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Characterization of fecal microbiota from a *Salmonella* endemic cattle herd as determined by oligonucleotide fingerprinting of rDNA genes☆

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ABSTRACT

The gastrointestinal (GI) tract microbiota is composed of complex communities. For all species examined thus far, culture and molecular analyses show that these communities are highly diverse and individuals harbor unique consortia. The objective of the current work was to examine inter-individual diversity of cattle fecal microbiota and determine whether *Salmonella* shedding status correlated with community richness or evenness parameters. Using a ribosomal gene array-based approach, oligonucleotide fingerprinting of ribosomal genes (OFRG), we analyzed 1440 16S genes from 19 fecal samples obtained from a cattle herd with a history of salmonellosis. Identified bacteria belonged to the phyla Firmicutes (53%), Bacteroidetes (17%), and Proteobacteria (17%). Sequence analysis of 16S rDNA gene clones revealed that Spirochaetes and Verrucomicrobia were also present in the feces. The majority of Firmicutes present in the feces belonged to the order Clostridiales, which was verified via dot blot analysis. β-Proteobacteria represented 1.5% of the bacterial community as determined by real-time PCR. Statistical analysis of the 16S libraries from the 19 animals indicated very high levels of species richness and evenness, such that individual libraries represented unique populations. Finally, this study did not identify species that prevented *Salmonella* colonization or resulted from *Salmonella* colonization.

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1. Introduction

Cattle are a natural reservoir for *Salmonella*, hosting a range of serovars with varying degrees of pathogenicity for both bovine and human hosts. Dairy herds in particular are susceptible, with actively shedding members in 27–31% of US herds (USDA-APHIS, 2003). It has been reported that *Salmonella* prevalence within a herd can range from <1 to 97% while varying over time (Huston et al., 2002a,b). These results indicate that within *Salmonella*-positive herds, some animals remain *Salmonella* free for extended periods. The role of the intestinal microbiota in this phenomenon is unknown. The objective of the current work was to examine inter-individual diversity of cattle fecal microbiota and determine whether *Salmonella* shedding status correlated with community diversity parameters.

We examined the microbiota from a herd of Holstein cattle with a heterogenous history of shedding *Salmonella*. These animals were unrelated, but had been cohabiting for at least 3 years. Thus, *Salmonella* shedding status was unlikely to derive from environmental or dietary factors. We examined 1440 bacterial 16S clones to provide a measure of the inter-individual variability of the intestinal

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microbiota and correlate that to *Salmonella* shedding status. Results indicated a predominance of Firmicutes, Bacteroidetes and Proteobacteria, with little redundancy at the genus/species level. Comparison of microbiota from *Salmonella* shedding and non-shedding individuals indicated similar richness and evenness parameters, although weak evidence indicated one species, *Ruminobacter amylophilus*, may correlate with *Salmonella* shedding status. These data indicate that species diversity within and between animals is sufficiently high that identification of *Salmonella*-protective microbiota will require significant effort using high-throughput sequencing methods and hundreds of animals.

2. Materials and methods

2.1. Media and culture conditions

Fecal samples from 19 Holstein cows from a farm in Minnesota, USA were taken via rectal palpation during a standard veterinary examination. Fecal samples were shipped to the laboratory on ice and either used immediately for DNA isolation or stored at −80 °C until used. Clone libraries were cultured in Luria–Bertani (LB) broth (MP Biomedicals, Solon, OH) containing 100 μg/ml ampicillin (Sigma, St. Louis, MO) and stored at −80 °C in Luria–Bertani (LB) broth (MP Biomedicals, Solon, OH) containing 30% glycerol and 100 μg/ml ampicillin (Sigma, St. Louis, MO). Enrichment culture of the samples did not alter the *Salmonella* status. Fecal sampling and culture for *Salmonella* was performed yearly prior to the community analysis. Feces (~1 g) were diluted 1:10 in PBS and plated on BGS plates (50 μl/plate). Presumptive colonies were subjected to *Salmonella* serogrouping agglutination using polyclonal antisera (DIFCO, Corpus Christi, TX). *Salmonella*-negative status was given to animals that were fecal culture negative for 3 consecutive years. Animals were considered *Salmonella*-positive if fecal cultures were positive in at least 2 of the 3 years.

2.2. DNA isolation

DNA was isolated from feces using the Qbiogene Bio101 Fast DNA kit (Irvine, CA) with some modifications to the manufacturer's instructions. Briefly, fecal samples (0.2 g) were combined with 1 ml L7 buffer (5.25 M guanidine hydrochloride, 50 mM Tris–HCl pH 6.4, 20 mM EDTA, 1.3% (w/v) Triton-X 100, and 1 mg/ml α-casein) with Lysing Matrix A (Boom et al., 1999). Lysis was performed with a Fast Prep FP120 (Bio101 Savant, Qbiogene, Irvine, CA) for 30 s at 5 m/s. The samples were incubated on ice for 5 min and centrifuged for 15 min at 13,200 rpm with an Eppendorf Centrifuge 54150 (Wesbury, NY). The resulting supernatant was transferred to a clean 1.5 ml eppendorf tube and 600 μl of bind matrix was added, mixed for 5 min, and centrifuged for 30 s at 13,200 rpm. The supernatant was discarded and the pellet was washed twice with 500 μl SEWS-M buffer with centrifugation between washes at 13,200 rpm. The resulting pellet was dried in a CentriVap concentrator (LabConco, Kansas City, MO) for 20 min, followed by resuspension with 100 μl 10 mM Tris–HCl pH 8. DNA was further purified by electrophoresis in a 1% agarose gel and DNA larger than 3 kb was excised and recovered using the QIAquick gel extraction kit according to manufacturer's instructions (Qiagen, Valencia, CA). DNA was further purified using a QIAquick PCR clean up kit according to manufacturer's instructions (Qiagen, Valencia, CA).

2.3. OFRG analysis

OFRG analysis was performed as previously described by Valinsky et al. (2004). Briefly, bacterial 16S rDNA gene clone libraries were constructed in *E. coli* DH5α using the USER Friendly Cloning Kit (New England Biolabs, Ipswich, MA) and PCR primers 27F (5′-GGGAAAGUAGRRTTTGA-TYHTGGYTCAG-3′) and 1492R (5′-GGAGACAUGBTACCTT-GTTACGACTT-3′) (Bent et al., 2006; Lane, 1991). PCR was performed in 20 μl containing 50 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 250 μM dNTPs, 0.5 mg ml^{-1} BSA, 400 nM forward and reverse primers, 1 μl fecal DNA, and 1.75 U Taq DNA polymerase. Amplification was carried out using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) with an initial denaturation at 94 °C for 5 min, cycling of 94 °C for 30 s denaturation, 48 °C for 40 s annealing, and 72 °C for 60 s extension, with a final elongation for 2 min at 72 °C. The number of amplification cycles varied between 15 and 30 cycles for each DNA sample, which was determined from the fewest cycles to generate a PCR product barely visible when 5 μl were examined on an agarose gel. A 1440-clone library was generated, with 75 or 76 clones representing each of the 19 bovine fecal samples.

The 16S rDNA gene clone library was PCR-amplified using primers UserOFRGFor2 (5′-TCGAGCTCAGGCGCGCC-TTAATTAAGCTGA-3′) and UserOFRGRev2 (5′-GCCAAGCTT-CCTGCAGGGTTTAAACGCTGA-3′) in reactions containing 50 mM Tris pH 8.3, 0.5 mg ml^{-1} BSA, 2.5 mM $MgCl_2$, 250 μM dNTPs, 400 nM forward and reverse primers, 1 μl cells, and 1.75 U Taq DNA polymerase (Bent et al., 2006). Amplification was performed with an initial denaturation at 94 °C for 10 min, 35 cycles of 94 °C for 1 min, 72 °C for 2 min, and a final elongation for 5 min at 72 °C. Amplicons were arrayed onto nylon membranes with a multi-blot replicator (V&P Scientific, Inc., San Diego, CA) as described previously (Valinsky et al., 2004). Membranes were hybridized overnight at 11 °C with a set of 10 nt, bacterial specific ^{33}P-labeled DNA probes (Valinsky et al., 2004). Two arrays were hybridized for each probe, stripped as described previously, and rehybridized with a universal probe 27F (5′-AGRRTTT-GATYBTGGYTCAG-3′). Hybridizations were visualized using a Typhoon Variable Mode Imager (Amersham Biosciences, Pittsburgh, PA) and hybridization signals were analyzed with Image Quant TL image analysis software, v2003 (Amersham Biosciences, Pittsburgh, PA). Fingerprints containing N (neither positive nor negative hybridization event), 1 (positive hybridization event), and 0 (negative hybridization event) were generated based on control clone hybridization intensities and Bayesian classification (Jampachaisri et al., 2005). Clone fingerprints containing more than 8 uncertain (*N*) classifications were discarded from further analysis. OFRG fingerprints were clustered using

Greedy Clique Partitioning (GCPAT; http://algorithms.cs.ucr.edu/OFRG/index.php). Evenness, Shannon's diversity index, and the number of operational taxonomic units (OTUs, defined as identical fingerprints) were also determined via GCPAT. Libraries of OFRG fingerprints from *Salmonella*-positive and *Salmonella*-negative cows were compared using the SONS software (Schloss and Handelsman, 2006).

2.4. Sequencing analysis

Two hundred and forty-two representative clones from OFRG clusters were sequenced. Clone sequencing was performed using primers UserOFRGFor2 (5′-TCGAGCTCAGGCGCGCCTTAATTAAGCTGA-3′), UserOFRGRev2 (5′-GCCAAGCTTCCTGCAGGGTTTAAACGCTGA-3′), 530F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Bent et al., 2006; Lane, 1991). Sequences were aligned and edited using Lasergene software (DNASTAR, Madison, WI). The sequences were compared to public databases using NCBI BLAST and the Ribosomal Database Project II (RDP)(Cole et al., 2005). Sequences were submitted to GenBank under accession numbers EU794074–EU794314. Three novel sequences were analyzed for chimeric structure using the program Pintail, and one sequence was removed as a result (Ashelford et al., 2005).

2.5. Dot blot analysis

Dot blot analysis was performed as described previously with modifications (Dore et al., 1998; Scupham et al., 2008). Briefly, 250 ng/μl of total DNA was diluted in DBEB (0.1 N NaOH and 1 mM EDTA) and applied to Hybond N+ nylon membranes (Amersham Biosciences, Piscataway, NJ) with a BioRad Bio-Dot dot blotter (BioRad, Hercules, CA). Twenty-five nanograms of PCR-amplified Clostridium group IV, group IX and group XIV 16S sequences, as well as those for Bacteroides, *Faecalibacterium* and *Mucispirillum*, were applied to the membranes as controls (Collins et al., 1994). DNA was cross-linked onto the nylon membranes with a UV Stratalinker 1800 (Stratagene, La Jolla, CA) at 70 mJ/cm^2. Probes Eub338, Erec482, Clept1240, Bacto1080, and Prop853 (Table 1) were ^{33}P-labeled by combining 1× kinase buffer, 1 unit T4 polynucleotide kinase, 1 μM probe, and 1.5 μl ^{33}P-ATP and incubating at 37 °C for 30 min (Amann and Ludwig, 2000; Amann et al., 1990; Franks et al., 1998; Sghir et al., 2000). Following a 1 h prehybridization in 5× Denhardt's buffer (5× SSC, 0.5% SDS, and 100 μg/ml salmon sperm DNA) ^{33}P-labeled probes were added to the membranes. Membranes were hybridized overnight at 45 °C and then washed twice for 15 min at 50 °C with primary wash buffer (0.5% SDS and 4× SSC) (Table 1). For ease of use with a 50 °C wash temperature, secondary washes were optimized as described previously (Scupham et al., 2008). Membranes were washed with the appropriate secondary wash buffer (Table 1) for 30 min at 50 °C followed by exposure to a storage phosphor screen for 5 h. The image was visualized on a Typhoon 9410 imager (Amersham, Piscataway, NJ). Hybridization is described as the ratio of signal from the specific probe divided by the signal from the universal bacterial probe Eub338. Reported results are averages of all 19 samples hybridized separately.

2.6. Real-time PCR

Real-time PCR was performed on the total DNA isolated from the fecal samples with iQ SYBR Green Supermix (Bio Rad, Hercules, CA) according to the manufacturer's instructions. Briefly, real-time PCR reactions were composed of 1× iQ SYBR Green Supermix, 400 nM β-proteobacteria primers Beta1F (5′-AGCCGCGGTAATAC-3′) and Beta1R (5′-GGTATCTAATCCTG-3′) or universal primers 342R (5′-CTGCTGCSYCCCGTAC-3′) and 27F (5′-AGRRTTTGATYBTGGYTCAG-3′). Primers Beta1F and Beta1R were designed for this study using the PRISE software (Fu et al., 2007). Real-time PCR amplification of the β-proteobacteria 16S rDNA gene was performed on an iCycler IQ5 optical system (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 51 °C 30 s, 72 °C for 1 min, and a final elongation for 5 min at 72 °C. Real-time PCR with the universal primers was carried out with an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 56 °C 30 s, 72 °C for 1 min, and a final elongation for 5 min at 72 °C. The number of 16S rDNA gene copies is described as the ratio of signal from the specific primers divided by the signal from the universal bacterial primers.

3. Results

3.1. OFRG analysis

OFRG results are reported in Tables 2 and 3. Analysis was performed on a total of 1440 clones from 19 Holstein cow fecal samples. Of the 1440 fingerprints generated from these clones, 240 fingerprints contained over eight uncertain (N) assignments and were discarded from further analysis, leaving an average of 63 (range, 58–69) clones per sample. OFRG analysis assigned the clones into the phyla Firmicutes, Bacteroidetes, and Proteobacteria

Table 1
Dot blot oligonucleotide probes and experimental wash conditions.

Probe	Sequence (5′–3′)	Experimental secondary wash buffer[a]	Reference
Clept1240	GTTTRTCAACGGCAGTC	0.5% SDS + 0.83× SSC	Sghir et al. (2000)
Bacto1080	GCACTTAAGCCGACACCT	0.5% SDS + 0.25× SSC	Dore et al. (1998)
Erec482	GCTTCTTAGTCARGTACCG	0.5% SDS + 0.63× SSC	Franks et al. (1998)
Prop853	ATTGCGTTAACTCCGGCAC	0.5% SDS + 0.2× SSC	Walker et al. (2005)
Eub338	GCTGCCTCCCGTAGGAGT	0.5% SDS + 0.75× SSC	Amann et al. (1990)

[a] 10× SSC was comprised of 1.5 M sodium chloride and 0.15 M sodium citrate, pH 7.0.

Table 2
Taxonomic distribution of 1200 rRNA 16S gene clones from *Salmonella*-positive and *Salmonella*-negative cow feces.

Taxon	No. clones[a]		
	Salmonella-positive	*Salmonella*-negative	Total
Firmicutes	**355 (52%)**	**301 (59%)**	**656 (55%)**
Phylum: Firmicutes	34 (5%)	34 (7%)	68 (6%)
Order: Clostridiales	53 (8%)	33 (6%)	86 (7%)
Family: Ruminococcaceae	236 (34%)	191 (37%)	427 (36%)
Genus: Lachnospiraceae Incertae Sedis	32 (5%)	43 (8%)	75 (6%)
Bacteroidetes	**130 (19%)**	**71 (14%)**	**201 (17%)**
Order: Bacteroidales	109 (16%)	61 (12%)	171 (14%)
Family: Porphyromonadaceae	21 (3%)	10 (2%)	31 (3%)
Proteobacteria	**116 (17%)**	**66 (13%)**	**182 (15%)**
Genus: *Rhizobium*	1 (<1%)	5 (1%)	6 (<1%)
Class: β-proteobacteria	36 (5%)	18 (4%)	54 (5%)
Genus: *Ralstonia*	73 (11%)	33 (6%)	106 (9%)
Class: γ-proteobacteria	6 (1%)	10 (2%)	16 (1%)
Unresolved	**88 (13%)**	**73 (14%)**	**168 (14%)**
Total	**689**	**511**	**1200**
Number OTUs[b]	497	635	1092
Shannon index (H')	6.20	6.42	6.96
Evenness (E)	1.0	1.0	0.99

[a] Percent represents the ratio of clones for each *Salmonella* positive/negative group.
[b] Operational taxonomic units, defined here as identical fingerprints.

Table 3
Percent phyla identified in the individual cattle feces.

Sample ID no.	Bacteroidetes	Firmicutes	Proteobacteria	Unresolved[a]	No. clones	Shannon index (H')	Evenness (E)
Salmonella-negative							
529	7.5[b]	5.4	1.9	3.6	58	4.09	1.0
546	5.2	6.7	3.3	6.3	67	4.22	1.0
547	5.2	7.1	2.4	8.0	69	4.22	1.0
553	4.7	7.5	1.9	5.4	67	4.21	1.0
568	2.3	7.1	2.4	7.1	62	4.21	1.0
604	2.8	6.4	4.9	7.1	64	4.16	1.0
633	6.6	6.6	1.5	7.1	66	4.12	1.0
1530	0.5	1.1	21.8	2.6	56	3.95	0.9
Salmonella-positive							
511	5.6	6.3	4.4	3.6	64	4.16	1.0
586	3.2	6.9	3.8	4.5	63	4.13	1.0
623	5.6	6.7	1.0	3.6	60	4.16	1.0
632	10.8	4.4	1.5	5.4	61	4.16	1.0
638	6.1	6.7	2.4	8.0	69	4.21	1.0
657	8.0	5.9	1.9	5.4	64	4.12	1.0
869	5.2	5.4	3.8	7.1	61	4.09	1.0
1290	3.7	1.6	17.5	3.6	58	3.91	0.9
1308	13.1	4.0	4.9	3.6	58	4.01	1.0
1445	1.4	2.1	20.9	2.6	62	4.02	0.9
1608	2.3	1.9	1.9	5.4	60	4.11	1.0
p-Value	0.2955	0.2024	0.8022			0.2525	0.7543
No. clones	213	658	206	112	1189[c]		

[a] Unresolved refers to clusters containing multiple phyla.
[b] Percents are the ratio of phyla of the individual fecal samples to total clones in phyla.
[c] Eleven misgrouped clones were not counted.

(Table 2). Firmicutes was the most abundant phylum present and the family Ruminococcaceae was most prevalent in this phylum (Table 2). All Bacteroidetes belonged to the order Bacteroidales, 15% (31/201) of which belonged to the family Porphyromonadaceae. The Proteobacteria were represented by 15% of the total clones (Table 2). Of the 1200 analyzed clones, OFRG was unable to classify 13% and these were subsequently designated as unresolved (Table 2). The 1200 clones included 1092 OTUs with a Shannon's diversity index (H') of 6.96 and evenness (E) of 0.995 (Table 2). Individual fecal samples all possessed a similarly high diversity, with H' between 3.91 and 4.22 and E = 0.993–1.0 (Table 3). No differences in phylogenetic richness or evenness were identified between the *Salmonella* shedding and non-shedding groups; 497 OTU were detected in the *Salmonella*-positive

sample (H' = 6.20, E = 0.997) while 635 OTU were detected in the *Salmonella*-negative samples (H' = 6.42, E = 0.995) (Table 2). Student's *t*-test of the number of Firmicutes, Bacteroidetes and Proteobacteria clones between the shedders and non-shedders all indicated *p*-values $\geq$0.202. Jaccard and Sorensen analyses of the OFRG data indicated that <10% of the OFRG fingerprints that derived from *Salmonella*-positive cows were also present in samples from *Salmonella*-negative cows. The non-parametric maximum likelihood estimator θN indicates low probability (<5%) that a given fingerprint present in one library is present in both.

3.2. Sequencing of OFRG clones

Sequencing was performed on 16S rDNA clones representing clusters throughout the OFRG dendrogram. A total of 242 16S rDNA gene clones were sequenced, representing the phyla Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, and Verrucomicrobia (Table 4). Clones were predominantly Firmicutes (50%, 120/242), and possessed greater than 80% similarity to published sequences (Table 4). The genera *Brochothrix*, *Caryophanon*, *Anaerovorax*, *Anaeroplasma*, *Mycoplasma*, and *Roseburia* were identified within the Firmicutes phylum and possessed a minimum of 92% similarity to cultured isolates (Table 4). The order Bacteroidales represented 25% (61/242) of the sequenced clones (Table 4). Genera identified using the RDPII classifier included *Bacteroides*, *Paludibacter* and *Alistipes*, although BLAST analysis indicated only low (<96%) sequence similarities to cultured isolates. The phylum Proteobacteria represented 20% (48/242) of the sequenced clones. Seventy percent (23/33) of the sequenced β-proteobacteria clones were *Ralstonia*, similar (96–99%) to the *Ralstonia insidiosa*, *Ralstonia pickettii* and *Ralstonia solanacearum* species. The γ-proteobacteria were comprised of the genera *Ruminobacter*, *Shigella*, *Acinetobacter*, and *Succinivibrio*. Clones representing the phylum Spirochaetes were all of the genus *Treponema*, some with 99% sequence similarity to *Candidatus T. suis* (Table 4).

3.3. Fecal bacteria quantification

Dot blot hybridizations were performed to validate the prevalence of taxa described by the OFRG data and to quantify the Bacteroidetes and Clostridia groups IV, IX, and XIV within the fecal samples (Table 1). Clostridium group XIV comprised 34% of the fecal bacterial 16S sequences while 24% derived from Bacteroides, 14% were Clostridium group IX and 11% were Clostridium group IV (Table 5). Hybridizations supported the OFRG calculation of no significant quantitative differences between the Clostridia or Bacteroidetes of the *Salmonella*-positive and *Salmonella*-negative groups of animals. Dot blot hybridizations were not sensitive enough to quantify the low numbers of β-proteobacteria, therefore real time PCR was performed. Real-time PCR revealed that the β-proteobacteria comprised an average of 1.5% of the bacteria in the 19 cow fecal samples, with no difference between the *Salmonella*-positive and *Salmonella*-negative samples (p = 0.7387).

4. Discussion

OFRG was used to determine the bacterial assemblage in feces from 19 Holstein cows with a heterogenous history of shedding *Salmonella* despite ≥3 years of cohabitation. No phylum-level differences were detected between OFRG libraries from *Salmonella* shedding and non-shedding cattle, but statistical analysis of OFRG fingerprints indicated that almost every fingerprint was unique, indicating high diversity at the genus/species level. In addition, sequence analysis of 242 clones representing OFRG clusters identified no identical clones. These results are consistent with recent results indicating unique microbial consortia in individual animals (Ley et al., 2008).

Two previous studies examined Holstein feces using 16S rDNA gene sequence analysis (Dowd et al., 2008; Ozutsumi et al., 2005). Ozutsumi et al. (2005) reported that their samples were composed of phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Spirochaetales. Dowd et al. (2008) also identified sequences from phylum Sphingobacteria. We did not identify Actinobacteria or Sphingobacteria but did identify Verrucomicrobia with low (92%) similarity to other uncultured clones from a variety of intestinal sources. Sequence analysis of our Spirochaetales clones identified anaerobic host-associated microbes of the genus *Treponema*. Multiple types of *Treponema* were identified, however, treponeme populations in cattle feces appear different from the populations in the rumen (Paster and Canale-Parola, 1982). One spirochaete sequence was 99% similar to a colitis-associated organism identified in swine feces, two had low (90–93%) identity to 16S sequences isolated from swine, one was 90% similar to *T. brennaborense*, a bovine dermatitis-associated microbe, and one was 99% similar to a spirochaete identified by the Ozutsumi analysis of bovine feces (Table 4) (Leser et al., 2002; Molbak et al., 2006; Ozutsumi et al., 2005; Schrank et al., 1999). Sequences by Dowd et al. (2008) have not yet been released to the public, thus comparison of our treponeme sequences to those previously reported is not currently possible.

Ralstonia 16S sequences comprised 11% of the clones from *Salmonella*-positive animals and 6% of clones from *Salmonella*-negative animals (Table 2). The *R. pickettii* group identified here is known to be an important nosocomial pathogen, and has been isolated from water, soil and clinical samples (Ryan et al., 2006). To our knowledge, this report is the first to identify it as a member of the fecal biota. To examine whether the OFRG based difference was real or a PCR artifact, a real-time primer pair specific for the β-proteobacteria was developed. SYBR green real-time PCR indicated that β-proteobacteria 16S genes comprised around 1.5% of the total, regardless of *Salmonella* status.

Analysis of the OFRG and sequence data identified one group potentially unique to *Salmonella*-negative samples, the γ-proteobacteria *R. amylophilus*. *R. amylophilus* is a starch-digesting species that is considered to be a primary amylolytic rumen species in cattle fed a high-starch diet (Anderson, 1995) (Table 4). A previous review suggests high-starch diets may exacerbate *E. coli* colonization

Table 4
Taxonomic distribution of 242 sequenced rDNA 16S gene clones.

Phylum	Class	Order	Family	Genus	No. clones (%)	Closest cultured relative
Firmicutes					**121** (50)	
Firmicutes					1	
	Bacilli	Bacillales	Listeriaceae	*Brochothrix*	1	*B. thermosphacta* M58798 99%
			Planococcaceae	*Caryophanon*	1	*B. silvestris* AJ006086 97%
	Clostridia				1	
		Clostridiales			2	
			Incertae Sedis XIII		3 (1)	
				Anaerovorax	1	
			Lachnospiraceae		5 (2)	
				Incertae Sedis	15 (6)	*R. schinkii* X94964 95%
				Roseburia	1	*R. faecalis* AY804150 93%
			Ruminococcaceae		77 (32)	
				Incertae Sedis	3 (1)	*B. capillosus* AY136666 95%
			Veillonellaceae		3 (1)	
	Erysipelotrichi	Erysipelotrichales			1	
			Erysipelotrichaceae		2	
	Mollicutes				1	
		Anaeroplasmatales	Anaeroplasmataceae	*Anaeroplasma*	2	*A. abactoclasticum* M25050 92%
		Mycoplasmataceae	Mycoplasma	*Mycoplasma*	1	*M. penetrans* L10839 100%
Bacteroidetes					**60 (25)**	
	Bacteroidetes	Bacteroidales			29 (12)	
			Bacteroidaceae	*Bacteroides*	10 (4)	*B. coprocola* AB200225 96%
			Porphyromonadaceae		11 (5)	
				Paludibacter	3 (1)	*P. propionicigenes* AB078842 87%
			Rikenellaceae	*Alistipes*	7 (3)	*A. finegoldii* AY643084 94%
Proteobacteria					**48** (20)	
	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	1	
		Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	1	*S. echinoides* AJ012461 99%
	Betaproteobacteria	Burkholderiales			1	
			Alcaligenaceae	*Sutterella*	3 (1)	*S. stercoricanis* AJ566849 95%
			Burkholderiaceae	*Ralstonia*	23 (10)	*R. insidiosa* AJ539233 99% *R. pickettii* DQ908951 98%
			Comamonadaceae	*Delftia*	2	*D. acidovorans* EF421406 100%
			Comamonadaceae	*Variovorax*	1	*V. paradoxus* DQ256487 99%
		Neisseriales	Neisseriaceae	*Neisseria*	1	*N. flavescens* L06168 98%
		Nitrosomonadales	Nitrosomonadaceae	*Nitrosospira*	1	*N. multiformis* L35509 99%
		Rhodocyclales	Rhodocyclaceae		1	
	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae		2	
				Ruminobacter	5 (2)	*R. amylophilus* Y15992 92%
				Succinivibrio	3 (1)	*S. dextrinosolvens* Y17600 96%
		Enterobacteriales	Enterobacteriaceae	*Shigella*	1	*E. coli* CP000802 99%
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	2	*A. lwoffi* U10875 98%
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	*Treponema*	**9** (4)	*Candidatus T. suis* AM284386 99%
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Subdivision 5	Incertae Sedis	**1**	*A. agarolyticus* AF075271 82%
Bacteria					**2**	
Total					**242**	

(Callaway et al., 2003). *R. amylophilus* was detected only in three of the eight *Salmonella*-negative cows, but may indicate a correlation between starch-digesting functional microbiota and protection from *Salmonella* colonization. Further studies will be necessary to support this idea.

The data presented here indicate no differences in the richness or evenness diversity parameters of 16S libraries made from the feces of *Salmonella* shedding and non-shedding cattle. In addition, phylum-level microbiota differences between *Salmonella*-positive

Table 5
Dot blot analysis of DNA isolated from 19 fecal samples. The data represents the averages of all 19 fecal DNA samples hybridized separately.

Probe	Collins group	Average proportion ± (standard error of the mean)[a]		*p*
		Salmonella-positive	*Salmonella*-negative	
Clept1240	IV	11.6 ± 1.3	11.2 ± 1.4	0.8942
Prop853	IX	15.1 ± 2.0	12.6 ± 1.8	0.7473
Erec482	XIV	35.9 ± 3.4	31.7 ± 3.1	0.5435
Bacto		24.3 ± 2.2	23.4 ± 1.3	0.8470

[a] The average proportion is the average of the ratios of the hybridization signals from the specific probes divided by the signals from the universal bacterial probe Eub338.

and Salmonella-negative cattle feces were not detected. The results do highlight the high level of genus/species level microbial diversity between individual hosts, and the difficulty of identifying microbes driving specific community functions. As demonstrated by strains of *Lactobacillus* with probiotic potentials ranging from null to immunostimulatory, microbes protective against *Salmonella* may have exclusionary functions with little correlation to current species concepts (Ljungh and Wadstrom, 2006). Thus, identification of CE strains may require significant effort additional to identification of promising species.

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