enrichment cultures (**Chapter 4**). Of the cultured bacterial representatives obtained from rumen content known to date (Creevey et al., 2014), those associated with rumen fibre degradation are represented by only be a handful of species.

Combining a culture dependent (in vitro) approach for the isolation of fibre-associated bacteria and a culture independent approach, i.e. 16S rRNA profiling of the fibre-associated microbiota, can expand our understanding of fibre degradation in the rumen. *Ruminofilibacter xylanolyticum* S1 (belonging to the Rikenellaceae) has caught our interest to target this genus for future fibre associated studies, as this genus was dominant in our MS enrichments (**Chapter 4**), suggesting its preference for maize derived fibres as substrates. Indeed

Ruminofilibacter xylanolyticum S1 was previously identified from an anaerobic biogas plant fed with maize silage, green rye and liquid manure (Krober et al., 2009).

Many attempts have been made to cultivate bacteria from the liquid fraction, however, the use of a solid surface as an attachment matrix in order to cultivate fibre associated rumen microbes has been limited. Most probably, the main reason for this is the difficulty of this approach to enrich microorganisms from a fibrous inoculum, as the microbes often remain attached to the fibres in the inoculum. Furthermore, taking samples of rumen fibrous material requires fistulated cows, which are a limited resource due to ethical reasons as well as high costs associated with maintaining fistulated animals.

Previously, Nyonyo et al. (2014) applied an isolation strategy where blended ruminal fibrous content and the liquid fraction was inoculated on an agar- or gellan gum modified basal medium with azo-carboxymethylcellulose as the carbon source (Nyonyo et al., 2014). This study yielded 129 isolates that grouped in 6 phyla with Firmicutes as the most represented. However, 19% of the isolates grouped in unclassified taxa at family level, indicating a largely uncharacterized biodiversity mostly within the family Lachnospiraceae. The cellulose degrading populations were believed to be derived from the fibre-adherent microbiota, and carboxymethylcellulase and xylanase acitivities were measured (Nyonyo et al., 2014). The rumen microbial communities associated with fibres have been previously shown to be dominated by Firmicutes and Bacteroidetes (Koike et al., 2003b). At genus level, *Ruminococcus, Fibrobacter, Pseudobutyrivibrio,* unclassified *Lachnospiraceae, Butyrivibrio, Streptococcus, Enterococcus, Saccharofermentans* and *Prevotella* were able to degrade filter paper as a measure of cellulose degrading capacity (Nyonyo et al., 2014).

The addition of methylcellulose inhibits bacterial attachment on fibrous particles (Kim et al., 2013). Treatment with methylcellulose has been shown to cause the detachment of *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* from cellulose fibres (Kudo et al., 1987). In this thesis, methylcellulose was used to specifically detach bacteria from rumen derived fibres **(Chapter 5).** In this way specific enrichments with fibre-associated bacteria could be developed. From these enrichments a novel *Propionibacterium* specie, *P. ruminifibrarum* strain JV5^{T}, could be isolated and further characterised.

Strain JV5^T produced propionate as a major end-product from glucose fermentation. As propionate can be an effective H_2 sink for reducing ruminal methane formation, the capacity of strain JV5^T to attach to fibres was assessed.

PROPIONIBACTERIUM RUMINIFIBRARUM: ITS POTENTIAL ROLE IN FIBRE ATTACHMENT AND DEGRADATION AND METHANE MITIGATION

With the novel strain *Propionibacterium ruminifibrarum* JV5^T in hand (**Chapter 5)**, we further investigated in vitro its ability to colonise and utilize silage derived sterilized grass fibres. The metabolites produced were measured after 24 hours of incubation, and bacterial cells were quantified and visualized on the fibre over the time course of incubation.

Scanning Electron Microscopy (SEM) revealed that strain JV5^T attached to the fibres and formed micro-colonies (Fig. 1). The micro-colonies typically formed in the cavities of the fibres (Fig. 1 A and B) or around damaged spots in the fibres (Fig. 1C). This characteristic formation of micro-colonies was previously observed for *Propionibacterium jensii* strain S1 on fruit fibres present in a cannery medium (van Schalkwyk, 2003). Furthermore, the fruit fibre - *P. jensii* complex was covered with an extracellular polymer matrix (EPS), and was stable even after five months of cold storage indicating that EPS is important in biofilm formation and stabilization (van Schalkwyk, 2003).

The physiological role of EPS can be: (i) cell protection from changes in temperature, pH and osmotic pressure, (ii) cell-cell interactions and (iii) adhesion to surfaces (Ruas-Madiedo et al., 2002; Donot et al., 2012). No EPS could be observed on the outside of strain JV5^T **(Chapter 5)**, although the liquid of batch grown cells on soluble substrates was viscous (visual observation). This might be indicative of EPS production as bacterial EPS can be secreted into the media or tightly attached onto the cell wall. The viscosity of the growth medium has been speculated to be associated with EPS (Cerning, 1990). Recently, it was found with *P. freundreichii* that EPS production was affected by medium composition and fermentation conditions (Belgrano et al., 2018). *P. freudenreichii subsp. shermanii* strain JS, which belongs to the dairy group of propionibacteria, also produces large quantities of EPS (Belgrano et al., 2018).

qPCR measurements of strain JV5^T indicated that no increase in the 16S rRNA gene copy numbers on silage derived grass fibres occurred within 1 h of incubation (Fig. 2),

suggesting that strain JV5^T requires a longer time to attach to fibres before subsequently multiplying and forming micro-colonies by 24 h. After 24 h, some acetate was produced indicating that the fibres served as a carbon source as well as an attachment matrix. These preliminary findings suggest a potential role of strain JV5^T in the utilization of dietary fibres in the rumen.

In the past, there have been few in vitro studies that investigated specifically fibre attachment or adherence (Cheng et al., 1984; Shinkai and Kobayashi, 2007). It is well documented that bacteria have a close association with plant cell wall materials (McAllister et al., 1990). This has been also clearly demonstrated in the past through microbiological examinations from grain (wheat and barley) and maize particles (McAllister et al., 1990). Digestive processes require physiologically complementing bacterial species that interact and form a consortium on the surface of plant tissues. Attachment of primary colonizers (*F. succinogenes, R. albus,* and *R. flavefaciens*) is pivotal for a sequential establishment of microbial consortia. Affinity of rumen microbes for specific plant or fibre components varies (Huws et al., 2014). For example, *Selenomonas bovis* employs non-specific attachment, while *Ruminococcus amylophilus* prefers to attach to the surface of starch granules. *Butyrivibrio fibriosolvens* is known to colonize the endosperm cell wall and digests starch granules (Mc Allister et al, 1994). Initial attachment to feed particles is established via the glycocalyx and associated binding proteins of the microbes. The primary colonizers and their hydrolytic enzymes attack the insoluble substrates creating pits and releasing soluble nutrients. This attracts the secondary colonizers from the fluid fraction to further ferment soluble substrates. These secondary colonizers many times attach to the primary colonizers forming micro-colonies resulting in a biofilm that performs the fibre degradation. The microbial species change depending on the available insoluble substrate. Microbial interactions with fibrous material or fibre components have been only been associated with *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* that are known to be involved in cellulose and hemicellulose degradation (Shinkai and Kobayashi, 2007). In the study of Shinkai and Kobayashi (2007), it was demonstrated using FISH analysis that the cell distribution of *F. succinogenes* and *R. flavefaciens* also changed depending on the plant tissues used as growth substrates. Moreover, numerous genes encoding enzymes necessary for plant fibre digestion have been isolated from *F. succinogenes, R. flavefaciens* and *R. albus*. To illustrate, *F. succinogenes* possesses 31 putative cellulase encoding genes, including ten members of glycosyl hydrolase family 5 (GH5), six members of GH8, nine members of GH9,

four members of GH45, and two members of GH51 and genes encoding proteins with a wide range of annotated hemicellulolytic activities including a large number of xylanases, arabinoxylanases, mannanases, curdlanases (β-1, 3 glucanases), licheninases (β -1, 4 glucanases), and xyloglucanases. These enzymes come from a range of GH families including GH10, GH11, GH18, and GH26) (Suen et al., 2011). These three bacteria, *F. succinogenes, R. flavefaciens* and *R. albus,* are the core microbiota associated with the fibrous fraction in the rumen. These bacteria are known to attach to fibrous fractions in the rumen within 5 to 15 minutes and subsequently form a biofilm (Edwards et al., 2017). Bacterial enzymes attack plant cell walls initially, and subsequently the more recalcitrant polymers. Moreover, cross-feeding takes place of fibre breakdown intermediates and end-products that are produced by the fibre degraders (Flint et al., 2008). This suggests that attachment might not necessarily mean that the attached bacteria are primary fibre degrading bacteria. This close proximity to the fibrous fraction for fibrolytic bacteria might be due to the complexity of enzymes involved in the degradation of polymeric cellulose (Flint et al., 2008).

The ability of *P.ruminifibrarum* to attach to silage derived grass fibres suggests that this isolate could serve as a potential direct fed microbial, which could offer an effective means of increasing propionate production (Jeyanathan et al., 2014) and as such contribute to reducing enteric CH_4 emissions. Recently, there have been two studies which showed the potential of several *Propionibacterium* strains to reduce CH_4 in vivo. In the first study, a number of *Propionibacterium* strains, namely *Propionibacterium acidipropionici* P169, *P. acidipropionici* P5, and *Propionibacterium jensenii* P54 were inoculated daily before feeding, however, failed to increase propionate proportions and showed no effect of treatment on total CH_4 emissions (Vyas et al., 2014). The strains could not persist or maintain themselves in the rumen and returned to pre-treatment levels nine hours after feeding. The follow up study ofthe same research group used ruminally cannulated heifers, which were fed different *Propionibacterium* strains (Vyas et al., 2016). The strains *Propionibacterium freundreichii* T114, *Propionibacterium theonii* T159 and *P. freundreichii* T54 were administered separately in different age grouped heifers. These microbials were added daily at the time of feeding directly into the rumen. Methane yield tended to be higher with the different *Propionibacterium* strains, and the relative abundance of total propionibacteria was higher with the inoculation of *Propionibacterium* T159 relative to the control heifers. The study of Vyas et al. (2016) concluded that the inoculation of *Propionibacterium* T159 decreased the ruminal acetate proportion, whereas *Propionibacterium* T114 increased the

acetate : propionate ratio. However, inoculated strains failed to lower total CH_4 emissions possibly due to the inability of *Propionibacterium* strains to elevate ruminal propionate concentrations (Vyas et al., 2016).

In another study 16 different *Propionibacterium* strains were screened in vitro and demonstrated that the potential of propionibacteria in reducing CH_4 emission was substrate dependent, with more reduction in CH_4 with maize as substrate (Alazzeh et al., 2012). To this end, it will be interesting to investigate the potential for *Propionibacterium ruminifibrarum* $JV5^T$ to mitigate methane emissions. To conclude, propionibacteria are promising candidates for direct fed microbials, but the current literature contains varying data on their impact in mitigating methane and increasing propionate.

KNOWLEDGE GAPS AND FUTURE RESEARCH

The vast majority of research worldwide focusses on optimizing milk production in dairy cattle, and meat production in beef cattle. One of the aspects that contributes to the above is the microbial digestion of feed in the rumen. The diverse rumen microbial community adapts to a wide assortment of dietary feedstuffs but also to management strategies. Understanding rumen microbiota composition and function, as well as its adaptation to changes in rumen environmental conditions, has global consequences ranging from GHG emissions to applied animal production. Conventional knowledge of rumen microbiology was based on anaerobic culture-dependent methods. Next-generation sequencing and other molecular, culture independent techniques have revealed novel features of the rumen microbiome (McCann, 2014). Single cell genomics today allows the study of individual cells and add to our understanding of the metabolic potential of individual microrganisms within their natural environment (Hornung et al., 2018).

In order to have a fundamental understanding of rumen microbiota and its interactions with the host in ruminal methanogenesis, high-throughput sequencing technologies have characterized numerous microbial OTUs/phylotypes based on 16S rRNA gene sequences. Combination of these high-throughput data describing the rumen microbiome with classical

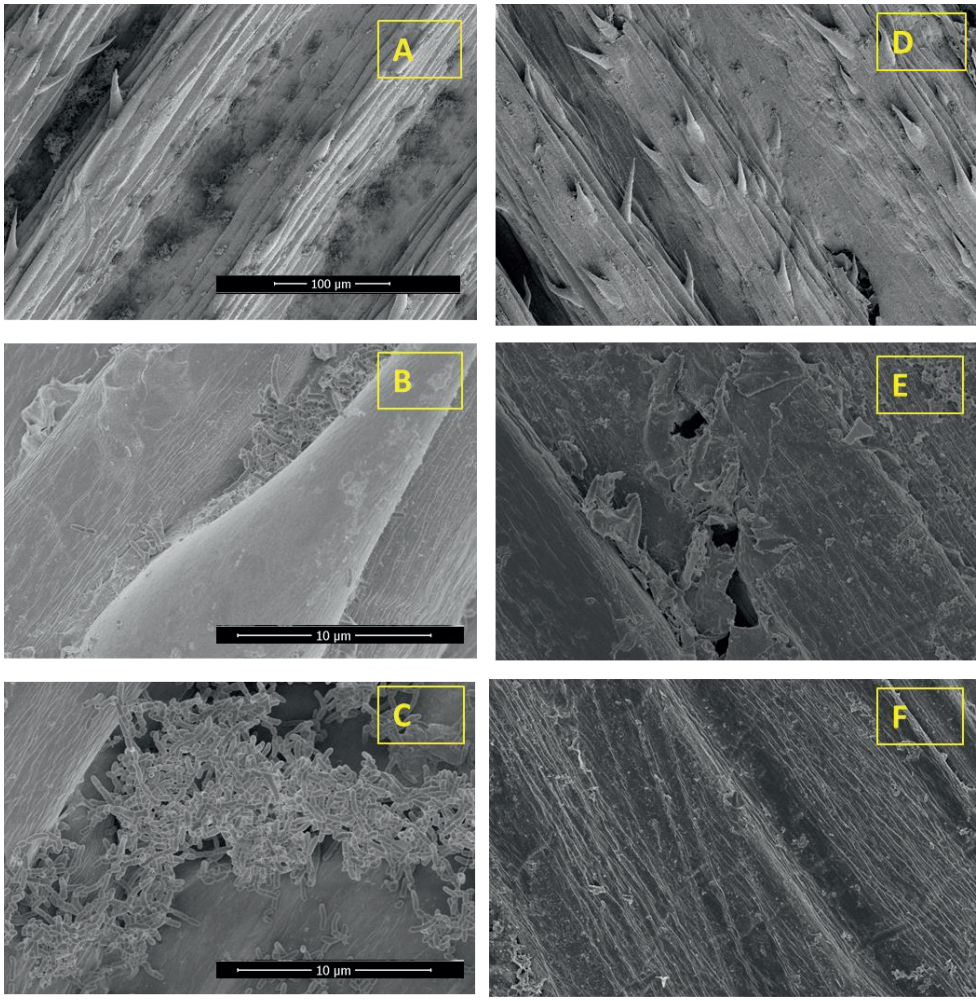

Figure 1. Scanning electron micrographs (SEM) of strain JV5^T micro-colonies attached to silage derived grass fibres after 24 hours of in vitro incubation in bicarbonate buffered mineral medium at 39ºC (A-C) and of control fibres after 24 h of in vitro incubation in the absence of JV5^T (D-F).

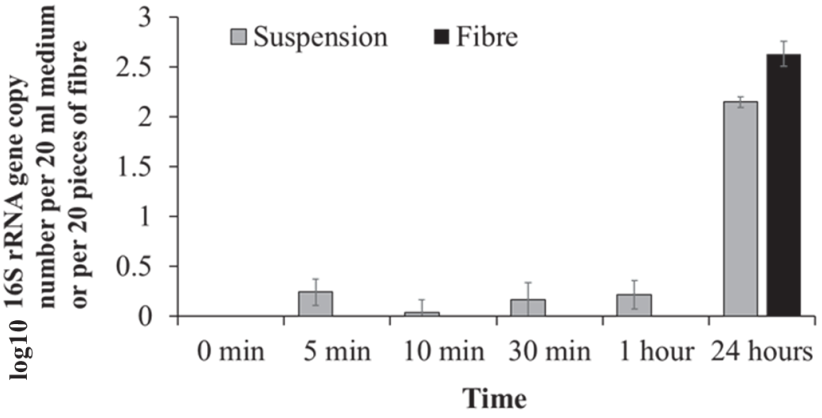

Figure 2. Quantification of Strain JV5^T in suspension and on the silage derived grass fibres. QPCR results are expressed per 20 ml of medium or 20 pieces of fibres, respectively. Error bars represent biological duplicates.

fermentations and animal performance parameters has yielded important advances and opened additional areas for further study. In addition, although dietary practices, supplements and additives that modify rumen microbiota composition for increasing feed efficiency have been identified, there is an increasing need to further understand the ecology of the rumen microbiome particularly in light of functional redundancy in the ecosystem. One way to more specifically address functionality of microbial ecosystems is through metagenomics and meta-transcriptomics. This allows to obtain genomes and make metabolic maps of the genes that are associated with methanogenesis or fibre degradation thereby providing the necessary basis for a comparative analysis at the functional level. Functionality must also be confirmed at the protein and metabolite level through meta-proteomics and meta-metabolomics.

To this end, investigations of the prokaryotic rumen metaproteome are challenged by the complexity of rumen samples, which requires specific sample preparation procedures to separate archaeal and bacterial cells from the residual matter prior to protein extraction. Moreover, humic compounds are present and interfere with the meta-proteomic workflow (Chourey et al., 2010; Heyer et al., 2013). Furthermore, such omics analysis would be most interesting to perform on the fibrous fraction as it is relatively unexplored. Recently, a functional gene array targeting fibre degrading activities in the rumen ecosystem, the Fibro-Chip, was developed (Comtet-Marre et al., 2018). Genes belonging to CAZyme families

known to contain cellulases and hemicellulases (GH5, GH9, GH10, GH11, GH43, GH48, CE1, and CE6) in the genome of the major rumen fibrolytic microorganisms including bacteria, fungi, and protozoa, were targeted. This tool can help to characterize the expression of the CAZyme genes of the rumen microbiota of the cow and help in elucidating roles of the different microorganisms in fibre digestion.

Using such omics tools, new genes could be identified encoding fibre degrading activities that can be a potential target to estimate fibre degrading capabilities. Nevertheless, it remains necessary to use an integrated approach towards assessing the importance of the different members of rumen microbiota to rumen productivity and the host animal.

The metabolism of complex carbohydrates and pathways associated with H_2 production have been recognized in gut microbiomes for efficient use of forage feeds and optimizing ruminal fermentation (Morgavi et al., 2012). The metabolism of methanogenic archaea, most of which are H_2 utilizers, is well known, and their numbers are estimated in different ruminants, rumen locations and time (Jansen and Kirs, 2008). Methane production is closely linked to the formation of H_2. Experiments such as the one described in **Chapter 3** would therefore benefit from measurements of ruminal fermentation, H_2 and CH_4 throughout the day to monitor the fermentation dynamics associated with dietary interventions, as previously performed by (van Lingen et al., 2017b). Moreover, H_2 measurements as a standard integrated tool next to the methane measurements would be beneficial.

Isolation of novel anaerobic fibre associated bacteria is relevant to obtain a deeper understanding of cow rumen physiology. In this thesis **(Chapter 5)**, we isolated *Propionibacterium ruminifibrarum* described as a potential fibre associated bacterium. Assessing the genome sequence of *P. ruminifibrarum* will help in identifying genes that encode hydrolytic enzymes for cellulose, xylan or starch degradation, as well as propionate synthesis. The efficiency for fibre degradation depends on the activity of the microbial enzymes produced by the rumen microorganisms. These enzymes include glycoside hydrolases (GH) active against the main plant structural polysaccharides (cellulose and hemicelluloses) and carbohydrate esterases (CE) which cleave polysaccharide substituents. Further growth experiments in microcosms could be started on dedicated polymeric substances, followed by RNA-seq analysis. This will provide improved in depth understanding of the metabolic capacity of the isolated novel species, and can contribute to assessing the suitability of strain $JV5^T$ to be applied as a probiotic to reduce ruminal methane.

The anaerobic fungi that are present in the rumen produce large amounts of H_2 along with CO_2, formate and acetate as metabolic end products depending on the diet. Methanogens in particular are found closely associated with fungal hyphae suggesting that fungal abundances might be related to methane emissions (Bauchop, 1981). A recent study by (Kittelmann et al., 2014) reported no difference in fungal community relative to methane emission. In contrast, a meta-analysis revealed that decreasing fungal abundance was associated with lower CH_4 emissions (Newbold et al., 2015). Therefore, it will be interesting to further study anaerobic fungi not only in terms of their role in fibre degradation, but also for their role in ruminal methane production (Edwards et al., 2017). Additionally, anaerobic fungi are known for fibre degradation as well, and therefore cultivating fungi and understanding their physiology is valuable and needs further attention.

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Thesis summary

Enteric fermentation in ruminants produces methane (CH_4) which is considered as a greenhouse gas (GHG). CH_4 that comes from enteric and manure fermentation, accounts for 106 Tg CH_4 per year from anthropogenic sources. Rumen micro-organisms (bacteria, anaerobic fungi and protozoa) break down complex compounds in the feed by hydrolysis to produce monomers which are fermented to volatile fatty acids (VFA), mainly acetate, propionate and butyrate in addition to other products like formate, hydrogen (H_2) and carbon dioxide (CO_2). Methanogenic archaea in the rumen utilize H_2 and CO_2 and other methyl containing compounds to produce CH_4. To increase understanding of ruminal methanogenesis, it is important to gain information on the initial colonizers of fibrous dietary components that are crucial for the attachment of the fibre associated microbiota to the feed and fibre degradation in the rumen. CH_4 is the end-product of feed degradation and that is not used by the ruminant itself but mainly eructated into the atmosphere. As this is posing a negative impact on the climate, a broad range of strategies like use of direct fed microbial and inhibitors of CH_4 production have been assessed with the aim of CH_4 mitigation. The main objective of this PhD project was to apply integrated cultivation based and molecular approaches to assess the microbial community composition and fibre attachment in relation to CH_4 emission.

In **Chapter 1** of the thesis, I summarize the currently available background knowledge on the role of microbiota in rumen feed degradation with a particular emphasis on CH_4 is presented. Furthermore, the composition and function of the different microbial communities associated with rumen fluid (RF) and the fibrous fraction (FC), the pros and the cons of culture dependent and culture independent methods are discussed. Lastly the current understanding of experimental, dietary and animal associated factors that influence rumen microbiota, fermentation patterns and enteric methanogenesis are reviewed. From the experimental factors affecting the rumen microbial composition discussed, **Chapter 2** addresses the potential bias of DNA extraction methods on the picture we can obtain of RF- and FC-associated microbial communities, using next generation sequencing based profiling of the bacterial, archaeal and fungal composition (16S rRNA gene and fungal ITS region). In addition, bacterial and archaeal numbers were assessed by 16S rRNA based qPCR. Most importantly, DNA extraction methods have an impact on the outcome of the downstream microbial community analysis, resulting in differences in absolute abundance, and relative abundances of specific community members. DNA extracted using the PBB method resulted in higher relative abundance of Ruminococcaceae than the FDSS method, whereas relative

abundance of Fibrobacteraceae was lower compared to the RBB method. Whilst the effect of DNA extraction method was limited compared to that of rumen fraction, differences due to both DNA extraction method and fraction were observed for certain taxa.

Following this experiment, in **Chapter 3** we explore the rumen microbiome in terms of its bacterial and archaeal composition and concentrations, in order to characterize the communities associated with different ratios of grass and maize silage rations diets and identify key microbial players associated with CH_4 measurements previously reported by van Gastelen et al. (2015). As these analyses focused on rumen fluid, anaerobic fungi were not measured. The changes in the rumen microbiota in response to dietary treatments having different grass/maize silage ratios (GS100, GS67, GS33, and GS0 wherein GS100 indicates 100% grass silage) after 10 and 17 days of feeding were assessed. The bacterial and archaeal composition changes were used to help understand ruminal VFA profiles and CH_4 measurements. In terms of ruminal VFA, no significant diet effects were found but the molar proportions of isovalerate were affected by time, being lower on day 17 than day 10. Diet affected bacterial concentrations, which were lower for the GS0 diet compared with the other three diets. There was no diet effect on archaeal concentrations. Bacterial and archaeal concentrations significantly increased from day 10 to day 17. This observed increase in bacterial concentrations was suggestive of an increase in fermentation, while the biological significance of increased archaeal concentrations with time could not be elucidated, as CH_4 emissions were only measured from days 12 to 17 in the study of van Gastelen et al. (2015). Several bacterial and archaeal genera could be associated with diet, but not with time. The bacterial families Succinivibrionaceae and Ruminococcaceae were associated with the maize silage diets, indicating their role in the lower CH_4 emissions observed before (van Gastelen et al., 2015).

Another research objective of this thesis was to explore the fibre associated microbiota as many are still uncultured and unidentified. To this end, in **Chapter 4** rumen fibre associated microbes were enriched from grass silage (GS) or maize silage (MS) fibres recovered from the bovine rumen. Enrichments used pre-autoclaved ruminal silage fibres (GS or MS, respectively, for each isolation source) as an attachment matrix in bottles containing different fibre related components (cellulose, amylopectin or xylan). All enrichments were incubated at 39 ºC for a 14 day period, with storage at 4 ºC for 3-4 weeks between transfers. Although overall activity in all the enrichments was low, fibre-degrading bacteria were

considered to be enriched as visual inspection of PE bottles from both the MS and GS enrichment series showed initial fragmentation or breaking up of the fibre structure. Sequencing analysis revealed that members of the genus *Ruminofilibacter* dominated all the MS SE5 bottles, whereas all GS SE5 bottles contained a more diverse community predominated by members of *Prevotella 1* and *Bacteroides*). The in vitro experimental approach of biweekly serial transfer seems likely to be important to enrich for fibre associated bacteria that are not favoured by shorter incubation times, such as e.g. *Ruminofilibacter*.

Chapter 5 describes a novel propionate producing bacterium, *Propionibacterium ruminifibrarum* strain JV5^T, isolated from the rumen fibrous content of a Holstein Friesian dairy cow. Characterization of this strain at genomic, biochemical and physiological level provided insights into the metabolic capacity of *Propionibacterium ruminifibrarum*. This species was able to utilize several sugars and sugar alcohols D-adonitol, galactose, glucose, inositol, DL-lactate, mannose, meso-erythritol, ribose and sorbitol mainly converting them to propionate and acetate, and succinate and/or formate in some cases. Furthermore, the ability of strain JV5^T to degrade representative plant carbon sources, i.e. cellulose, xylan or starch, was tested and we observed that strain JV5^T could not directly use plant polymeric components, but probably can associate with fibres to use the compounds that are released by primary degraders.

Finally, **Chapter 6** provides an integrated overview and discussion of results obtained in the research described in this thesis as well as findings from other studies. In this context, preliminary results regarding the ability of the novel isolate strain JV5^T to attach onto silage derived grass fibres are presented and discussed in light of the potential for this isolate to be developed into a probiotic feed supplement. In addition, this chapter provides future directions of research on rumen microbial management to mitigate CH_4 and improve ruminal fermentation.

Appendices

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With this, I thank you all once again and hope we all keep in touch in future.

About the author

Jueeli D. Vaidya was born in Pune, India brought up in Mumbai, India. After completing her secondary education, she received her Bachelor's degree in Life Sciences at St. Xavier's College, Mumbai, India in July 2010. Following month August 2010 she moved to the Netherlands for her Master degree education. In 2012 she completed her Master's degree in Cellular and Molecular Biotechnology, after which she started as a PhD candidate at the laboratory of Microbiology under the supervision of Prof. Hauke Smidt. She will defend her thesis in November 2018.

List of publications

1. Vaidya JD, van den Bogert B, Edwards JE, Boekhorst J, van Gastelen S, Saccenti E, Plugge, CM and Smidt H (2018) The Effect of DNA extraction methods on observed microbial communities from fibrous and liquid rumen fractions of dairy Cows. Front. Microbiol. 9:92. doi: 10.3389/fmicb.2018.00092
2. Van Lingen H, Edwards JE, Vaidya JD, van Gastelen S, van den Bogert B, Saccenti E, Bannink A, Smidt H, Plugge C & Dijkstra J (2017) Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. Frontiers in Microbiology 8. doi: 10.3389/fmicb.2017.00425

3. Vaidya JD, Van Gastelen S, Smidt H, Plugge CM and Edwards JE (2018) Characterization of dairy cow rumen bacterial and archaeal communities associated with grass silage and maize silage based diets (submitted)

4. Vaidya JD, Hornung BVH, Smidt H, Edwards JE and Plugge CM (2018) Characterization of *Propionibacterium ruminifibrarum* sp. nov., isolated from the cow rumen fibrous content (submitted).

Overview of completed training activities

Discipline specific courses/meetings	Graduate school/Institute	Year
Ruminomics summer school, Piacenza, Italy	Rowett-INRA	2012
4th TI Food and Nutrition Annual Conference	ACTA Research Centre, VU Amsterdam	2013
Ruminomics one day workshop, Aberdeen, UK	Rowett-INRA	2014
Rowett-INRA Gut Microbiology conference	Rowett-INRA	2014
TI Food and Nutrition Annual Conference	Nutricia Research Danone	2014
16th Gut day symposium	Gut Flora foundation	2014
Scientific Spring meeting KNVM	KNVM	2014
Arb	Wageningen University / Ribocon	2014
University Konstanz research excursion	Wageningen University/Konstanz University	2016
International conference on Biogas Microbiology (ICBM-3) conference	Wageningen University	2017
General courses		
VLAG PhD week	VLAG	2012
Writing and presenting a scientific paper	WGS	2013
Project and time management	WGS	2013
Scientific writing	WGS	2013
Optionals		
VLAG research proposal	VLAG	2012
PhD study tour	Microbiology dept, WUR	2013
Weekly group meetings	Microbiology dept, WUR	2012-2017
TIFN group meetings	TIFN	2012-2017

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