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FOODBORNE PATHOGENS AND VIRULENCE IN THE MICROBIOME OF CATTLE GROWN NATURALLY VERSES CONVENTIONALLY

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Abstract – Foodborne illness and virulence factors (VF) are public health concerns present at variable abundances in beef microbiome communities. This study assessed abundance of beef pathogens and VF in feces of conventionally- and naturally-raised cattle. Forty-eight pens of cattle, 24 pens per treatment, were included in the study. Composite fecal samples were collected from each pen, DNA was extracted, and shotgun metagenomic DNA libraries were prepared and sequenced. Kraken was used for pathogen identification and the Virulence Factor Database was used to identify VF. No statistical difference (P>0.05) was detected between conventionally- and naturally-raised cattle in pathogen load or VF abundance. Results indicated that cattle diet does not play an important role in food safety risk.
Key Words – bovine, metagenomics, pathogenicity

INTRODUCTION

Foodborne illness is a public health and economic concern. The World Health Organization (WHO) estimated that 600 million illnesses and 420,000 deaths resulted from foodborne pathogens in 2010 [1]. Furthermore, WHO estimated that, in 2010, the global burden of foodborne illness was 33 million Disability Adjusted Life Years [1]. In 2011, bacterial pathogens were estimated to cause 39% of foodborne illnesses and 64% of foodborne deaths annually in the United States [2]. Virulence factors (VF) are genetic traits that allow microorganisms to establish themselves within a host, and/or escalate symptoms of disease [3]. Virulence factors are present in some meat bacterial pathogens, such as *Salmonella spp., Escherichia coli* O157:H7, and non-O157 Shiga Toxin-producing *E. coli*. Beef feedlots and abattoirs are common sources of foodborne pathogens, and some outbreaks are caused by pathogens with known VF [4, 5]. Natural cattle, defined here as cattle not exposed to antibiotics or growth promotion technologies during rearing, have gained popularity recently due to consumer's beliefs that these products are, among other things, safer and healthier than conventional beef [6]. Culture-based studies to quantify differences between naturally- and conventionally-raised cattle have reported few and generally inconsequential differences in pathogen loads [6, 7]. Here, we used next generation sequencing and microbial metagenomic techniques to investigate the ecological and possible food safety impacts of natural vs conventional cattle rearing systems.

MATERIALS AND METHODS

Study population

Forty-eight (N=48) pens (average pen size = 180; range 45 to 285) of cattle from commercial feedlots throughout the United States and Canada were included in the study, 24 from conventional systems and 24 from natural systems across five feedlots at different times of feeding (beginning of feeding period through immediately prior to slaughter). No cattle pens selected for inclusion showed pen-level signs of systemic disease.

Sample collection

One composite fecal sample was collected from each pen by combining feces collected by hand from 20 areas along crossing diagonals of each pen [8]. Composite fecal samples were collected in sterile bags and transported on ice to Colorado State University (Fort Collins, CO) for further processing.

DNA extraction

Ten grams of each fecal composite sample was thawed and DNA was extracted using the Mo-Bio PowerMax Soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA) following manufacturer's protocols. Quality and concentration were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Samples were concentrated with ethanol precipitation until reaching a concentration ≥20 ng/µl DNA.

Shotgun sequencing

A one hundred microliter aliquot of DNA from each sample was delivered to the Genomic and Microarray Core at the University of Colorado's Health Science Center for library preparation and sequencing (Denver, CO). Sequencing libraries were constructed using an Illumina TruSeq DNA Library Kit (Illumina, Inc. San Diego, CA). Eight samples per lane were run on an Illumina HiSeq 4000 (Illumina, Inc. San Diego, CA) with a 2×150 inserts.

Bioinformatics

Raw sequence reads were trimmed using Trimmomatic [9]. Trimmed sequences were then aligned to the *Bos taurus* and draft *Bos indicus* genomes using the Burrows-Wheeler aligner (BWA) default settings [10]. Reads that classified to either of these genomes were removed. Non-bovine reads were then classified with Kraken [11] for pathogen identification and aligned to the core Virulence Factor Database (v. 2.11.17) using BWA at default parameters for VF identification [12]. Alignments to the VFBD were parsed with an 80% gene fraction threshold [8].

Statistical analysis

The critical value was set at α=0.5. Foodborne bacterial pathogens included in the analysis were: *Campylobacter* spp. (average of *C. jejuni, C. coli*, and *C. fetus*), *Clostridium perfringens*, generic *Escherichia coli* (as a marker for pathogenic enteric bacteria), *Salmonella enterica*, *Listeria monocytogenes*, and *Staphylococcus aureus* (a marker for toxigenic strains). Pathogen counts per million reads were estimated as described in Yang *et al.* [8] and natural versus conventional cattle were compared using PROC MIXED in SAS (v. 9.3).Virulence factor counts were normalized both VF nucleotide length/ amount of bacteria present in each sample [13] and cumulative sum scaling [14]. Log fold change in number of alignments to VF were assessed with zero-inflated Gaussian mixture models 'fitZig' function and limma's 'makeContrasts' function [14]. Additionally, non-metric multidimensional scaling (NMDS) ordination using Euclidean distances on both sets of normalized VF counts were calculated and the 'anosim' function in the Vegan Package in R was used to assess differences between natural and conventional samples.

RESULTS AND DISCUSSION

Sequencing

The average number of raw reads in each sample was 26.5M (Range 2.1 to 42.9M) with an average Phred score of 33.76, (a score of 30 is associated with 99.9% accuracy).

Pathogens

Over 75M reads were assigned to one of the pathogens or indicators of pathogenic bacteria across all samples, with 88% of these reads assigned to generic *E. coli*. All samples included in the analyses contained at least one read that aligned to each pathogen of interest. When pathogen abundance for composite fecal samples of cattle reared under conventional vs natural systems were compared by pathogen, there was no difference (P>0.05) in counts per million reads (Figure 1).

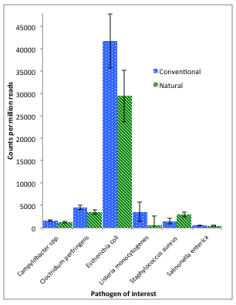

Figure 1. Comparison of least square means with standard error of log read counts per million reads of the investigated pathogens/indicators between naturally raised and conventionally raised feedlot cattle feces, there were no differences (P>0.05) between feeding method.

Virulence factors

A total of 94,691 reads were assigned to 110 VF-related genes in 37 samples (23% of pens contained no identifiable VF). Of the 37 pens with VF identified, there was an average of 2.2 (95% CL 0.95 to 3.38) VF present per 100 bacteria. Four superfamilies were identified in the samples: adhesion (58.7%), iron acquisition (23.5%), secretion (17.5%), and toxin (0.3%). All VF identified were associated with gram-negative organisms; though the database used contained twice as many gram-negative VF as gram-positive VF so this may have introduced some bias. No statistical difference (P>0.05) between sample-level VF composition of naturally and conventionally reared cattle was observed from either 16S/VF length or CSS normalized counts (Figure 2).

Industry impact

In feedlots with no disease, there was no difference (P>0.05) in pathogen load or VF composition in composite fecal samples obtained from naturally- and conventionally-raised cattle when assessed using shotgun metagenomic sequence data. Also, in a given sample, only an average 2.2 VF were present per 100 bacteria regardless of feeding system. As a result, conventional versus natural feeding programs are likely not a major driver in foodborne pathogen load or VF.

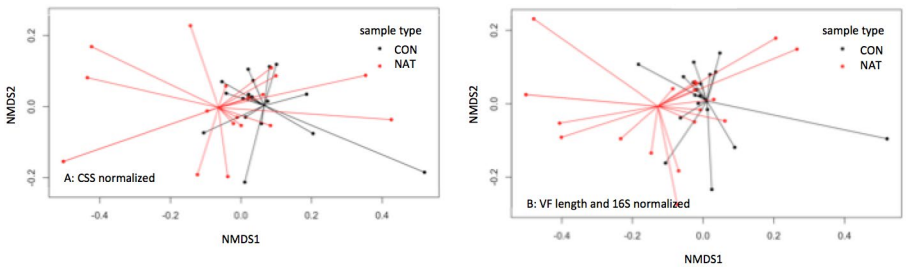

Figure 2. Non-metric multidimensional scaling (NMDS) ordination plots of fecal sample virulence composition by both (a) cumulative sum scaling (CSS) and (b) Virulence Factor (VF) length/16S gene normalization, there is not significant sample separation between natural (NAT) and conventional (CON) cattle (CSS normalization: stress=0.088, R=0.035, P=0.11; 16S normalization: stress=0.110, R: 0.051, P=0.06).

Advantages of next generation sequencing approach

Next generation sequencing (NGS) of fecal samples provided for a unique perspective not previously employed in this type of comparison. Using a culture-free approach allowed for multiple comparisons of pathogen load within a sample, as well as quantification and comparison of relative abundance. One of the most compelling uses of NGS is the ability to study VF and other bacterial properties in both pathogenic and commensal bacteria in transfer of VF and other genes associated with increased cell survival, such as antibiotic resistance, has been well documented [15]. Transfer of these genes is not exclusive to pathogens; commensal bacteria can harbor and transfer genes to pathogens [16]. As a result, studies should not be confined to pathogenic or opportunistic bacteria, but should include the entire community of bacteria being studied. This ecological approach adds a dimension that can be used in tandem with traditional techniques to identify and combat VF and other genetic modifications that make pathogens of greater risk to the public.

Challenges associated with next generation sequencing

Due to the 'newness' and continually declining cost of NGS, there are still challenges associated with employing these methods. One of the most obvious limitations is incompleteness of public databases used to identify bacteria and VF. If certain bacterial strains and/or VF have not yet been sequenced, classified, and indexed within a database, there is no way to find it using a database-centered bioinformatics approach. Additionally, while DNA shotgun sequencing allows identification of gene presence within a DNA sample, it does not enable assessment of gene expression.

CONCLUSIONS

Feces of naturally and conventionally fed cattle did not differ significantly in pathogen or VF abundance. So, dietary management of cattle may not be a major driver of risk for pathogen dissemination and foodborne illness. The ability to use NGS allows a new means to gain ecological insight into bacterial communities of interest.

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