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An eight-year investigation of bovine livestock fecal microbiota

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ABSTRACT

Cattle represent a major source of fecal contamination worldwide. Understanding the natural variation of the bovine livestock fecal microbiota is therefore important. For this reason we addressed the yearly differences of the fecal microbiota for bovine livestock reared in the same geographical region from 1999 to 2007 – analyzing a total of 300 samples representing a range of experimental regimes. The aim of our work was to determine the effect of year visa experimental regime of the bovine livestock fecal microbiota. We used a newly developed high-throughput 16S rRNA sequencing approach (a single mixed Sanger sequence was generated per sample) in combination with deep pyrosequencing. We found that similar feeding and treatment regimes for different years showed major differences in the fecal microbiota, suggesting other factors important for shaping the fecal microbiota than those experimentally controlled. *Ruminococcaeae*, *Peptostreptococcaceae*, *Acinetobacter*, *Escherichia/Shigella*, *Lachnospiraceae* and *Lactobacillales* were the main taxa associated with the yearly fluctuations. Furthermore, we found that fecal samples with high levels of *Lactobacillales*, *Ruminococcaea* and *Lachnospiraceae* had the most even species distributions. The *Peptostreptococcaceae* and *Acinetobacter* dominated samples, on the other hand, showed a few highly dominant taxa. Testing of neutrality showed that the evenly distributed samples were explained by a neutral mode (that the assembly of the microbiota was random), while for the other samples there were overrepresentation of the dominant species (indicating bacterial-bacterial nice competition). We therefore propose that there are natural yearly fluctuations of the bovine livestock microbiota – both with respect to ecology and composition. This knowledge will have impact on our management of fecal bacteria in the environment, since it is very difficult to predict risk based on historical data.

1. Introduction

Intensive agriculture and extensive land usage have altered the global dispersal of fecal bacteria (Rapp, 2010). Bovine livestock (*Bos primigenius*), with a world population of approximately 1.5 billion, represent a major source of fecal contamination worldwide. Understanding the factors shaping the bovine fecal microbiota would therefore be important for understanding the global spread and epidemiology of fecal bacteria (Sinton et al., 2007). To our knowledge, no long-term investigations of fecal microbiota within bovine livestock have yet been conducted.

Recent 16S rRNA gene deep-sequencing studies of the bovine fecal microbiota have revealed a highly diverse composition where: breed, gender, diet, age, or weather could not explain the microbiota differences between

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individuals (Dowd et al., 2008; Durso et al., 2010). Natural yearly fluctuations in the microbiota, however, have not yet been investigated. To address this question a long-term study of a large number of individuals reared under different conditions is needed. Such studies have until now been limited both by the availability of high-throughput analytical methods, and the availability of suitable historical fecal material from sufficient number of individuals.

A conception has been that freshly frozen feces is the only valid material for DNA analyses. This has until now precluded the use of historical material, since these have commonly been stored at ambient temperature for a few hours before freezing. Recent reports, however, state that fecal samples can in fact be stored up to 14 d at ambient temperature without major changes in bacterial DNA profiles (Lauber et al., 2010), and that only minor changes occur within 12 h (Roesch et al., 2009).

We have developed a microbiota analytical approach that enables large-scale population screenings (Trosvik et al., 2007; Zimonja et al., 2008). The principle is to generate mixed 16S rRNA gene sequences, and to use a multivariate statistical approach to resolve the sequences (a schematic representation of the technique is given in Suppl. Fig. S1). The result of this analysis is a concentration profile of the main bacterial groups in the samples analyzed. Using the mixed sequencing techniques in combination with deep pyrosequencing the aim of our work was to determine the effect of year visa experimental regime of the bovine livestock fecal microbiota with respect to environmental shedding. This was achieved by a temporal characterization of the dominant fecal microbiota in samples taken from either cows or steers (*Bos p. taurus*) undergoing metabolic studies at Trawsgoed Research Farm, Aberystwyth, UK, between 1999 and 2007. In total over 300 samples were taken for analysis.

We present results showing major yearly differences of the fecal microbiota. We argue that these were caused by transitions between neutral and non-neutrally evolving fecal microbial communities.

2. Materials and methods

2.1. Animals, diet and feeding regimes

All animals sampled were undergoing metabolic evaluation of different forages at a purposefully designed metabolism unit. Only one sample was collected from each animal. All cows were Holstein-Friesian dairy cows and all steers were Hereford × Friesian castrated bullocks. The animals were offered either: grass (typically perennial ryegrass *Lolium perenne*), clover (typically white *Trifolium repens* or red clover *T. pratense*) or a combination of grass and clover. The diets were presented as either: freshly cut, conserved as silage or as hay. In addition the cows received 1–2 kg per milking (2–4 kg/d) of standard 18% protein dairy concentrate (containing rolled barley, sugar beet pellets, and soybean meal; Clynderwen and Cardigan Farmers Ltd, Aberystwyth, Ceredigion, UK). During the collection period animals were fitted with urine and feces separators for a period of 6 d (Lee et al., 2009). Fecal samples were collected daily and stored frozen (−20 °C) before a homogenized bulked 6 d collection of the 5% daily output was produced and stored at −20 °C. All animals had free access to water and mineralized salt licks (Baby red horse lick, Rockies, Winsford, Cheshire, UK). An overview of all the samples is given in Table 1.

2.2. Direct sequencing of the microbiota

DNA isolation and purification was carried out using an automated procedure with silica particles (Bioclone Inc., San Diego, CA) as described earlier (Skanseng et al., 2006). DNA from the fecal samples were amplified and sequenced using the direct sequencing approach as described by Trosvik et al. (2007). Briefly, this involves 16S rRNA gene amplification using primers targeting universally conserved regions of the gene flanking V3 and V4 (Nadkarni et al., 2002), and sequencing using a sequencing primer targeting an internal universally conserved region. The direct sequencing spectra was aligned and processed with use of MATLAB (MathWorks, Natick, MA), as previously described (Zimonja et al., 2008).

The mixed sequences were resolved using a multivariate statistical approach, as schematically shown in Fig. 1. We used Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) as an iterative approach (algorithm) to find the matrices of concentration profiles and pure component spectra from the mixed sequence spectra. The algorithm firstly identifies rank, or dimensionality of the data. In the next step the pure spectra and concentrations of these are identified, assuming closure of the data. The method is described in detail by Zimonja et al. (2008). We used the implementation of MCR-ALS in Unscrambler version 9.8 (Camo, Woodbridge, NJ). The final number of components selected was determined by the shape of the explained variance curve. When additional components did not increase the explained variance no further components were added.

The concentration profiles for the components identified by MCR-ALS were clustered hierarchically using average linkage with Manhattan distances. The Manhattan distance between two points is the distance measured along axis at right angles, while average linkage is the distance between two clusters computed

Table 1
Summary of animals and dietary treatments which yielded the fecal samples.

Year	Sex		Diet			Feeding regime		
	M	F	Grass	Clover	Grass and clover	Fresh	Silage	Hay
1999	–	26	7	12	7	–	26	–
2000	16	2	8	8	2	2	16	–
2001	13	–	9	4	–	7	6	–
2002	33	6	25	11	3	6	30	–
2003	3	17	20	–	–	–	20	–
2004	22	11	28	5	–	–	33	–
2005	17	74	72	19	–	55	36	–
2006	14	49	48	15	–	17	46	–
2007	–	34	5	14	15	15	15	4