Sveriges lantbruksuniversitet
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Department of Animal Nutrition and Management

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Abbreviations

GHG	Greenhouse Gas
PCR	Polymerase Chain Reaction
SAO	Syntrophic acetate oxidating (bacteria)
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
TRF	Terminal restriction fragment
VFA	Volatile fatty acids

Abstract

The newborn calf that has not been fed colostrum lacks immune defense and ruminal microorganisms needed for digestion of polysaccharides from plants and cellulose. Establishment of a gastrointestinal microbiota is vital. This study aimed to shed light on the establishment of the methanogenic and acetogenic community in young calves.

Nine naturally born bull calves at the age of 2 or 7 days were used for collecting fecal samples and intestinal tissue and digesta from various segments of the gut. In addition, 31 heifer calves of various ages (2 days - 6 months) were used only for fecal sampling. DNA was isolated from the samples and polymerase chain reaction (PCR) used to amplify the methyl coenzyme-M reductase (mcrA) genes for detection of methanogens and the formyltetrahydrofolate synthetize gene (FHS) for the acetogens.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis was performed on PCR products generated from methanogens and acetogens. T-RFLP does not give any data about which species that are present in the microbial community. In order to get more information on species level, clone libraries were created from representative samples from both methanogens and acetogens. Fecal samples from one 6 months old and one 2 weeks old calf were chosen for generation of a library for the methanogens whereas for the acetogens, four libraries were created from a fecal sample from a 6 month old calf and feces, rumen- and cecum digesta from a 2 days old calf.

Gel electrophoresis of the PCR confirmed presence of methanogens in 21 faecal samples from calves 14 days of age and older. Not all of the samples from 14 and 28 days old calves were positive for methanogens. However, from 6 weeks and older the presence of methanogens seemed to be established and all samples contained methanogens.

Analysis of the acetogens gave positive results for both faecal, GI digesta and tissue samples. Interestingly the tissue that contained acetogens came mainly from rumen and abomasum samples from 7 days old calves. This may indicate that the acetogens primarily adhere to the tissue of those regions but pass through the rest of the GIT and get picked up in digesta samples from other sites.

T-RFLP analysis on the methanogenic community composition revealed a shift according to age of the calves. The PCA analysis of the T-RFLP data revealed two clusters of samples from calves at 2-8-weeks age and a third cluster of samples from 6-months old calves. Among the acetogenic samples there was a large spread among 2-day calves whereas the 6-month old calves clustered more closely in the PCA plot. Which indicates that the within group similarity in the microbiota increases as the calves get older.

The results from this study indicate that the methanogens are not present in young animals. Acetogens were found in intestinal and faeces samples from calves 2 days and older. The method could not confirm whether the acetogens were homoacetogenic or not.

1 Introduction

The newborn calf is subjected to many challenges as it enters the world. Because of its lack of immune defense and ruminal microorganisms needed for digestion of polysaccharides from plants and cellulose, ingestion of colostrum and establishment of a gastrointestinal microbiota is important for its survival (Hammon & Blum 1998). The documentation of the microbial gastrointestinal development of the calf has been limited but is making breakthrough thanks to new molecular techniques (Malmuthuge et al. 2015).

The early development of the microbiota in ruminants have previously been investigated, primarily in lambs (Fonty et al. 1987; Morvan et al. 1994) and the focus has been mainly the microbiota in rumen (Li et al. 2012; Leahy et al. 2010). The forestomach of ruminants does not produce digestive enzymes but in adult ruminants they contain an extremely large number of microorganisms that degrade organic matter in the anaerobic environment. The microbiota is fermenting cellulose and hemicellulose and provides the animal with more than two thirds of its daily energy requirement, mostly by volatile fatty acids (VFA). They are also an important source of protein as the dead cells are broken down and absorbed in the small intestine. However in the calf the forestomach is poorly developed until 2-3 weeks after birth and the calf gets its energy from digesting the cow's milk in the abomasum (Sjaastad et al. 2010). The microbial population consists of bacteria, archaea, fungi and protozoa (Malmuthuge et al. 2015). Most of the archaea are methanogens belonging to the phylum Euryarchaeota. They utilize hydrogen (H_2) and carbon dioxide (CO_2) from bacterial fermentation to produce methane. The most common genera found in ruminants are *Methanobacterium* and *Metanobrevibacter* (Kim et al. 2011).

Methane is an important green-house gas (GHG); it is 21 times more potent than CO_2 and has a big impact on global warming (Buddle et al. 2011). Ruminants kept for meat and milk production contribute to a prominent part of methane emissions from human activity (Martin et al. 2010). Methane production in the rumen occurs mainly by methanogens and it affects the animal that loses 2-12 % gross energy (Johnson & Johnson 1995). The methanogens have been found in the rumen of lambs as early as 1-3 days after birth (Skillman et al. 2004).

Acetogens are a diverse functional group of microorganisms who also utilize H_2 and CO_2, in the process of reductive acetogenesis with acetate as end product (Joblin 1999). In contrast to methane, acetate can be utilized as a nutrient by ruminants. However, in the hydrogen reducing process, acetogens is often outcompeted by methanogens (Morvan et al. 1994). More recent studies have found that acetogenic microorganisms encoding functions of the Wood-Ljungdahl pathway, in which acetate is formed, do not always produce acetate. They are so called non-

homoacetogens. This makes it harder to know if the acetogens detected in a sample actually produce acetogen or only carry the gene (Henderson et al. 2010a). In newly born lambs acetogens are the predominant hydrogen utilizer but after a few days they are replaced by methanogens (Lopez et al. 1999). Acetogens have been seen as a possible solution to the environmental problems caused by GHG from methanogens. By replacing methanogens by acetogens in the process of VFA formation the expectation is to reduce methane emission (Lopez et al. 1999; Gagen et al. 2010; Morvan et al. 1996).

1.1 Methods for analyzing microbial communities

To make a valid analysis of the ruminal microbiota, preparation and selection of methods is important and small changes might have large impact on the results. Techniques such as polymerase chain reaction (PCR) and terminal restriction fragment length polymorphism (T-RFLP) makes it possible to isolate DNA and map microbiological communities present in different environments (Matsui et al. 2008). Culturing bacteria is not yet possible for many of the bacteria in ruminants and pure-culture isolates seem to give an incomplete picture of the authentic communities (Whitford et al. 1998; Leaphart & Lovell 2001).

1.1.1 DNA marker genes

When investigating the presence of microorganisms from specific functional groups, PCR is a powerful tool. It amplifies DNA by repeated heating and cooling cycles of a premix consisting of DNA template, DNA polymerase, and forward and reverse primers. The primers are specific and complementary for the target sequence (Atlas & Steffan 1991).

Today's research on ruminant microbiota is quite limited in comparison with for instance the biogas field that is engaged in defining microbiological communities with efficient methane producers (Luton et al. 2002; Müller et al. 2013). Consequently, some methods can be borrowed and used in an agricultural context.

The 16S ribosomal RNA gene is the most common gene used in bacterial sequencing. However, it is not specific for either methanogens or acetogens. To make analysis faster, specific primers that target the genes coding the wanted trait is preferable (Luton et al. 2002; Sirohi et al. 2013).

The methyl coenzyme-M reductase (mcrA) gene is specific for methanogens and suitable as a marker gene when looking into methanogen diversity in ruminants gastrointestinal tract (GIT) (Sirohi et al. 2013). This marker gene has been verified by Luton and coworkers who tested it as a marker gene on material from landfill, and found a great diversity of methanogens from all five recognized orders (Luton et al. 2002).

When looking into the world of acetogens there is still a lot to uncover. Since acetogens are such a sprawling group it is tricky to find a suitable marker gene (Matsui et al. 2008). In an attempt to investigate the genetics of Syntrophic acetate oxidizing bacteria SAOB, Müller and coworkers created a primer pair targeting the formyltetrahydrofolate synthase (FTHFS) gene by

comparing genes from a number of acetogens and non-acetogens and identified conserved

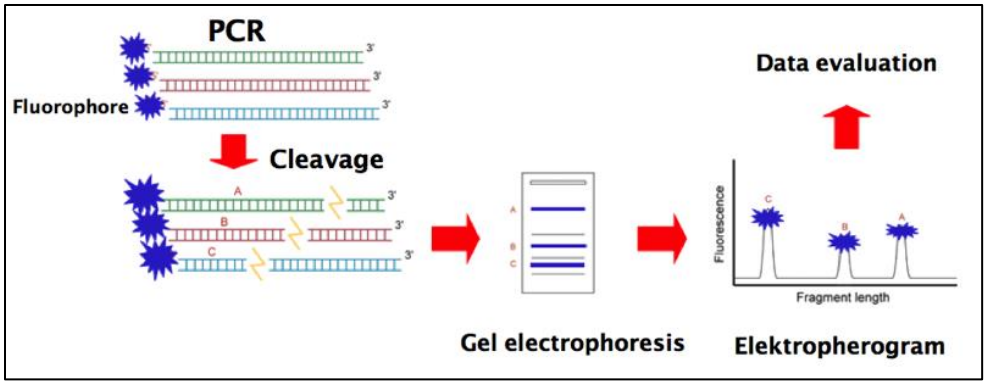

Figure 1. Schematic picture of T-RFLP procedure.

stretches that they used for designing primers targeting acetogens. The study also discovered that acetogens when growing in syntrophy with methanogens, can reverse the acetate production in the Wood-Ljungdahl pathway and produce H_2 and CO_2 instead (Müller et al. 2013).

1.1.2 T-RFLP

Terminal-restriction fragment length polymorphism (T-RFLP) is a method for fingerprinting microbiological communities after PCR amplification. It has proven to be an adequate, high-throughput, culture-independent method for mapping the microbiological community structure in various environments (Hartmann & Widmer 2008). It works according to the principle that a fluorescent primer labels the PCR product, which is then digested by a restriction enzyme. The terminal labelled ends are detected during electrophoresis by an automated sequencer and the fragment sizes of different species create heterogeneous terminal fragment length (TRF') profiles. By registration of the intensity of the fluorescence a relative abundance of the TRF's can be calculated dividing the area of each peak by the total peak area in the sample. The raw T-RFLP data are visualized in an electropherogram, which can be compared visually with other samples (figure 1) (Dicksved 2008). There is however risks of misinterpretation and biases in the T-RFLP depending on contamination and technical settings (Hartmann & Widmer 2008).

Since T-RFLP does not give any data about which species that are present in the microbial community a clone library can be created in order to give more information on species level (Matsui et al. 2008).

This study aimed to shed light on the establishment of the methanogenic and acetogenic community in young calves. Mucosa and digesta samples from multiple sites along the GIT of two and seven days old calves, as well as fecal samples from calves up to six month of age were tested for presence and composition of the methanogenic and acetogenic community.

2 Materials and methods

2.1 Animal handling and sampling

Uppsala animal ethics committee approved all the experimental animal procedures used in this study, reference number C44/14.

This thesis is a part of a project called "*The importance of the colostrum feeding routine for the development of the microbiota in the gastrointestinal and respiratory tract in newborn dairy calves".* The samples were collected during October and November 2015. Nine naturally born bull calves from dairy cows were used for collecting GIT and fecal samples. In addition, 31 heifer calves of various ages (2 days - 6 months) were used only for fecal sampling. The calves used for GIT sampling were, as part of the project, prevented suckling from their mothers and instead given their first meal of colostrum through bottle or oesophageal tube within 4 hours after birth. When fed the first meal some of the calves were moved to individual pens in another part of the stable and one group were allowed to stay with their mother and suckle for 24 h. The calves that were moved were fed milk from a teat bucket until euthanasia at maximum 7 days of age. The different treatments were not taken into account in this study due to few replicates for each treatment.

Five calves were euthanized with pentobarbital at 2 days and four at 7 days of age. Tissue and digesta samples were taken from rumen; abomasum, duodenum (at plica duodenocolica), ileum (at plica ileocecalis), cecum and colon (at plica duodenocolica) in all animals after the GIT had been separated from the animal. Fecal samples were taken from these calves before euthanasia.

Digesta samples were collected directly from the GI-site after it had been cut open with sterile scissors, using either sterile Pasteur pipettes or inoculum loops, depending on the texture. The mucosa samples were collected by attaching the tissue to sterile steel plates followed by a rinsing step with sterile saline solution and subsequently mucus was collected using a scalpel.

In addition, fecal samples were collected from rectum of heifer calves at day 2, 7, 14, 28, 6 weeks, 8 weeks and 6 months. All calves were fed milk until 6 weeks of age and by 8 weeks they were fed with silage, hay and concentrate.

All test tubes with samples were kept on ice before put in freezer at - 80 °C.

2.1.1 DNA-extraction

DNA was extracted from GIT and fecal samples using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The extraction procedure followed the standard protocol for isolation of DNA from stool for pathogen detection with a few optimizing modifications. The 2 ml micro-centrifuge tubes were replaced with bead beating tubes with 0,1 mm Zirconium/silica beads and samples were homogenized in a super homogenizer (Precellys, Bertin Corp. USA) at 8000 RPM for 2 * 1 min and kept on ice for 5 min in-between. The volumes of reagents and lysate were doubled in the downstream steps in order to optimize yield from extraction. The samples were stored at -20 °C until PCR.

2.1.2 PCR

PCR was used to amplify the mcrA genes for detection of methanogens and formyltetrahydrofolate synthetase (FHS) for the acetogens. The PCRs was performed using DreamTaq PCR chemistry (ThermoFisher Scientific, Waltham, USA). For methanogen detection and amplification the mcrA-primers (Table 1) were used (Luton et al. 2002). For the acetogens, the selected primer pair was 3-SAOfhs (Table 1), developed by Müller and co-workers for detecting the formyltetrahydrofolate synthetase (fhs) gene of syntrophic acetate oxidating bacteria (SAO) (Müller et al. 2013; Matsui et al. 2008). In both primer pairs the forward primer was fluorescently marked with 6-Fam. The 3-SAOfhs primer pair was designed to have a greater range of detection than previous published primers, which missed several known acetogens (Anna Schnürer, personal communication). Each PCR run was complemented with a negative control and a positive PCR control. The negative control was dH_2O and the positive was for methanogens an approved positive sample of rumen liquid and for acetogens a co-culture of *Tepidaerobacter acetatoxydans, Clostridium ultunese, Syntrophaceticus schinkii* and *Methanoculleus bourgensis* where the DNA had been purified.

The McrA genes was amplified using the following PCR conditions: initial denaturation at 95 °C for 5 minutes followed by 40 cycles of 94 °C for 30s, 55°C for 45 s and 72 °C for 60 s. The PCR was finalized by an extension at 72 °C for 10 min. The SAOfhs genes was amplified under the following PCR conditions: initial denaturation at 94 °C for 5 min, followed by 11 cycles of 60 s at 94 °C, 60 s at 63 °C (with a degradation of 1 °C for every cycle until 53 °C was reached) and 60 s at 68 °C. Then the program continued with 30 cycles of 60 s at 94 °C, 53 and 68 °C followed by a final elongation for 20 min at 68 °C (Müller et al. 2013). The PCR products where confirmed by gel electrophoresis in 1% agarose gel and 1kb DNA-ladder.

Table 1. PCR primers used in the project and their sequences.

	Primer name	Primer sequence	Reference
Acetogens	3-SAOfhs-fw	CCNACNCCNGCHGGNGARGGNAA	
	3-SAO-rev	ATRTTNGCRAADGGNCCNCCRTG	Müller *et al* 2013
Methanogens	mcrA-fw	GGTGGTGTMGGATCA-CACARTAYGCWACAGC	Luton *et al* 2002
	mcrA-rev	TTCATTGCRTAGTWGRTAGTT	
Clone library	M13	GTAAAACGACGGCCAGT	ThermoFisher
	M13-RV	CAGGAAACAGCTATGAC	Scientific, Waltham, USA

2.1.3 T-RFLP

T-RFLP analysis was performed on PCR products generated from methanogens and acetogens. For the methanogens, PCR products were digested for two hours in 37°C with the restriction

enzyme BMVAI and for the acetogens, AluI was used. The cleaved product where diluted 10-fold and distributed to Uppsala Genome Center for T-RFLP analysis in an ABI3730XL DNA Analyzer (Applied Biosystems) using MapMarker® as internal size standard. The generated T-RFLP profiles were visualized by Peak Scanner Software v1.0 (Applied Biosystems), and exported to Microsoft Excel, where the relative amount of the peaks was calculated and peaks with an abundance of less than 2% were excluded. The remaining peak values where rounded to nearest integer and a data matrix was created in Microsoft Access and exported back to Excel. Alignment of the peak data that where adjusted manually in order to correct for peaks that by error separated into different TRFs. In addition, to reduce complexity in data, all peaks only present in three or less samples were deleted.

2.1.4 Cloning and sequencing

Clone libraries were created from six representative samples from both methanogens and acetogens. Fecal samples from one 6 months old and one 2 weeks old calf were chosen for generation of a library for the methanogens whereas for the acetogens, four libraries were created from a fecal sample from a 6 month old calf and feces, rumen- and cecum digesta from a 2 days old calf.

After PCR and control of the samples on 1% agarose gel, the amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Next the PCR products were cloned into TOPO® TA Cloning vectors (ThermoFisher Scientific, Waltham, USA) which were transformed into competent *Escherichia coli* cells. The bacteria were plated and after incubation 10 colonies per sample, i.e. "transformants" were selected PCR amplified using cloning vector primers M13F and M13R (Table 1). The PCR-program was: annealing at 94ºC for 3 min, 35 cycles of 94 ºC 40 s, 55 ºC 40 s and 72 ºC 60 s, extension at 72 ºC for 7 min. The product was sent to Uppsala Genome Centre for sequencing. The chromatogram file generated from the sequence analysis data was analyzed by FinchTV (Geospiza inc., Seattle, USA) and the sequences were matched against known bacterial sequences in the Genbank database using BLAST.

2.1.5 Statistical analysis

The statistical analysis was made in statistical software (PAST 3.0), (Hammer & Harper 2005) using principal component analysis (PCA) to identify samples with similar TRF profiles.

3 Results and discussion

3.1 Methanogens

Gel electrophoresis of the PCR confirmed presence of methanogens in 21 faecal samples from calves 14 days of age and older, see table 2. Not all of the samples from 14 and 28 days old calves were positive for methanogens. However, from 6 weeks and older the presence of methanogens seemed to be established and all samples contained methanogens.

Table 2. Results from gel electrophoresis of methanogenic PCR products. Numbers in brackets is total number of analyzed samples and without brackets is the number of positive samples.

	Age						
Methanogen samples	2 days	7 days	14 days	28 days	6 weeks	8 weeks	6 months
Rumen tissue	0 (5)	0 (4)	-	-	-	-	-
Rumen digesta	0 (4)	0 (3)	-	-	-	-	-
Abomasum tissue	0 (5)	0 (3)	-	-	-	-	-
Abomasum digesta	0 (3)	0 (3)	-	-	-	-	-
Duodenum tissue	0 (5)	0 (4)	-	-	-	-	-
Duodenum digesta	0 (5)	0 (3)	-	-	-	-	-
Ileum tissue	0 (5)	0 (4)	-	-	-	-	-
Ileum digesta	0 (4)	-	-	-	-	-	-
Cecum tissue	0 (5)	0 (4)	-	-	-	-	-
Cecum digesta	0 (3)	0 (3)	-	-	-	-	-
Colon tissue	0 (5)	0 (4)	-	-	-	-	-
Colon digesta	0 (4)	0 (1)	-	-	-	-	-
Faeces	0 (12)	0 (8)	4 (6)	1 (2)	2 (2)	6 (6)	8 (8)

No detection of methanogens in neither intestinal nor faecal samples from calves younger than 2 weeks was confirmed. This does not necessarily mean that methanogens are not present before

two weeks of age, but their occurrence might be too low to be detected by the methods used in this study. Alternatively, the primer did not pick up the species occurring in the samples, but that is quite unlikely. Other studies have shown presence of cellulolytic bacteria and other organisms involved in rumen functions of adult animals in 1 day old calves (Fonty et al. 1987; Jami et al. 2013; Rey et al. 2014). In lambs methanogens have been detected at day 1-3 after birth (Skillman et al. 2004). Lamb and calves are of course of different species and probably have species-specific GI characteristics although they are both ruminants, which explain why we could not detect any methanogens in the younger calves. The increase of positive samples from 14 days and forward is in line with the result from Rey and co-workers who noted in their trial that rumen bacterial composition changed at day 15 and a lot of the earlier detected families declined or were

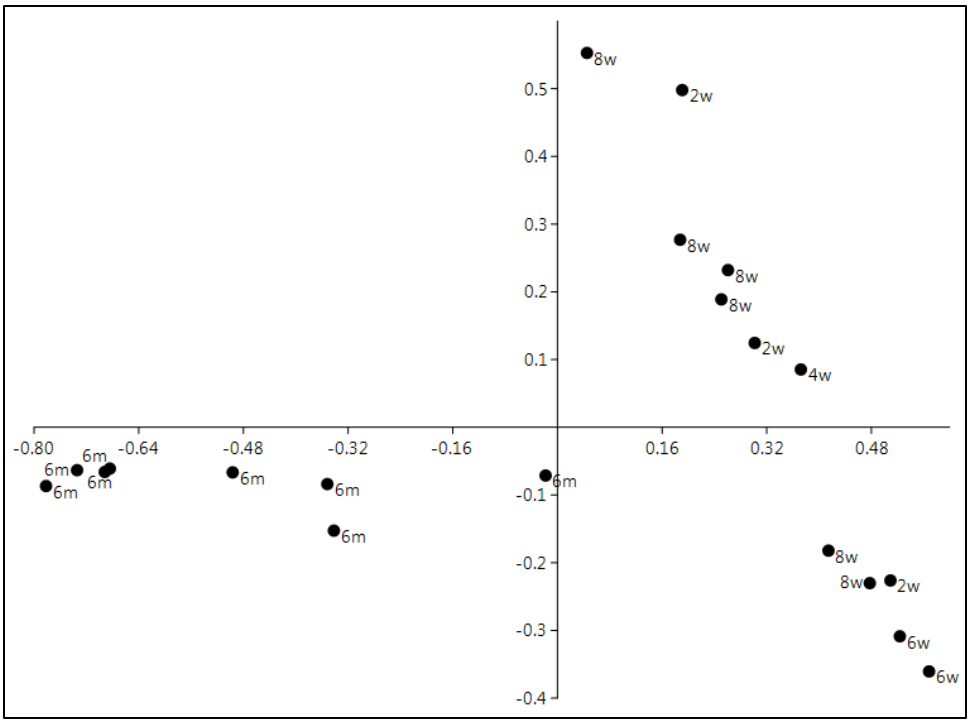

Figure 2. PCA plot showing clustering of samples according to their TRF profiles. The data was generated from faecal samples of 2 weeks (2w) - 6 months (6m) old calves.

no longer detectable (Rey et al. 2014). Morvan et al. found methanogens by culture dependent analysis on samples from lambs at 1 week of age which is comparable to our study (Morvan et al. 1994).

There seem to be a remarkable change between facultative and obligate anaerobic bacteria at about 2 days of age, where the obligate anaerobes increase and the facultative anaerobe bacteria decrease (Jami et al. 2013; Morvan et al. 1994). T-RFLP analysis on the methanogenic community composition revealed a shift according to age of the calves. The PCA analysis of the T-RFLP data revealed two clusters of samples from calves at 2-8-weeks age and a third cluster of samples from 6-months old calves, see figure 2. The 6 months' calves seem to cluster vertically to

the left and the younger calves clustered in a slanting line on the right with a break in the middle that could not be explained by age or diet. The clusters of the 2-8-week calves did not show any clear within-group pattern. The shift in methanogenic community structure was probably dependent on the transition from milk to solid feed, which starts at 6 weeks. At 8 weeks the calves were weaned and it has been documented that feed affect the rumen microbiota (Jami et al. 2013; Kocherginskaya et al. 2001; Lukás et al. 2007). At 6 months' age the calves had been on a uniform steady diet for a while which might explain the conformity of the methanogenic community. The break in the line of the 2 weeks - 8 weeks old calves is difficult to explain. It is possible that it has to do with some parameters that we did not accounted for in this study or it might be related to individual differences.

Visualizing the methanogen TRF's by stacked columns showed a clear shift in individual methanogen communities between 2-6 week (n=6), 8-week calves (n=6) and 6-month old calves (n=8). Especially 5 TRF's were distinguishing from the rest; 51, 144, 375, 471 and 491. TRF 471 was the only TRF that occurred in almost all (18/20) samples. It was more abundant in the younger calves but showed a clear decline in the older ones from an average of 68 % of total

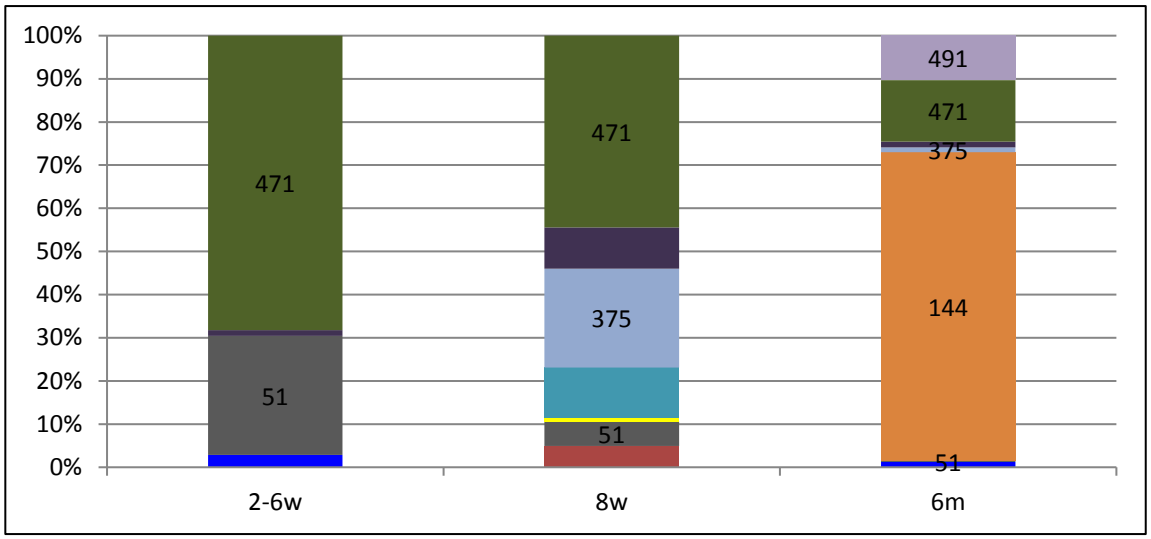

Figure 3. Bar plots of the average methanogenic TRF profile from 2-6 weeks, 8 weeks and 6 months old calves. Different colours represent TRFs, where the size of the bar represent relative abundance. The numbers associated with the bars indicate TRF sizes.

TRF-profile in calves of age 2-6-week, to 48% in 8-weeks old calves, to 19 % in 6-months old calves, figure 3. TRF 51 had a quite high abundance in 2-6 weeks old calves, but occurred in only two 8-week and one 6-month old calf at a low abundance. TRF 144 and 491 was present in all of the 6-month calves but none of the younger ones. The average composition of methanogens within different age groups is shown in figure 3.

Analysis of the sequence data from the clone libraries of the methanogen PCR products showed similar results as TRF data with different species composition between the 6-month and the 2-week old calf. The sequences from the library generated from the 6-month old calf were mostly matched with *Methanocorpusculum labreanum* (88% homology) whereas from the 2-

week old calf, they matched with *Methanobrevibacter smithii* (98%) and with the mcrA-gene from *Methanobrevibacter sp.* D5 96 %. The low score for the 6-month calf might devolve upon that the sample contain a new subspecies not yet documented in BLAST. The expectations would be to find species from one of the more common genera; *Methanobrevibacter*, *Methanomicrobium, Methanobacterium,* or *Methanosarcina* (Whitford et al. 2001; Jarvis et al. 2000). The clone libraries were based on samples from only one individual per age group. This might entail that a non-representative calf was selected. The number of transformants analyzed was limited which can influence the results. The TRF's could have been connected to their corresponding species through research in databases or by running T-RFLP on the transformants that got sequenced. This did however not fit inside the frame for this project.

Table 3. Results from sequence analysis from methanogenic clone libraries.

	Results from BLAST	
Sample animal	Methanogens	Homology (%)
6 month calf faeces	Methanocorpusculum labreanum Z, complete genome	88
2 week calf faeces	Methanobrevibacter smithii ATCC 35061 , complete genome	98
	Methanobrevibacter sp. D5, mcrA gene	96

3.2 Acetogens

Analysis of the acetogens gave positive results for both faecal, GI digesta and tissue samples (Table 4). Interestingly the tissue that contained acetogens came mainly from rumen and abomasum samples from 7 days old calves. This may indicate that the acetogens primarily adhere to the tissue of those regions but pass through the rest of the GIT and get picked up in digesta samples from other sites. According to earlier cultivation based studies the detection of rumen acetogens may range from undetectable to $1{,}2 * 10^9$ per g of rumen content (Henderson et al. 2010a), which would explain why we did not detect acetogens in all samples.

T-RFLP analysis was also performed to study the structure of the acetogenic community. A few samples that were unclear in the gel electrophoresis of the PCR, where still included and gave results in the T-RFLP. Analysis of the PCA plot from TRF data from faecal samples (figure 4) revealed a large spread among 2-day calves whereas the 6-month old calves clustered more closely. The number of samples in the different age groups varied which might have affected the spread, but the indication is still that the within group similarity in the microbiota increases as the

Table 4. Results from gel electrophoresis of acetogenic PCR products. Numbers in brackets is the total number of analysed samples and without brackets, the positive samples.

	Age						
Acetogen samples	2 days	7 days	14 days	28 days	6 weeks	8 weeks	6 months
Rumen tissue	0 (5)	2 (4)	-	-	-	-	-
Rumen digesta	5 (5)	3 (4)	-	-	-	-	-
Abomasum tissue	1 (5)	2 (3)	-	-	-	-	-
Abomasum digesta	3 (5)	2 (4)	-	-	-	-	-
Duodenum tissue	1 (5)	0 (4)	-	-	-	-	-
Duodenum digesta	3 (5)	3 (4)	-	-	-	-	-
Ileum tissue	0 (5)	0 (4)	-	-	-	-	-
Ileum digesta	2 (5)	0 (0)	-	-	-	-	-
Cecum tissue	0 (5)	1 (4)	-	-	-	-	-
Cecum digesta	3 (5)	2 (4)	-	-	-	-	-
Colon tissue	1 (5)	0 (4)	-	-	-	-	-
Colon digesta	4 (4)	1 (1)	-	-	-	-	-
Faeces	11 (12)	8 (8)	5 (6)	2 (2)	1 (2)	6 (6)	8 (8)

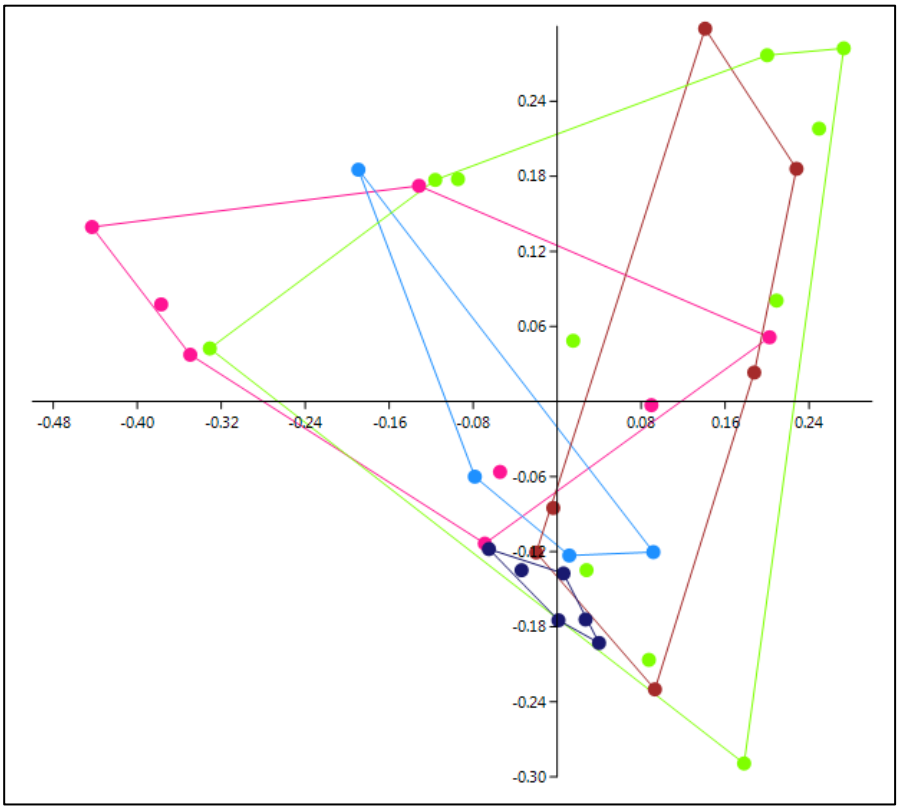

Figure 4. PCA plot displaying the clustering of samples based on their faecal acetogenic community structure. Samples from 2 days old calves show a much greater spread compared to 6 months old calves. Green = 2-day, pink = 1-week, light blue = 2-week, brown = 8-week, dark blue = 6-month

calves get older just like Jami et al. (2013) concluded in their study of the bovine microbiota over time. Three TRF's; 67, 95 and 640 were present in almost all samples (26, 25 and 24 respectively out of 27 samples), some TRF's were present in only 2-day or 6-month samples indicating that there are age and/or feed dependent acetogenic species in the bovine GIT. In figure 5, the average TRF profile for 2-day (n = 11) and the 6-months (n = 6) old calves are displayed.

Clone libraries were created to study which species of acetogens that could be found in the samples. The clone library matched different species for 6-month and 2-day samples (table 5). The clone library from the 6-month sample provided sequences that only matched with *Eubacterium siraeum*, but with low homology scores (73%). The clone libraries for the 2-day samples had sequences matching at least two different species (*Lactobacillus johnsonii*, *Clostridium perfringens*) and with higher homology scores than in the 6-month library.

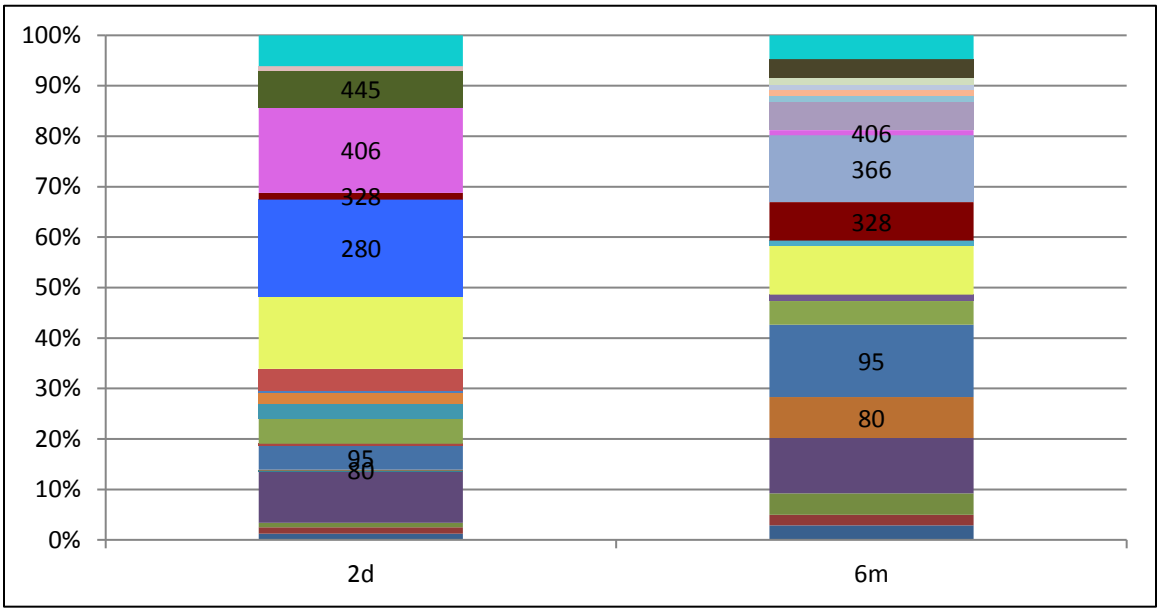

Figure 5. Bar plots of average TRF profiles of the acetogenic community from 2-day and 6-month samples.

The FTHFS gene might also occur in organisms that are not true acetogens. Many organisms carrying the FTHFS gene do not produce acetogen, for example many sulphate reducers (Leaphart et al. 2003). The ones that carry the gene and only produce acetogen is called homoacetogens (Henderson et al. 2010b). This can explain the BLAST search result. It is also important to emphasize that the clone library contain sequences from only two individuals, which might not be representative, especially not the 6-month faeces that is from a single individual. In a study by Henderson et al the importance of primer combinations where highlighted. They used different primers to amplify different regions of the FTHFS gene and no single primer pair could amplify the FTHFS gene from all known homoacetogens that they used for control (Henderson et al. 2010a). This means that we might have gotten a different result if we had used more primers. In the same study they also tried to find genetic residues that would distinguish rumen homoacetogens from other acetogens. Lovell and Leapheart have identified distinguishing

Table 5. Results from sequence analysis of the clone libraries from acetogens.

	Results from BLAST	
Sample animal	Acetogens	Identic (%)
6 month calf faeces	*Eubacterium siraeum* 70/3 draft genome	73
2 day calf faeces	*Lactobacillus johnsonii* DPC 6026, complete genome	99
	Clostridium perfringens SM101, complete genome	85
2 day calf rumen	*Clostridium perfringens* ATCC 13124, complete genome	99
	Lactobacillus johnsonii DPC 6026, complete genome	96
2 day calf cecum	*Lactobacillus johnsonii* DPC 6026, complete genome	99

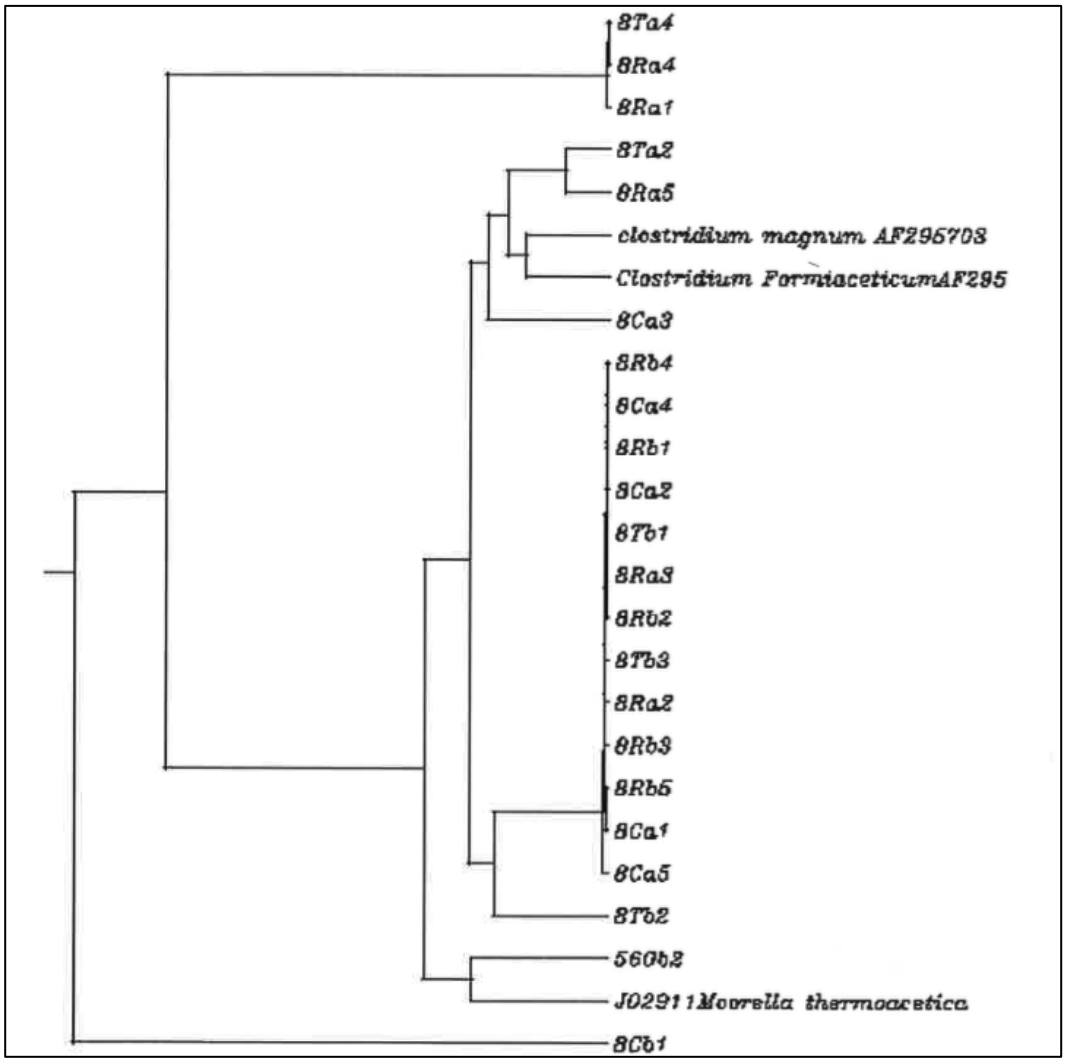

Figure 6. Phylogenetic tree of known homoacetogens and sequences from samples from faeces, rumen and cecum of a 2 day old calf and faeces from one 6-month-old calf.

markers for homoacetogens from natural habitats but it seem in the Henderson study that it would be more difficult to find the same patterns in rumen isolates (Lovell & Leaphart 2005; Henderson et al. 2010a).

We made an attempt to put our acetogenic sequences into a phylogenetic tree with known homoacetogens to get an idea if the sequences shared homology with known acetogens (Figure 6). We found that our sequences had a quite diverse composition where some of the sequences shared homology with known acetogens and some did not. Most of the sequences generated from the clone libraries clustered on a separate branch with no homoacetogen representative on the same branch. The sequence from the 6-month-old calf had the clearest connection to *Moorella thermoacetica*. Two samples from the 2-day old calf, faeces and rumen, clustered close to *Clostridium magnum* and *C. formiaceticum.* The tree would be easier to interpret if it was supplemented with more known acetogens, unfortunately that did not fit in the framework of this study.

Analysis of the data from the acetogenic intestinal samples, using PCA (figure 7), showed that the spread of the samples did not follow a clear pattern. Five samples from one calf (No 7) clustered together in two groups. One group consisted of rumen tissue and digesta and cecum digesta and the other abomasum tissue and duodenum digesta. Samples from calf 9 are all

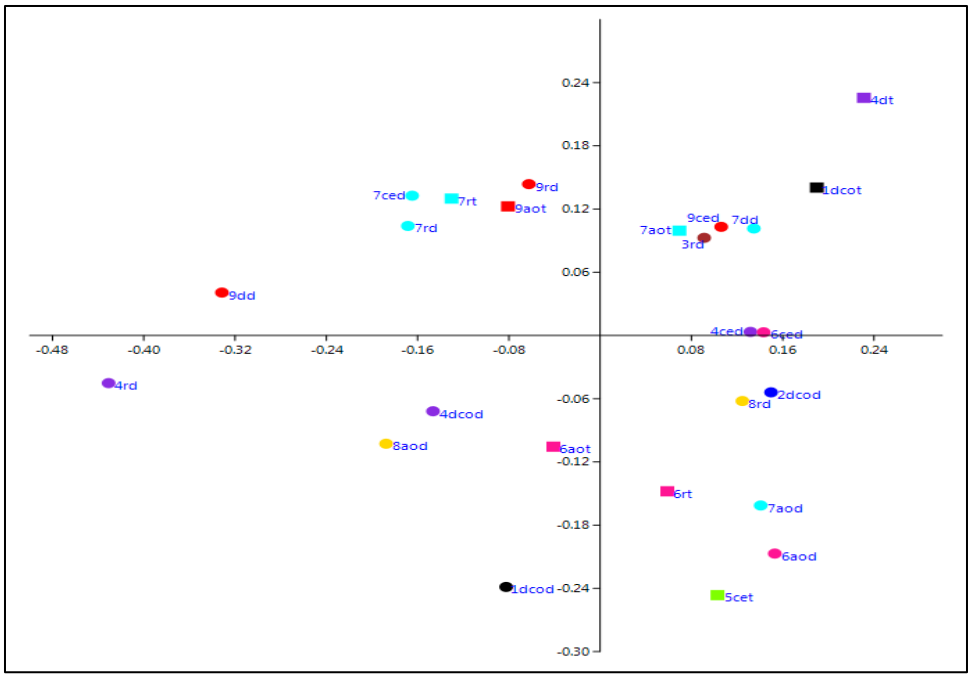

Figure 7. PCA plot of TRF's from intestinal samples from 2 and 7 days old calves. Dot = tissue, square = digesta. 1=black, 2=blue, 3=brown, 4=purple, 5=green, 6=pink, 7= turquoise, 8=yellow, 9=red.

spreading above the horizontal axis rumen digesta and abomasum tissue samples cluster together and cecum digesta is not far away.

Three samples from calf 6; abomasum tissue, rumen tissue and abomasum digesta were also clustering on the plot. This indicates not only an individual pattern but also that rumen and abomasum share a similar acetogenic and methanogenic structure in the same individual. Two samples were excluded from the PCA-plot because they were outliers. When looking into the data behind the bar plots of the TRF's (figure 8) it is clear that the two differ so much from the others because they only have two TRF's, while the other samples have at least four. This is not visible in the picture because the bars 2 rumen, 7 abomasum, 7 cecum and 2 colon are visualizing the average of 2-3 samples. The explanation to the variation between the samples is presumably that the microbiological development varies a lot between individuals at this young age and the

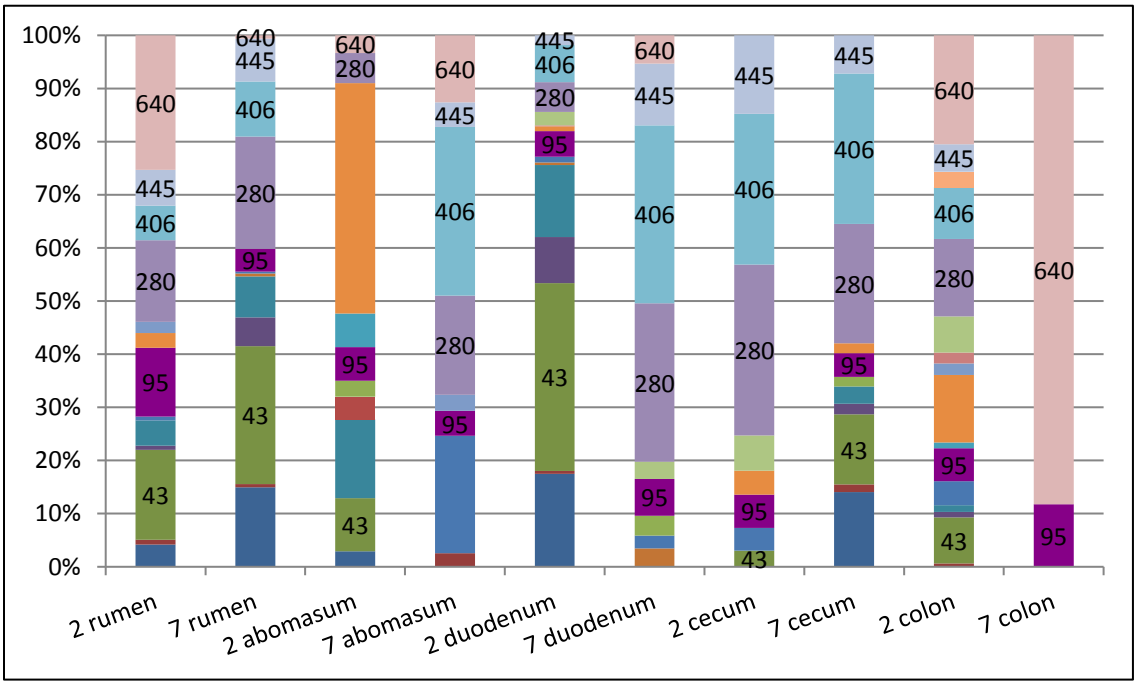

Figure 8. TRF-profiles visualized by bar plots, digesta samples from 2 and 7 days old calves. The bars; 2 rumen, 7 abomasum, 7 cecum and 2 colon shows the average of 2-3 different samples.

primers do not intercept all species present. Something might also have gone wrong during sampling or extraction of DNA.

The bar plots of the GIT tissue and digesta samples (figure 8 and 9) illustrate the TRF profiles and how the abundance of different TRF's is varying in different samples. Most TRF's are present in all samples, also in faeces, but in varying proportions, whereas a minor fraction of the TRFs are restricted to specific samples. Both of the rumen samples in figure 8 are quite dissimilar and the samples from duodenum and cecum are quite alike except duodenum from 2 days. Some differences can be seen between digesta samples in 2 and 7 days old calves and the samples from the 2 days old seems a bit more variable.

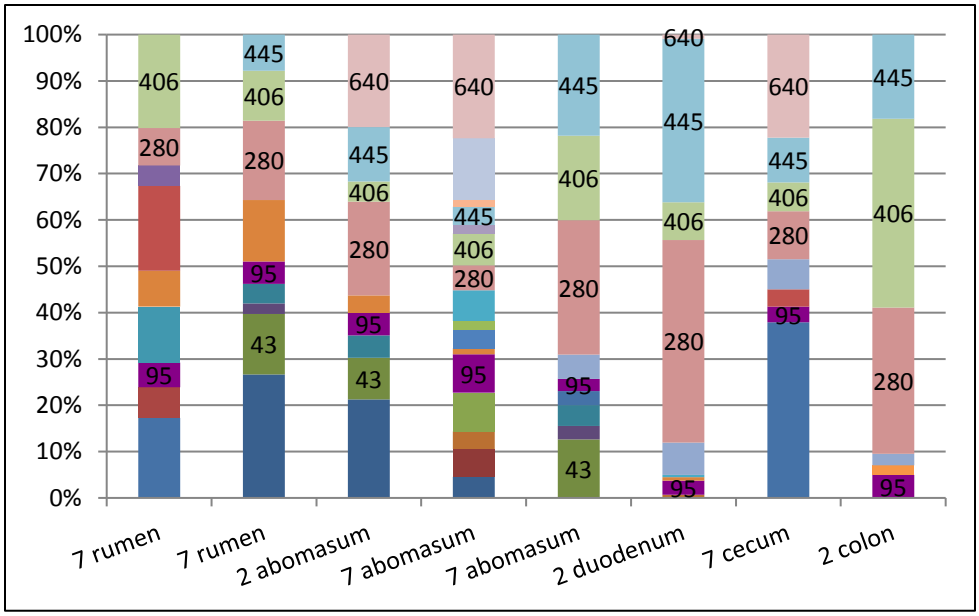

Figure 9. Bar plots of the TRF profiles of tissue samples from the intestinal samples.

Figure 9 illustrates the few positive tissue samples. They exhibit less TRF's than the digesta samples and seem less variable. In figure 10 bar plots from samples from one 7 days old calf illustrates that there are similarities within an individual. Interestingly the faecal samples distinguish a little from the intestinal samples, indicating that faecal samples might not be telling the whole truth of the GIT microbiota.

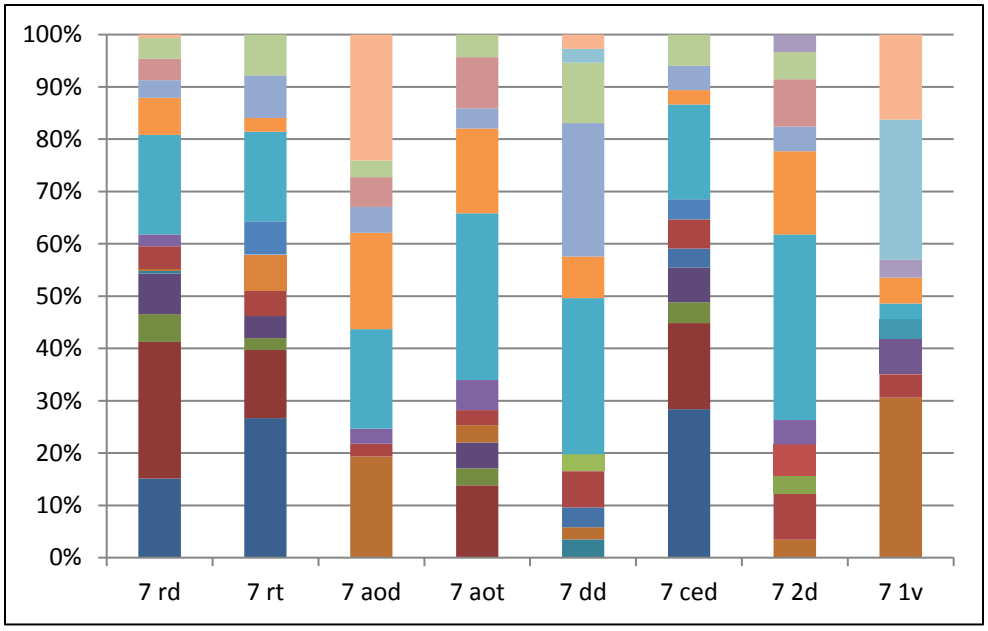

Figure 10. Barplots showing TRF's from calf 7. Rumen digesta and tissue, abomasum digesta and tissue, duodenum digesta, cecum digesta and faeces from day 2 and 7.

4 Conclusion

The results from this study indicate that the methanogens are not present in young animals. No methanogens were detected in the intestinal samples and not in the faecal samples before two weeks of age. Acetogens were found in intestinal and faeces samples from calves 2 days and older. The method could not confirm whether the acetogens were homoacetogenic or not.

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