# *On the Relationship between Spillovers and Bundling*

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# Microbial diversity in the digestive tract of two different breeds of sheep

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**Running header:**

Microbes in digestive tract of sheep breeds

## Abstract

Aims

This work aims to determine the factors which play a role in establishing the microbial population throughout the digestive tract in ruminants and is necessary to enhance our understanding of microbial establishment and activity.

Methods and Results

This study used Terminal Restriction Fragment Length Polymorphism (TRFLP) to investigate the microbial profiles of 11 regions of the digestive tract of two breeds of sheep (Beulah and Suffolk). TRFLP data revealed that the regions of the digestive tract were highly significantly different in terms of the composition of the bacterial communities within three distinct clusters of bacterial colonisation (foregut, midgut and hindgut). The data also show that breed was a significant factor in the establishment of the bacterial component of the microbial community, but that no difference was detected between ciliated protozoal populations.

Conclusions

We infer that not only are the different regions of the tract important in determining the composition of the microbial communities in the sheep, but so too is the breed of the animal.

Significance and Impact of Study

This is the first time that a difference has been detected in the digestive microbial population of two different breeds of sheep.

## Key words:

Sheep; microbes; diversity; digestive tract; TRFLP

## Introduction

Since ruminants lack the enzymes necessary for complete digestion of the plant material they ingest, they depend upon a symbiotic relationship with the microbes of their digestive tract in order that the complex carbohydrates in plants may be digested. The microbes secrete a range of enzymes, which degrade the plant

material and initiate fermentation of polysaccharides to volatile fatty acids and lactate, which can then be absorbed across the digestive tract wall and provide energy sources for the host animal. In return, the host animal provides the microbes with an initial physical disruption of the plant material via mastication, and a homeostatic environment.

It is already well-established that the microbial (bacterial, fungal, archaeal and protozoal) composition of the rumen can be manipulated by changes in dietary environmental stimuli; e.g. roughage: concentrate ratio; addition of antibiotics; protein content (e.g. Eadie, 1962; Yanez Ruiz *et al.*, 2008). In addition to dietary effects on the microbes present in the rumen, studies which have been performed on the microbial communities of other herbivores (e.g. horse, rabbit) make it clear that there are different microbial populations in the different species (e.g. Daly *et al*., 2001; Abecia *et al*., 2005). These inter-specific differences have raised several questions concerning the impact of the host animal on the composition of the microbial community. For example, it is generally believed that in many species the host's digestive immunological and endocrinological status can influence the composition of the microbial population colonising the digestive tract (Ouwehand *et al.*, 2002; Freestone and Lyte, 2010; Buddington and Sangild, 2011), and it has been suggested that the genetic composition of the host may influence the composition of the gut microbiota (Toivanen *et al.,* 2001). In addition to inter-species differences, it has been suggested that there may also be intra-species variation. For example it has been found that bacterial populations from faecal samples in genetically identical human twins have a higher level of similarity compared to unrelated individuals (Zoetendal *et al*., 2001; Stewart *et al*., 2005). This similarity is not due to twins having the same current environmental conditions, as marital partners, who were

living in the same environment and having comparable feeding habits, showed low similarity (Zoetendal *et al*., 2001). Thus there is evidence that suggests that the host's genetic background plays a role in the establishment of differences in the microbial population in the gut between individuals from within the same species (Shi *et al.*, 2008). However the extent the host animal's genetics plays a role in the establishment of the microbial population within the digestive tract of ruminants is unknown.

It is already known that in ruminants different breeds cope better in different environments and thus different forage conditions relative to other breeds. For example hill and mountain breeds of sheep such as Cheviot, Welsh Mountain, Blackface and Swaledale breeds survive in harsh conditions with very poor quality pasture. Conversely lowland breeds of sheep such as the Border Leicester, Bluefaced Leicester and Suffolk tend to be farmed in less harsh conditions and on lush pasture. While the hill breeds are known to survive and thrive on lowland pastures, it is generally accepted that attempts to maintain a lowland breed of sheep in harsh hill conditions often results in them not thriving as they are unable to cope with the poor quality pasture and harsh weather conditions. In part this may be due to abiotic factors (e.g. the weather) impacting on genetic factors such as the difference in fleece characteristics. It is however unclear if there are differences in the microbial community of lowland and hill sheep which might play a contributing role in their ability to utilise the poor-quality forage.

Shi *et al*. (2008) investigated the effect of host species on the microbial community in the rumen of goats. The study analysed the microbial community in the rumen of three goat species (Boer, Inner Mongolia and Nanjiang yellow goats) that were grazed in the same environment. It was found that there was a greater

interspecies variation in bacterial populations compared to intraspecies variation, indicating that the bacterial community in the goat rumen is species-specific. This suggests a host-specific effect on the bacterial community in the goat's rumen in the absence of environmental or dietary factors.

It appears that genetic background along with environmental factors influence the gut microbiota, but due to the lack of research and data it is unclear to what extent host genetics may influence the establishment of the microbial population that inhabit the digestive tract of the ruminant. Therefore the aim of this experiment was to investigate differences between two breeds of ruminants on the composition of the microbes that inhabit the digestive tract, using sheep as a representative of ruminant species.

## Materials and Methods

*Sheep*

Yearling ewes of two different breeds (n=4 for each breed) were used in this study; Beulah Speckled-Faced (which are native to Wales and have been bred to cope with relatively harsh upland conditions) were used as an example of a hill breed and Suffolks (which are used as a terminal sire breed) were used as an example of a lowland breed. All animals were bred and reared on Aberystwyth University farms.

The animals were weighed at the beginning of the experiment and at weekly intervals during the course of the work. The average initial body weight of the Beulahs was 38.2 ± 2.47 kg; and an average 52.8 ± 3.27 kg for the Suffolks. The animals were housed in individual pens on sawdust bedding with contact allowed between neighbouring animals. Animals were fed a uniform diet of grass pellets (Badminton grass cubes, Badminton Feeds, Station Mill) at maintenance level,

based on body weight, in two equal meals at 09:00 and 16:30 daily. Salt licks and water were continuously available.

*Collection of digesta samples*

All animals were fed the grass pellet diet for 9 weeks prior to euthanizing by the on-site Named Animal Care and Welfare Officer. Animals were euthanized in August 2010 and digesta samples were collected in 35 mL tubes, from 11 regions (rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, caecum, large colon, small colon and rectum) of the digestive tract within 20 min of death and were stored at -80°C for later analyses.

In addition samples from the rumen and reticulum were placed in formalin to allow ciliates to be counted.

*DNA extraction and purification*

DNA extraction was carried out using QIAamp® DNA Mini Stool Kits (Qiagen Ltd.; West Sussex, England) following the manufacturer's standard instructions with the exception of increasing the initial incubation from 70°C to 95°C for 5 min, which was found to give optimal yields from a range of digestive tract organs (Douglas, 2013). The lysis temperature was increased as the manufacturers suggest that this step helps with the lysis of Gram-positive bacteria which have previously been shown to be difficult to lyse, as these are known to be present in large numbers in the digestive tract.

Once extracted, the purity and concentration of the DNA was assessed using a BioTek Epoch Spectrophotometer System to measure the absorbance at 260nm, as well as the $A_{260}$:$A_{280}$ ratio.

*PCR amplification*

A partial region of the 16S *rRNA* gene was amplified using the bacterial primers *27F* (5'-AGA GTT TGA TCC TGG CTG AG-3') and *1389R* (5'-AGG GGG GGT GTG TAG AAG-3'), which have been described previously as being able to amplify DNA from a diverse range of bacteria (Hongoh *et al.*, 2003; Weisburg *et al*., 1991). A Cy5-label was attached to the 27F primer to allow it to be used for TRFLP analysis. PCR was performed in a reaction cocktail containing 0.25 µl of each primer (50 µM stock concentration), 12.5 µl ImmoMix$^{TM}$ (Bioline), 11 µl of molecular grade water and 1 µl of DNA template to make a final volume of 25 µl. Amplification was performed with the following steps: 95°C hot start for 10 min; 25 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min); 1 cycle (94°C for 1 min, 55°C for 1 min, 72°C for 5min) and a final hold at 4°C. Amplicons were analysed by electrophoresis on a 1% [w/v] agarose gel to verify the presence of an amplicon of the anticipated length; around 1300-1400 base pairs (bp).

*TRFLP analysis*

For terminal restriction fragment length polymorphism (TRFLP) analysis, PCR products were digested using restriction enzymes *Hae* III*, Hha* I and *Msp* I (New England, Biolab) in single enzyme digestions using 2.5 U for *Hha* I and 1.25 U for *Msp* I and *Hae* III. Reactions were performed using 50 ng of amplicon sample, together with the appropriate buffer provided by the manufacturer and molecular grade water to make a final volume of 50 µl. Samples were digested at 37°C for 5 hours and reactions were terminated by incubation at 80°C for 15 min. Samples were cleaned in 96-well plates by ethanol precipitation.

Digested fragments were separated on a CEQ™ 800 Genetic Analysis capillary sequencer (Beckmann Coulter, USA). The sizes of the fluorescently labelled fragments were determined by comparison with the internal size standard 600 (Beckmann Coulter). Relative peak heights of each terminal restriction fragment (TRF), and by inference phylotype, were determined for samples within the size range 60-600 bp. Data were normalized by applying a threshold value for relative abundance within individual samples and only TRFs with higher than 0.5% of the maximum value relative abundances were included in the remaining analyses.

TRFLP data were initially organized in MS Excel and classified relative abundance based upon bin sizes of 1 bp with the assumption that each bin comprised a discrete TRF/OTU. PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) respectively were used to conduct permutation multivariate analysis of variance (PERMANOVA) (Anderson, 2001) and canonical analysis of principal coordinates (CAP) (Anderson and Willis, 2003) on Bray–Curtis distances of fourth-root log of T-RFLP abundance data. PERMANOVA was conducted using default settings with 9999 unrestricted permutations, while CAP was conducted using default settings (Edwards *et al*., 2011). Dendrograms were generated using Fourth root and S17 Bray Curtis similarity, using default settings.

*Protozoal analysis*

Protozoal samples which had been stored in formalin were diluted to appropriate concentrations and then counted by microscopy. Representatives of five different ciliate genera were identified and counted (*Entodinium* spp., *Diplodinium* spp., *Epidinium* spp., *Isotricha* spp. and *Dasytricha* spp), as well as total ciliate

numbers. Sample numbers were compared by ANOVA for organ differences (rumen versus reticulum), breed differences and breed*organ interactions.

*FTIR analysis*

Frozen samples were transferred in duplicate onto an FTIR microplate. Samples were dried overnight at 50ºC, then analysed by FTIR using a Vertex 70 spectrophotometer. Data were recorded for every wavenumber (spectral range 4000 $cm^{-1}$ to 660 $cm^{-1}$), giving over 3,500 data points per sample. Since it was unknown which wavenumbers contributed most to the differences between samples, data had to be analysed by multivariate analysis. FTIR data were analysed by Principal Component Analysis (PCA) followed by Discriminant Function Analysis (DFA) using MATLAB (R2009a) software, using a cross validation to exclude the bias of pre-knowledge to the data set. This was done by splitting the data into training and testing sets, the training set being given prior knowledge to create the model and the test set of data projected in with no prior knowledge.

# Results

*T-RFLP analysis*

Numbers of RF peaks meeting the cut-off threshold ranged between organs, with the greatest found in the rumen samples (mean = 151 + 15.9) and the smallest in the abomasum samples (mean = 27 + 2.4).

PERMANOVA analysis of TRFLP data showed a significant difference in bacterial community structures between breeds ($P$ = 0.02) and within regions of the digestive tract ($P$=0.0001) but no significant interaction between these factors

(*P*=0.50). This point can also be seen diagrammatically in Figure 2 following CAP analysis. In addition the P-values for comparison of samples between different pairs of organs are shown in Table 1.

TRFLP data revealed significant differences between communities from most of the regions of the digestive tract. In those cases where comparisons showed a difference, values were significant (P<0.001), and the only areas where no differences were observed (P>0.05) were: omasum versus rumen and reticulum; duodenum versus jejunum; and all combinations of the organs of the hindgut (caecum, large colon, small colon and rectum). These differences were consistent with clustering data from both the Bray Curtis similarity dendrogram (Figure 1) and CAP analysis (Figure 2) i.e. division between foregut, midgut and hindgut regions, which then split more clearly by organ when organs from a particular region were analysed together (Figure 3).

*Protozoal analysis*

Analysis by t-tests showed no differences (P>0.05) between protozoal counts between organs (rumen versus reticulum) or breeds (Suffolks versus Beulahs). This was true for all values for each of the individual genera, based on microscopic identification of morphotypes (*Entodinium*, *Diplodinium*, *Epidinium*, *Isotricha* and *Dasytricha*), and for total numbers of protozoa.

*FTIR analysis*

Analysis of FTIR data by PCA and DFA from ruminal, caecal and faecal samples showed a trend towards segregation by organ (Figure 4) but no split by breed, suggesting that the major metabolites being produced from the different

regions by the sheeps' digestive tracts do not have a breed specific difference associated with them.

## Discussion

There has been extensive work carried out on environmental and dietary effects on the microbial composition of the digestive tract of ruminants, however there has been very little, if any, work done on the host genetic and region effect on the composition of the microbial population within the tract. To our knowledge this is the first comparison of breed effect on bacterial diversity in the digestive tract of sheep. Previous work (e.g. Kocherginskaya *et al*., 2001; McEwan *et al*., 2005; Yanez *et al.*, 2010) has been focused on the dietary and environmental impacts on the microbial population within the digestive tract, with the main focus generally being in the rumen.

From the TRFLP data PERMANOVA showed that the region of the tract contributes highly significantly with regards to the microbial community present. There appears to be a cluster separation of the microbes inhabiting the non-glandular foregut, midgut and hindgut which is also represented in the dendrogram (Figure 1) and CAP (Figures 2). This regional split was as expected, as it is assumed that the different regions of the tract are anatomically and physiologically different and so it would be likely that they would have different microbial communities inhabiting them.

Physiological differences have been reported previously for different parts of the digestive tract, e.g. differences in pH (Ørskov *et al*., 1972). Here we also report differences in the metabolites present in different groups of organs. These however are not sufficiently great to promote bacterial populations which are radically different

between certain organs. For example any differences between the bacterial populations of the rumen and reticulum, or between those in the jejunum and ileum, are relatively small in comparison between the organs of the small intestine versus those of the non-glandular foregut. As a consequence of these intra-regional differences being small, against a backdrop of inter-regional variation, it is possible that differences between the bacterial populations in organs with similar function (e.g. rumen versus reticulum) may not be resolved when comparing all organs within the digestive tract (Figure 2). For this reason CAP analysis was repeated using only organs from a single region of the tract (Figure 3).

When comparisons were performed by CAP analysis between organs within regional parts of the tract it was possible to separate the bacterial populations based on their original source organ. This was true for the populations in the non-glandular foregut (Figure 3A), the abomasum and small intestine (Figure 3B) and the hindgut (Figure 3C). These observations substantiate the suggestions made above that the level of variation between bacterial populations in organs which are functionally similar is too low to be detected against a background of differences between bacteria from more diverse backgrounds. Thus these data also demonstrate that there are population differences between the different organs, even between relatively similar ones.

In addition to graphically demonstrating these differences based on CAP analysis, an organ effect on the bacterial population was also detected by use of PERMANOVA ($P$=0.0001). Moreover, although there was no obvious difference between breeds detected by the use of either CAP or the Bray Curtis similarity dendrogram, one was detected when PERMANOVA was performed ($P$ = 0.02). In the same way as the organ-effect was masked by the regional effect (Figures 2 and

3), comparing P-values for both the breed effect and the organ effect graphically suggests that the breed effect is masked against a higher background of organ effect.

This work is the first which shows a difference in the microbial population of the digestive tract in two breeds of sheep. The differences are restricted to the bacteria, with no differences in the protozoal population. The composition of the diet was identical for the 9 weeks prior to sample collection, giving the microbial population time to adapt to the feed, and so should not have a dietary impact on any differences detected. Early life factors have been reported to influence the microbial community of adult sheep (e.g. Yanez *et al.*, 2010). The two groups of animals were all reared on the university farm, but were not kept together prior to the experiment, so earlier life factor cannot be ignored as playing a part in the observed differences in the bacterial populations. In humans individuals who are closer related to each other have higher levels of similarity in terms of their gut microbial community relative to the general population (e.g. Zoetendal *et al*., 2001; Stewart *et al*., 2005). In the same way, it might be expect that domesticated animals would follow a similar pattern, with animals which are more closely related (e.g. those within one breed) having higher levels of similarity than those from other sources (e.g. different breed). This is the first time that a breed difference has been shown in the digestive tract of the sheep, and as far as we are aware, in any domesticated animal.

It is also worth noting that although a difference between breeds was detected in the bacterial population of the digestive tract, no such difference was observed in the protozoal population, many of which predate on the bacteria within this environment. Likewise, the metabolites from within digestive tract, while they could be differentiated by source organ, could not be split by breed. Therefore, we

propose that there is a breed effect on the bacterial population of the digestive tract, but that the role carried out by these different bacteria results in similar metabolites being produced in all animals investigated.

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## Conflict of Interest

The authors are unaware of any conflict of interest within this work.

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**Figure 1**

A dendrogram producing using Bray Curtis similarity of bacterial T-RFLP peaks in the amplicons of 16S *rRNA* gene which had been digested using *Hha I*, *Hae III* and *Msp I* (the dendrogram shows amalgamation of data from all three enzymes). The identity of the sheep from which samples were collected is shown as a number at the start of the label (1, 2, 5 and 6 were Suffolks; 3, 4, 7 and 8 were Beulahs), whilst the region of the tract is denoted by a letter rumen (a), reticulum (b), omasum (c), abomasum (d), duodenum (e), jejunum (f), ileum (g), caecum (h), large colon (i), small colon (j) and rectum (k). The regions of the tract (midgut and abomasum, non-glandular foregut and hindgut) are indicated.

**Figure 2**

CAP plot for all digesta samples from all parts of the digestive tract for bacterial T-RFLP for all 3 restriction enzymes (*Hae* III, *Msp* I and *Hha* I) combined. In general the samples can be split into three regions: non-glandular foregut (rumen, reticulum and omasum); abomasum and small intestine; and hindgut.

Figure 3

(A) CAP plot for digesta samples from the rumen, reticulum and omasum (non-glandular region of the foregut) from all animals for bacterial T-RFLP for all 3 restriction enzymes (*Hae* III, *Msp* I and *Hha* I) combined. The first digit indicates the breed of animal (1, 2, 5 and 6 were Suffolks; 3, 4, 7 and 8 were Beulahs); and the second digit indicates the organ (1 = rumen; 2 = reticulum; 3 = omasum).

(B) CAP plot for digesta samples from the abomasum, duodenum, jejunum and ileum from all animals for bacterial T-RFLP for all 3 restriction enzymes (*Hae* III, *Msp* I and *Hha* I) combined. The first digit indicates the breed of animal (1, 2, 5 and 6 were Suffolks; 3, 4, 7 and 8 were Beulahs); and the second digit indicates the organ (4 = abomasum; 5 = duodenum; 6 = jejunum; 7 = ileum).

(C) CAP plot for digesta samples from the caecum, large colon, small colon and rectum from all animals for bacterial T-RFLP for all 3 restriction enzymes (*Hae* III, *Msp* I and *Hha* I) combined. The first digit indicates the breed of animal (1, 2, 5 and 6 were Suffolks; 3, 4, 7 and 8 were Beulahs); and the second digit indicates the organ (8 = caecum; 9 = large colon; 10 = small colon; 11 = rectum).

**Figure 4**

PCA plot of FTIR samples from different regions of the digestive tract. The first digit denotes the source of material (1= rumen; 2 = caecum; 3 = faeces) and the second digit (subscript) denotes the animal from which the sample was isolated 1-8 (1, 2, 5 and 6 were Suffolks; 3, 4, 7 and 8 were Beulahs). In addition, Suffolks are shown in blue and Beulahs are shown in red.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Rumen | | | | | | | | | | |
| Reticulum | 0.031 | | | | | | | | | |
| Omasum | 0.129 | 0.430 | | | | | | | | |
| Abomasum | 0.0001 | 0.0001 | 0.0002 | | | | | | | |
| Duodenum | 0.002 | 0.0002 | 0.0002 | 0.0004 | | | | | | |
| Jejunum | 0.0002 | 0.0001 | 0.0002 | 0.0006 | 0.090 | | | | | |
| Ileum | 0.0001 | 0.0001 | 0.0002 | 0.0001 | 0.0001 | 0.002 | | | | |
| Caecum | 0.0001 | 0.0002 | 0.0004 | 0.0001 | 0.0002 | 0.0001 | 0.0002 | | | |
| Large Colon | 0.0001 | 0.0001 | 0.0002 | 0.0001 | 0.0002 | 0.0002 | 0.0001 | 0.958 | | |
| Small Colon | 0.0001 | 0.0001 | 0.0003 | 0.0001 | 0.0004 | 0.0002 | 0.0001 | 0.969 | 0.989 | |
| Rectum | 0.0001 | 0.0003 | 0.001 | 0.0004 | 0.0003 | 0.0003 | 0.0007 | 0.876 | 0.911 | 0.991 |
| | Rumen | Reticulum | Omasum | Abomasum | Duodenum | Jejunum | Ileum | Caecum | Large Colon | Small Colon |

**Table 1**

Pairwise comparisons of P-values for different organs are shown. Values which are not significant (P<0.05) are shaded.