Fecal Microbiome in the Bovine Animal

James E. Wells, Minseok Kim, Larry A. Kuehn, and James L. Bono*

INTRODUCTION

Microbes are the cause of spoilage, food loss and foodborne disease, but for centuries microbes also have played an important beneficial role in food production for humans. Microorganisms are important for nitrogen fixation in legumes, are used to ferment milk for making dairy products, are used to pickle and preserve, and are used to ferment grains into alcoholic beverages. Animal production also relies on beneficial microbes to ensile nutrient-rich feedstuffs and, in the case of ruminants, as a source of nutrients. The rumen has been one of the best-studied microbial niches, and the benefits of the symbiotic relationship between host and microbes are clear. The host provides a warm nutrient-rich environment in the pre-gastric rumen compartment, and the microbes ferment ingested feedstuffs into energy, protein, and vitamins that are used by the host. However, the benefits of microbes are not limited to the rumen and the ruminant animal.

Animals harbor microorganisms on the skin and inside the body. In the animal, the gastrointestinal tract (GIT) is colonized throughout by a variety of microorganisms, most of which are bacteria. Bacteria may be microscopic in size, but the number of individual bacteria cells outnumbers the number of host cells 100-1000 to 1 (Touhy et al., 2009). A consequence of the diversity and number of bacteria in the GIT is the potential expansion of the host genetic potential by exploiting these bacteria. Identifying these bacteria and the role they may play in host well-being and performance has proven difficult until recently. Traditional microbiology relied on time-consuming specialized isolation and identification techniques that were tedious and difficult to perform. Molecular biology provided a simpler approach by exploiting conserved genes, such as the 16s rRNA gene, found in all bacteria. Simply amplifying, cloning and sequencing the gene allows one to analyze the bacterial diversity by analyzing the sequence variations of the clones. Subsequent technologies, such as high-throughput sequencing, has removed the cloning barriers for amplicon analyses and allows for collection of larger datasets, albeit with shorter sequence reads. Sequencing of amplicons does have some bias introduced with the primers used in the amplification steps, but improvements in sequencing are removing these barriers by allowing direct sequencing of total DNA in a sample.

GASTROINTESTINAL TRACT AND FECES

The mammalian GIT begins with the mouth and ends with the anus, and includes the esophagus, stomach, and intestines. The gastric stomach empties into the intestinal tract, which includes the small intestine, cecum (when present), large intestine, and colon. The intestinal tract is important for digestion and absorption of nutrients by the host, and this nutrient rich environment is conducive to colonization by bacteria. Much of the mass of feces is of microbial origin. Bacteria in the intestinal tract and in feces have been studied for decades, and the physiology of numerous isolated bacteria has been documented (Krause and Khafipour, 2011). However, much of this research with intestinal bacteria has concentrated on opportunistic pathogens and their role in disease.

In ruminants, the stomach has evolved into compartments (rumen, reticulum, and omasum) that allow pregastric fermentation of feedstuffs; which then empty into the abomasum, or gastric stomach. The bovine rumen has been well studied in regards to microbial form and function, particularly for animals fed forage diets (Russell and Rychlik, 2001). However, the intestinal and fecal bacteria of cattle have not been well documented.

MICROBIOME

Microbiome, in the purest sense, is the community of microorganisms in a habitat, and includes not only bacteria but also protozoa, fungi and archea. Early research used microscopy to describe the observed community

James E. Wells
PO Box 1066
USDA-ARS
U.S. Meat Animal Research Center
Clay Center, Nebraska 68933-0166
USA
jim.wells@ars.usda.gov

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members and used enrichment and isolation to study individual inhabitants. Bacteria are typically the predominant inhabitants, and characterization of isolated strains proved useful to describing the taxonomy of the inhabitants and understanding the ecosystem. However, isolation of bacterial strains often proved difficult because of synergies between different bacteria species, unknown nutrient requirement(s), slow growth rates, or low abundance in the system.

In bacteria, the 16S rRNA gene product (1500-1600 bp in size) is required for translation of RNA transcripts into proteins. This ubiquitous gene is common to all bacteria and has a high degree of conservation relative to other conserved bacterial genes. As a consequence, the 16S rRNA gene sequence and strain specificity has served as an ideal candidate to not only evaluate evolutionary relatedness, but to also monitor bacterial populations in a microbial habitat. In addition, the 16S rRNA gene has 10 highly conserved regions that has allowed the primer targeting of smaller sequence reads when full length reads are not technically feasible.

When using 16S rRNA gene sequences to study habitats, one has to first determine the identity for each of the sequences. Microbial ecologists have studied the phylogeny and established a taxonomy or relatedness for bacteria from an individual species to domain (Figure 1; adapted from Schloss and Handelsman, 2004). For hierarchial classification into a species, identity of the partial or nearly full length reads for the sequence typically must be 97%. Some bacteria, such as *Escherichia coli* and *Salmonella enterica* have nearly 99% identity, but were originally classified into different species because of differences in pathogenic traits. Species are classified into a genus, family, class, and phylum at 95, 90, 85, and 80% identity, respectively. With this hierarchy, microbiologists can sort and analyze sequence data into different levels of relatedness, or taxa grouping.

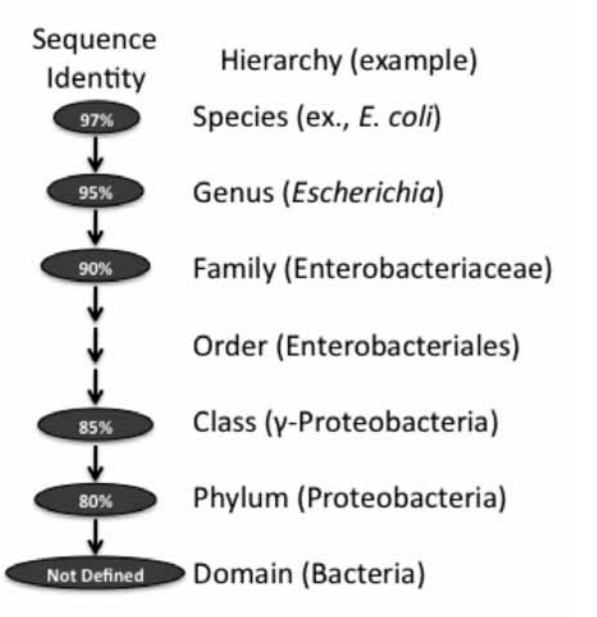

Figure 1. Percentage of sequence identity and taxonomic level for phylogenetic analysis of bacteria. The hierarchy from species to domain of Escherichia coli is shown as example.

In recent years, interest in the microbial communities associated with the human body has increased (The Human Microbiome Project Consortium, 2012a). In samples collected from oral cavities, skin, vagina, and stool of human subjects and analyzed for microbial DNA sequences, the stool samples contained the largest variety of unique bacteria species and genes. It is estimated that the diversity of functional genes from the microbial communities may be 100 to 1000 times that of the host (The Human Microbiome Project Consortium, 2012b).

The use of high throughput next generation sequencing (NGS) has dramatically improved the depth of understanding of microbial niches. However, comparison across different studies is difficult (Wells et al., 2014). The most common sequencing approaches used for these studies have been for short sequence reads using 454 (Roche Diagnostics Corporation, Banford, VT) and Illumina (Illumina, Inc., San Diego, CA) platforms, but only the Illumina platform is currently supported. Newer approaches utilize single molecule sequencing analysis and include the IonTorrent (Life Technologies Corporation, Grand Island, NY) and the PacBio (Pacific Biosciences, Menlo Park, CA) platforms, but these platforms have large error rates on a single sequence pass. The IonTorrent platform is low cost but generates shorter sequence reads, whereas the PacBio platform does have potential for sequencing longer reads and ability to perform circular sequencing to improve sequencing error rates.

As noted above, the 16S rRNA gene has 10 conserved regions and one can utilize primers targeting these regions to amplify DNA containing one or more of the 10 variable regions. The amplified DNA, or amplicon, is then sequenced directly using NGS. The resulting sequence data set is cleaned of bad sequence reads by denoising and chimera checking (Wells et al., 2014). The resulting high quality sequences can be assigned to a taxonomic group (Figure 1), or the sequences can be grouped into operational taxonomic units (OTU) based on a level of sequence similarity (typically 97% identity) and the consensus sequence assigned to a taxonomic group. Because of different approaches and bioinformatic analyses, comparison of microbiome results across different studies is difficult, particularly at the bacterial genus and species levels.

The use of primers targeting conserved regions to amplify variable regions of the 16S RNA gene from bacteria is not perfect. Each conserved region shares sequence identity across most species of bacteria, but not all bacteria; thus there are potentials for amplicon biases and this can lead to underrepresentation of some bacterial groups.

In an example from the human microbiome project, primers that worked well to identify bacterial species from the fecal samples did not work well for skin samples (Shafquat et al, 2014). Metagenomic approaches that shotgun sequence all of DNA in a sample using NGS techniques not only removes much of the bias observed with amplicon sequencing but also provides sequence information of protozoa, fungi, and viruses in the habitat. However, metagenomic sequence data can be dominated by host genomic DNA, and the potential for identifying unique bacterial inhabitants or observing host associations with less abundant microbes is limited due to read volume compared to the amplicon approaches targeting the 16S RNA gene for microbial identification.

BOVINE DIET AND MICROBIOME

The GIT of mammals is sterile at birth and colonization begins as the neonate passes through the birth canal and continues as the infant ingests microbes from the environment. In non-ruminant animals, ingested microbes must pass the acidic stomach before colonizing the intestines, and the composition of the mother's milk can drive GIT colonization. After birth, Proteobacteria, such as *Escherichia coli*, may predominate, but the microflora rapidly shifts to lactic acid bacteria due to milk consumption (Varel and Wells, 2005). In ruminants, the story is more complex because of the rumen. The rumen microbiota develops slowly after birth. In a NGS study targeting the 16S rRNA gene variable regions 3 to 5 (V3-V5), at 14 d of age genera belonging to the phyla Bacteroidetes, Firmicutes, and Proteobacteria accounted for nearly 98% of the bacterial community (Li et al., 2012). The phyla Bacteroidetes increased in abundance and the phyla Proteobacteria decreased by 12 m of age. Specifically, the phyla Proteobacteria represent nearly 20%, 10%, and >1% of the microbes in the rumen on d 14, d 42, and 12 m of age.

The development of the bovine fecal microflora appears to be faster than in the rumen. In a study targeting the 16S rRNA gene variable region 4 (V4), the phyla Bacteroidetes was most abundant followed by Firmicutes and Proteobacteria (45, 31, and 21%, respectively) for dairy calves receiving pasteurized milk (Edrington et al., 2012) at 1 w of age. At 2 w of age, these predominant phyla did not change much, but by 4 w of age, the phyla Proteobacteria represented only 6.9% of the community and less than 3% at 6 m of age. Interestingly, this same study analyzed data from calves fed non-pasteurized milk and found that pasteurization resulted in a less variable population of microbes at 6 m of age. In a different study targeting variable regions 1 and 2 (V1-2) of the 16S rRNA gene in fecal samples collected from dairy calves from 1 to 7 w of age, the phyla Proteobacteria was less abundant than the Firmicutes (Oikonomou, et al., 2013). However, the authors did note that abundance of the genus *Fecalibacterium* was positively associated with less diarrhea and higher body weights for the calves. Once the microbial community is established in the GIT after weaning and the animal has adapted to a solid diet, questions remain regarding effects of diet and are there potential benefits of certain inhabitants.

Using traditional bench-top microbial techniques to isolate bacteria, *Escherichia coli*, a species of the bacterial phylum Proteobacteria, was most frequently isolated from digesta and feces of adult cattle (Maki and Picard, 1965). However, when proper anaerobic isolation techniques were applied, the predominant bacterial genera isolated from feces belonged to the two phyla Firmicutes and Bacteroidetes (Krause and Khafipour, 2011). Comparison of reported data from several analyses of 16s rRNA sequence indicated that the bovine fecal microbiota is diverse, but like humans the genera belonging to the phyla Firmicutes and Bacteroidetes are the predominant flora observed for cattle feces across most studies (Wells et al., 2014).

In recent years, several reports have assessed the composition of the bovine fecal microbiota from dairy and beef cattle using 16S rRNA gene sequencing. Using amplicon cloning and sequencing of 11,171 near full-length 16S rRNA gene clones, Durso et al. (2010) identified 7 phyla groups in samples from 6 beef heifers fed a low concentrate diet. The phyla Firmicutes, Bacteroidetes, and Proteobacteria collectively accounted for 96.7% (92.8, 29.5, and 4.4%, respectively) of the microbial OTUs observed. The phyla Tenericutes and Verrucomicrobia were next abundant OTUs, but only found at 0.6 and 0.05%, respectively. The genus *Prevotella* was the most abundant taxon and ranged from 7 to 40% of the sequences and 45 genera were observed across the 6 animals. The genus *Prevotella* was least variable at 5.7-fold difference, whereas the genus *Anaerostipes* was the most diverse at over 14-fold difference across all six animals. Overall, there appeared to be much variation from animal-to-animal, even though the heifers had been housed in the same feedlot pen for 8 weeks prior and were fed as a group the same diet.

The effect of diet on fecal microbiome can be even more significant. Once again using amplicon cloning, Durso et al. (2012) sequenced 20,002 near full-length 16S rRNA gene clones from feces of 20 beef steers cattle fed either a corn-based finishing ration or a diet where 40% of the corn was replaced with WDGS on a dry matter basis. Previous research had demonstrated that feeding wet distillers grain with solubles (WDGS) at high levels altered the fecal environment and was associated with increased shedding of *Escherichia coli* O157:H7 (Wells et al., 2009). In the microbiome study by Durso et al. (2012), 10 phyla were observed from the 14,591 OTUs and many of the observed taxa groups representing 42 genera were present in all samples. However, only 4.5% of the identified OTUs (97% cutoff) were observed in fecal samples for animals across both diets. Multiple OTUs can be observed for the same species, and this latter result indicated that while species were observed across all animals there was potential variation in grouping at the sub-species. Some of the more abundant genera associated with the corn

diet were *Blautia*, *Faecalibacterium* and *Sarcina*, whereas the more abundant genera associated with the WDGS diet were *Ruminococcus, Desulfonispora, Alistipes, Hydrogeoanaerobacterium* and *Phocaeicola*. Shifts in microflora also were observed, with *Prevotella* higher with the corn diet and *Bacteroides* higher with the WDGS diet. The WDGS diet lead to over-representation by the genera *Sporacetigenium* and *Anaerovorax,* which may be due to components in the diet.

Using NGS, Rice et al. (2012) was able to sequence deeper into the fecal microbiome of 20 animals fed 1 of 5 stream-flaked corn based diets that varied in the level and type of distillers grains that were fed. In this study, 24 phyla types were observed from the 127,530 OTUs that were sequenced utilizing amplicons of the variable region 4 (V4). Using NGS, the diversity was more apparent with 80 different species accounting for 91% of the sequence. As observed by Durso et al. (2012), diet affected the abundance of *Ruminococcus, Desulfonispora* and *Hydrogeoanaerobacterium*, but in this latter study by Rice et al. (2012) diet also affected the genera *Clostridium, Oscillibacter, Tannerella, Parabacteroides, Pseudoflavonifractor, Acetovibrio, Ethanoligenens, Selenomonas,* and *Barnesiella*.

Shanks et al. (2011) were the first to observe potential variation in the fecal microbiome across multiple types of diets and locations. Using NGS targeting the variable region 6 (V6), the authors were able to generate 633,877 sequences from feces of 30 beef cattle fed either forage, processed grain or unprocessed grain diets. Although the sequences were short reads (66 base pairs), the authors observed much variation in the taxa groupings at the phyla level due to diet. It was clear the microbiota community as a whole differed due to diet. However, like many previous studies, the lack of observations for each diet precluded the identification of a core microbiome at the species level.

A recent report from the U.S. Meat Animal Research Center has provided greater detail about the animal-to-animal and dietary variations for the fecal microbiota in beef cattle (Kim et al., 2014). Amplicon sequence data for the 16S rRNA gene variable regions 1-3 (V1-3) was collected from individual animal composited samples from 426 animals fed high (83% corn), moderate (67% corn), or low concentrate (33% corn silage) type of diet. The number of usable sequence reads per sample varied from less than 900 to nearly 15,000, and 333 individual samples had ≥2000 sequence reads that were ≥500 nucleotides in length. Nearly 2.15 million sequence reads were obtained. Typical to most studies, sequence data was classified directly into taxa groups for initial analyses.

When classified at the phylum level by Kim et al. (2014), there were 21 phyla observed. Firmicutes was the most abundant across all diets (Table 1), and abundance differed for all three diets. Interestingly, the moderate concentrate diet had the lowest abundance for Firmicutes.

Table 1. Relative abundance of dominant phyla in feces from 333 cattle fed diets differing in levels of concentrate.

	Percent of Total Sequences		
Phylum Classification	**High Concentrate**	**Moderate Concentrate**	**Low Concentrate**
Firmicutes	70.2	50.3	76.9
Bacteroidetes	1.8	37.4	12.8
Proteobacteria	2.4	6.0	1.3
TM7	10.9	0.2	0.6
Actinobacteria	2.9	0.1	1.8
Cyanobacteria	0.1	1.1	0.1
Verrucomicrobia	1.2	0.1	0.1
Unclassified Bacteria	10.1	4.5	6.3

Fecal Bacteroidetes differed by nearly 20-fold across the three diets, and had the highest abundance in the moderate concentrate diet. The Bacteroidetes were the second most abundant phyla for the high and moderate concentrate diet, and fifth most abundant phyla for the low concentrate diet. The Proteobacteria were less variable (4.5-fold difference), and this phylum was third most abundant for the moderate gain diet and fourth most abundant for the high and low concentrate diets. The phyla TM7 and Actinobacter both varied across diets nearly 50- and 10-fold, respectively, and these were more abundant in the low concentrate diet where they were second and third most abundant phyla present. Unclassified bacterial sequences accounted for 6.3, 4.5, and 10.1% of the total sequences for high, moderate, and low concentrate diets, respectively.

At the genus level in the study by Kim et al. (2014), there were 434 genera observed but only 22 genera had abundance greater than 0.5% for one or more of dietary treatments (Table 2). *Prevotella* was the most abundant genus which ranged from 0.09 to 14.4% across the diets, with moderate concentrate diet having the highest abundance. *Oscillibacter* and *Turicibacter* were the next most abundant genera and only varied 15-fold and 7-fold, respectively, with both genera being more abundant in the high concentrate diet. *Roseburia*, *Fecalibacterium*, and *Coprococcus* genera were more than 2% abundance in the collective data, and all three genera were lowest in the low concentrate diet. With the exception of the putative TM7 genera *incertae sedis*, the low concentrate diet did not have a dominant genus in high abundant like the other two diets. Within each phylum grouping, sequences that could not be sorted into a specific genus were binned into unclassified groupings at family or order levels. More than 50% of the sequences could not be binned into a genus level and were sorted into unclassified bins for each diet. Based on total sequences from samples for each diet, unclassified Firmicutes were nearly 30% with the moderate concentrate diet and 60% for the low concentrate diet. The inability to classify some of the data is

Table 2. Relative abundance of dominant genera in feces from 333 cattle fed diets differing in levels of concentrate.

Genus Classification	Phylum	Percent of Total Sequences		
		High Concentrate	Moderate Concentrate	Low Concentrate
Prevotella	Bacteroidetes	2.2	14.4	0.1
Oscillibacter	Firmicutes	8.1	4.6	0.7
Turcibacter	Firmicutes	8.4	1.3	4.4
Roseburia	Firmicutes	4.2	3.9	0.4
Fecalbacterium	Firmicutes	1.8	4.1	0.1
Coprococcus	Firmicutes	2.9	2.4	1.0
Gen. incertae sedis	TM7	0.6	0.2	10.9
Succinivibrio	Proteobacteria	0.4	4.5	0.1
Clostridium	Firmicutes	2.9	1.1	1.3
Blautia	Firmicutes	1.7	0.2	0.1
Bacteroides	Bacteroidetes	0.8	1.0	0.1
Lactobacillus	Firmicutes	1.5	0.3	0.1
Parabacteroides	Bacteroidetes	0.6	0.9	0.1
Streptophyta	Cyanobacteria	0.1	1.1	0.1

partially a reflection of the database and the classifier, but also a reflection of how little we know about the types of bacteria in the bovine intestinal tract.

The community structure and diversity of the bovine fecal microbiome can be analyzed using OTUs analysis (Kim et al., 2014). With this method, sequences are sorted into closely related groupings based on sequence similarity and a consensus sequence is used for taxa assignment. The 2,149,008 sequence reads grouped into 176,692 OTUs (at 0.97 similarity cutoff), whereas when directly classified as discussed above there were only 454 genera observed. Within the OTUs groups, 56% of the groups had only one sequence read (singleton), indicating that there is potential diversity of bacteria at low abundance in this large data set. The moderate concentrate diet exhibited the highest number of OTUs, indicating the highest amount of diversity. The remaining 78,166 OTUs represented sequences that were present multiple (two or more) times in the samples, and when plotted on a Venn diagram only 33,743 OTUs (43.2% of the OTUs with multiple reads) were observed in samples for more than one diet. Only 2,359 OTUs were present for samples from all three diets (Figure 2), suggesting that the bovine microbiota is highly diverse and dependent on the diet. The number of shared OTUs was highest for the high and moderate concentrate diets, and the low concentrate diet had the lowest shared OTUs. The results overall indicate that there is not a core fecal microbiome based on bacterial types and that association analysis between the microbiota and host phenotypes need to account for diet.

The role fecal microbiota may play on *E. coli* O157:H7 colonization and prevalence has not been reported. In

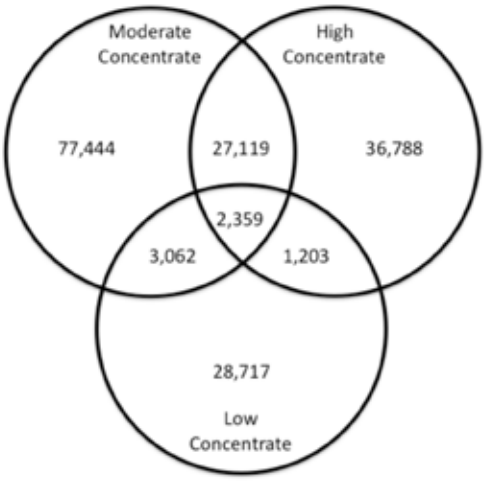

Figure 2. Venn diagram showing the number of OTUs shared within and across diet groups for cattle fed a high, moderate, or low concentrate diet typical to beef production.

the data from Kim et al. (2014), the community structure did not differ between animals not shedding and shedding *E. coli* O157:H7 when analyzed within diet. In addition, no single taxa or OTU was strongly associated with shedding. Using linear regression with the same sequence data, Kim et al. (unpublished data) have looked at OTUs that shift with pathogen shedding and observed that 31, 51, and 49 OTUs weakly correlated with pathogen prevalence for low, moderate, and high concentrate diets, respectively. Most of the OTUs were positively correlated with *E. coli* O157:H7, but some OTUs were negatively

correlated. None of the OTUs associated with *E. coli* O157:H7 were present in all three diets. Combinations of OTUs may be more important, but the role of multiple OTUs on pathogen shedding needs to be determined.

CONCLUSIONS

The fecal microbiome is important to host health. The bovine fecal microbiota is complex and the composition of the microbiome can vary with diet. Diet appears to have a greater affect on the fecal microbiome than animal-to-animal variation. Current studies have provided insight into the microbial populations, but most studies have only analyzed the predominant microflora. Metagenomic approaches to determine total DNA sequence may indicate that bacterial biochemical pathways are more important than specific microbial composition in relation to host phenotypes. However, deeper microbiome sequencing may be needed to determine if bacterial populations in lower abundance compete with pathogenic bacteria and play a role in host colonization.

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