Developing Efficient Probiotics for Microbiota of Diarrhea-Resistant Livestock

Final report for GS19-206

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Graduate Student:

Peixin Fan

Major Professor:

Dr. Kwangcheol Jeong
University of Florida

Project Information

Summary:

Calf diarrhea is one of the biggest challenges in both the dairy and beef industry worldwide. In the U.S., between 4 and 25 percent of calves die from diarrhea each year. The increased intestinal permeability and disturbance of gut microbiota are key factors leading to the pathogen-induced diarrhea. Brahman cattle contribute substantially to beef production in the southern regions of the US through crossbreeding, due to their heat tolerance and disease resistance. The heat stress can induce the damage of intestinal barrier dysfunction, but it is largely unclear whether the heat-tolerant Brahman calves have a more integrated intestinal epithelium that contributes to their resistance to diarrhea. Our preliminary studies found that fewer pathogens and mucin-degrading bacteria but more beneficial butyrate-producing commensals colonized in the gut of preweaning Brahman calves compared with Angus calves. Fecal microbiota transplantation from diarrhea-resistant livestock has been reported to relieve diarrhea of recipients. Here, we raised a hypothesis that gut microbiota of Brahman calves contributes to diarrhea resistance and strong intestinal mucus barrier through suppressing pathogenic bacteria and mucin-degrading bacteria. In this project, we first evaluated the differences in gut microbiota structure between Brahman and Angus cattle throughout the production lifecycle using a cohort multibreed Angus-Brahman (MAB) herd using the 16S rRNA gene sequencing. Consistent with preliminary studies, the beneficial butyrate-producing bacteria were enriched in cattle with more Brahman composition. Meanwhile, we collected the fecal samples from 91 3~5-week-old beef calves, and conducted 16S rRNA gene sequencing to understand the differences in gut microbiota composition between healthy and diarrheic calves. We found that the diarrheic cattle contained less diverse gut microbiota and harbored less relative

abundance of butyrate-producing bacteria, such as Roseburia, Faecalibacterium, and Odoribacter, but higher abundant pathogens, including Campylobacter and Fusobacterium. Bacterial strains were isolated from preweaning calves, and one of them were characterized as a new bacterial species as Streptococcus vicugnae. These results shed light to reducing the calf diarrhea by promoting the diversity of gut microbiota and developing probiotics from butyrate producers.

Project Objectives:

- To compare the gut microbiota composition between disease-resistant Brahman cattle and fast-growing Angus cattle
- To Characterizing the gut microbial composition and function between healthy and diarrheic calves
- To isolate potential probiotic strains from diarrhea-resistant Brahman calves and test their antimicrobial activity
- To evaluate the efficiency of potential probiotic strains on the reduction of diarrhea and investigate its functional mechanisms in terms of regulation on the gut microbiota structure and intestinal mucus barrier function

Cooperators

- Dr. Franciscco Peñagaricano

  fpenagarican@wisc.edu

Research

Materials and methods:

Evaluation of the breed effects on gut microbiota throughout the production life cycle of Multibreed Angus-Brahman (MAB) cattle.

The 16S rRNA gene amplicon sequencing data were previously obtained from the multibreed Angus-Brahman (MAB) population throughout life. Briefly, fecal samples from 239 preweaning calves, 195 postweaning calves, and 105 fattening calves were collected for conducting the 16S rRNA gene sequencing. Differences in Bray-Curtis distances among breed groups were analyzed using a permutational multivariate analysis of variance (PERMANOVA) with the beta-group-significance command. Breed group was set as fixed effect in the model. To evaluate the effect of breed composition on specific gut bacteria, multiple linear regression models were fitted using breed composition, age, and sex as explanatory variables, and log-transformed relative abundance of core bacterial taxa or IgG1 levels as responsive variables.

Characterizing the hindgut microbiota of healthy and diarrheic preweaning beef cattle

Animal management

The multibreed Angus-Brahman calves were naturally born on pasture at the Beef

Research Unit (BRU) in Waldo, FL during the calving season. During the preweaning stage, calves were transferred to Santa Fe River Ranch Beef Unit, and were raised with their dams on the bahiagrass (Paspalum notatum) pastures.

Sample collection

Fecal samples were collected from 91 beef calves ranging in age from 21 days to 35 days as previously described. Briefly, each fecal sample was collected from the rectal-anal junction (RAJ) using two sterile cotton swabs. Swabs with fecal samples were placed in a 15 mL conical tube on ice and were transported to the laboratory within 1 hour for further processing. Each swab sample was resuspended in mixture of 2 mL of Luria-Bertani (LB) broth and 2 mL of 30% glycerol. The fecal solution was then split into four 2 mL tubes and frozen in an ultra-low freezer at -80°C.

Observation of fecal samples

The morphology of feces was observed to detect whether the feces are watery or bloody. The calves that had normal solid feces were considered healthy calves. The calves that had had feces that are either watery, or pale in color and wet, or bloody were considered abnormal calves.

16S rRNA gene amplicon sequencing

Fecal samples were thawed on ice and homogenized. Then 1 mL of each sample was used for DNA extraction using QIAamp PowerFecal DNA kit according to the manufacturer's instructions (Qiagen, USA). To understand bacterial community, a dual-index sequencing strategy was used (REF). Briefly, the V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with dual-index primers. The PCR amplification reaction consisted of 1 µL forward index primer (10 mM), 1 µL reverse index primer (10 mM), 1 µL 10 ng/µL DNA template, and 17 µL Pfx AccuPrime master mix (Invitrogen, USA). Amplification was initiated with denaturation for 5 min at 95 ℃, followed by 30 cycles of 95 ℃ for 30 s, annealing at 55 ℃ for 30 sec and extension at 72 ℃ for 1 min, with a final elongation for 5 min at 72 ℃. The amplicons were purified and normalized using the SequalPrep plate normalization kit (Invitrogen, USA). The same amount of barcoded V4 amplicons from each sample were pooled to construct the DNA library.

Bioinformatic analysis for 16S rRNA gene amplicon sequencing

The 16S amplicon sequencing data were analyzed with version 2 of the Quantitative Insights into Microbial Ecology (QIIME 2) pipeline. Briefly, paired-end raw reads were imported, and the quality of the initial bases was evaluated according to the Interactive Quality Plot. The sequence quality control was performed with the Divisive Amplicon Denoising Algorithm (DADA2) pipeline implemented in QIIME 2, including steps for filtering low quality reads, denoising reads, merging the paired-end reads, and removing chimeric reads. The phylogenetic tree was generated using the align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin of QIIME 2. The sequencing depth was normalized to 10,080 sequences per sample. The Shannon index and Bray-Curtis distance were measured by the core-metrics-phylogenetic method. All amplicon sequence variants (ASVs) were classified into the bacterial taxonomy using the q2-feature-classifier plugin of QIIME 2 and the SILVA 138 database (https://www.arb-silva.de/documentation/release-1381/).

Co-occurrence network analysis

To investigate bacteria-bacteria interactions in the gut bacterial community of 1-month-old calves, co-occurrence events of core bacterial genera that were present in at least 50% of normal and abnormal fecal samples were evaluated in the network interface, respectively, using pairwise Spearman's rank correlations (rs) based on the relative abundance according to the previous study (Fan et al., 2020).

The Spearman rank correlation was analyzed using Hmisc within RStudio (version 1.1456). A significant rank correlation between two bacterial genera (rs > 0.2 or rs < - 0.2, FDR-adjusted *P*-value < 0.05) was considered as a co-occurrence event. The network was visualized using the Force Atlas algorithm in the interactive platform Gephi 0.9.2 (http://gephi.org). In the network, nodes represented different genera, and edges indicated significant correlations between nodes. The size of the nodes represented the degree of connection, and the thickness of edges indicated the strength of the correlation.

Isolation of Lactobacillus spp. strains and antimicrobial activities

The normal and abnormal feces were plated after 10-fold serial dilution in PBS on MRS agar (Difco, USA) to determine the concentrations of lactic acid bacteria (LAB), then plates were anaerobically incubated at 37 ℃ for 48 hr using the GasPak EZ Anaerobe System (BD, USA). To isolate *Lactobacillus* strains from the normal feces, colonies with different morphologies were randomly selected and purified on the same solid media to isolate pure colonies. A total of 79 colonies were isolated from fecal samples of healthy calves. Genomic DNA of the isolated colonies were extracted by bead beating method, as previously described (Ma et al., 2021). Then, to speciate the isolates, the 16S rRNA gene was amplified using the universal primer pair KCP812 (5'-CAG GCC TAA CAC ATG CAA GTC-3') and KCP813 (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*. 1998). Amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen, USA). Purified PCR products were sequenced in Genewiz (South Plainfield, USA), and the 16S gene sequences were identified by BLAST searching against National Center for Biotechnology Information (NCBI) 16S ribosomal RNA sequences database.

Antimicrobial activity of the *Lactobacillus* isolates was assessed against *Escherichia coli* KCJ2K2616, *E. coli* K88 KCJ4567, and *Salmonella enterica* Typhimurium KCJ187. For antimicrobial activity of the isolates, the agar well diffusion method was used with slight modification (Espeche et al., 2009;Boranbayeva et al., 2020). Briefly, antimicrobial activities of filter (0.22 μm) sterilized supernatants of 18 hr cultures of *Lactobacillus* spp. were tested against pathogens described above. Overnight cultures of pathogens (10^7 CFU) were mixed with soft LB agar medium (0.75% agar) and solidified. Then, 100 μL of the supernatants was loaded in the agar well. After overnight incubation at 37 ℃, the diameters of inhibition zones were measured. *Lactoplantibacillus plantarum* (formerly known as *Lactobacillus plantarum*) KCJ4051 was used as a positive control (Yu et al., 2021), and each strain was tested in triplicate.

Statistical analysis

Differences in alpha diversity and antimicrobial activity were analyzed using Student's *t*-test or a one-way analysis of variance (ANOVA) followed by an F-test and Tukey's HSD test for pairwise comparison of multiple means using the GraphPad prism. Differences between Bray-Curtis distances were analyzed using a permutational multivariate analysis of variance (PERMANOVA) with the beta-group-significance command in QIIME 2 pipeline.

A Linear discriminant analysis Effect Size (LEfSe) analysis was applied to identify unique bacteria in normal and abnormal feces based on the non-parametric factorial Kruskal–Wallis sum-rank test. An effect size of 2 was considered as significant difference.

Research results and discussion:

The difference in gut microbiota structure between Angus and Brahman throughout

the production lifecycle

Figure 1

Gut microbiota structure was significantly influenced by breed composition in preweaning (Fig. 1A), postweaning (Fig. 1B), and fattening (Fig. 1C) stages, showing greater dissimilarity with increasing genetic distance, regardless of the growth stage. Breed composition effects on gut microbiota, analyzed with combined microbiota of all three stages together, showed that the gut microbiota structure of calves was significantly different among the six BGs (Fig. 1D, $p = 0.001$). The greatest difference in microbiota structure was observed between BG1 and BG6 ($p = 0.015$), the calves of which had the greatest genetic distances; this indicates that the effects of host genetics are not specific to certain growth stages, but are universal throughout life.

To identify specific bacterial genera affected by breed composition, associations between breed composition and the $\log_{10}$ transformed relative abundance of core bacterial taxa were evaluated using multiple linear regression models that included the explanatory variables of age, sex, and breed composition. At the genus level, the relative abundances of 36 (52.2%) out of 69, 32 (40%) out of 80, and 31 (37.3%) out of 83 core bacterial genera were significantly associated or showed tendency with breed composition in preweaning, postweaning and fattening calves, respectively (Fig. 1E). Among the bacterial genera, the relative abundance of Oscillospira, Roseburia and Sutterella showed positive associations with Brahman composition throughout life (Fig. 2E). Interestingly, Oscillospira ($h^2 = 0.46$) and Sutterella ($h^2 = 0.42$) showed relatively high heritability estimates (Fig. 1F), which indicates their colonization is dramatically influenced by host genetics,while Roseburia ($h^2 = 0.21$) seems to be more susceptible to environmental conditions.

The prevalence of diarrheic calves in the early preweaning MAB calves

To detect the diarrheic rate in young MAB calves, the morphology of feces collected from 91 3~5-week-old calves was observed and recorded. Among the 91 fecal samples, 74 were normal solid, 6 were mild watery with pale color, 8 contained blood, 2 were severe water, and 1 was severe bloody.

The microbiota diversity between healthy and abnormal feces

Figure 2

To detect whether healthy calves contained more diverse gut microbiota, the bacterial richness reflected by the number of ASVs and bacterial evenness reflected by Shannon index were compared between calves that had normal solid feces and those that had abnormal feces. As shown in Fig. 2, the number of ASVs was higher in healthy calves compared to abnormal calves (Fig. 2A, $P = 0.017$), and the several watery feces and severe bloody feces contained the lowest number of ASVs compared with normal solid feces, mild watery, and mild bloody feces (Fig. 2B). Although the Shannon index did not show a significant difference between healthy and abnormal calves, the severe water feces and severe bloody feces showed a lower Shannon index compared with normal solid feces. These data suggest that a more diverse gut microbiota is beneficial to prevent diarrhea.

The distinct microbiota composition in abnormal feces compared with healthy feces

Figure 3

To characterize the gut microbiota between diarrheic and healthy calves, we compared the microbiota composition based on the morphology of feces. The principal coordinates analysis (PCoA) plot based on the Bray-Curtis distance did not show a significant difference between healthy calves and calves with abnormal

feces (Fig. 3A, $P = 0.214$). However, a separation between the severe watery or severe bloody feces with normal solid feces along the Axis 2 was observed (Fig. 3B). More specifically, pathogenic bacteria Fusobacteria, Campylobacter, Tyzzerella, Veillonella were enriched in either severe watery feces or severe bloody feces, while potential butyrate-producers Oscillospirace, which had higher relative abundance in disease-resistant Brahman cattle, were more abundant in healthy calves. These data support the isolation of potential butyrate-producing bacteria to relieve diarrhea.

Bacterial-bacterial interactions predicted by co-occurrence network

Figure 4

To investigate whether there are differences in bacterial-bacterial interactions in the gut microbial ecosystems, the co-occurrence networks were analyzed using the 59 and 54 core bacterial genera that were present in more than 50% of the normal and abnormal fecal samples, respectively. The normal feces had 8 unique core bacterial genera including *Actinomyces*, *Christensenellaceae* R-7 group, *Collinsella*, *Coprococcus*, *Erysipelatoclostridiaceae* UCG-004, *Incertae Sedis*, *Oscillospiraceae* NK4A214 group, RF39 and *Rhodospirillales* uncultured, whereas the abnormal feces had only 4 unique core bacterial genera including *Coprobacter*, *Intestinimonas*, *Streptococcus*, and *Tyzzerella*. The normal feces had more bacteria-bacteria interactions (n = 656) compared to the abnormal feces (n = 272) based on Spearman correlations (*P*adjust < 0.05, $r_s > 0.2$ or $r_s < -0.2$). For the normal feces, the network was grouped into three modules according to the inner interactions among the nodes (Fig. 4A). In module 1, *Oscillospira*, which had higher relative abundance in normal feces, was the hub among the 22 core bacterial genera, having 38 significant correlations with other bacteria (Fig. 5A). Notably, *Oscillospira* was negatively associated with *Campylobacter*, which showed high relative abundance in the severe watery feces, and *Escherichia-Shigella*, which contain multiple pathogenic species that can cause diarrhea in young calves. In module 2, *Oscillospiraceae UCG-005* was the hub among the 20 core bacteria, having 38 correlations with other bacteria. *Oscillospiraceae* UCG-005 showed negative associations with *Veillonella*, which was enriched in watery feces (Fig. 3E), as well as *Campylobacter* in Module 1. In module 3, *Lachnospiraceae* UCG-004 associated with watery feces was the hub among the 17 core bacteria and it was negatively correlated with the *Christensenellaceae* R-7 group in module 1, which was belonged to the unique core bacteria of the normal feces. Importantly, a significant negative correlation was also observed between beneficial bacteria *Lactobacillus* and *Escherichia-Shigella*.

For the abnormal feces, the bacterial-bacteria interaction network was also grouped to three modules, with module 1 being the dominant, but the number of nods and correlations were significantly less compared to normal feces (Fig. 4B). The [*Eubacterium*] *coprostanoligenes* group was the hub among the 38 core bacteria, with 26 significant correlations with other bacteria. Notably, the negative associations between potential beneficial bacteria, *Oscillospira* and *Lactobacillus,* and opportunistic pathogens, *Campylobacter* and *Escherichia-Shigella*, which were detected in the normal feces, were not observed in the abnormal feces, suggesting that beneficial bacteria may suppress diarrheagenic pathogens in normal healthy gut microbiota.

Identification of Lactobacillus spp. strains and their antimicrobial activities

Figure 5

As we discovered higher abundance of *Lactobacillus* in normal feces (3.12%) compared to mild watery feces (2.02%) and severe watery feces (1.82%), as well as

negative correlations between *Lactobacillus* and *Escherichia-Shigella* in normal feces, we isolated *Lactobacillus* strains and evaluated their antimicrobial activity against pathogens to test if these strains may contribute to the GI tract health. The number of LAB in normal feces was significantly greater than that of abnormal feces ($P = 0.0198$) (Fig. 5A), suggesting the concentrations of LAB might be associated with the gut health in the calves. In addition, 79 *Lactobacillus* spp. strains were isolated from the normal feces, and they were identified as *Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri*), which was the most predominant species in the normal feces (Fig. 5B). We also identified *Lactobacillus johnsonii*, *Lactobacillus amylovorus*, and *Ligilactobacillus animalis* (formerly known as *Lactobacillus animalis*) in the normal feces (Fig. 5B). To test if these strains may suppress pathogenic strains as suggested in Figure 4, we conducted antimicrobial activity of these strains against *E. coli* and *S*. Typhimurium, which can cause calf diarrhea (Borabayeva *et al*., 2020). All *Lactobacillus* spp. strains inhibited the growth of 3 pathogens (Fig. 5C). Notably, *L. reuteri* and *L. johnsonii* showed clear inhibition that was similar to that of well-known probiotic strain *L. plantarum* against all test pathogens (Fig. 5C). *L. reuteri* and *L. johnsonii* were the most and second most abundant *Lactobacillus* species in healthy calves (Fig. 5B), suggesting these *Lactobacillus* spp. may affect gut health positively by suppressing the colonization of pathogenic bacteria in the gut of preweaning calves.

Participation Summary

Educational & Outreach Activities

1 Journal articles

1 Other educational activities: Training the undergraduate about understanding the gut microbiota between healthy and diarrheic calves

PARTICIPATION SUMMARY:

6 Ag professionals participated

Education/outreach description:

With the support of the SARE grant, we have published a peer-reviewed article on a high-impact Nature Publishing Group Journal ISME J (Impact factor: 9. 18), which title is "Host genetics exerts lifelong effects upon hindgut microbiota and its association with bovine growth and immunity". The paper reported the difference in gut microbiota between disease-resistant Brahman cattle and fast-growing Angus cattle throughout the production lifecycle, and the connections between specific commensal bacteria and animal health. We have also submitted another research article to Frontiers in Microbiology, which title is "The gut microbiota of newborn calves and influence of potential probiotics on reducing diarrheic disease by inhibition of pathogen colonization". The paper is now under review and is about the role of gut microbiota in preventing the diarrhea in newborn beef cattle and isolation of potential probiotics from diarrhea-resistant calves.

We have also trained one undergraduate student for observation of fecal samples to characterize diarrhea and analyze the high-throughput microbiota data using advanced bioinformatic tools. We plan to present our findings on the EPI research

day early next year. We also would like to share our research results with farmers and the industry community during Field Day events once Cov1d-19 is controlled well.

Project Outcomes

1 New working collaboration

Project outcomes:

We have published a research article on the high-impact Nature Publishing Group journal ISME J. We have isolated 4 species of *Lactobacillus* spp. strains, including *L. reuteri, L. animalis, L. johnsonii, and L. amylovorus* from healthy calves, which showed strong antimicrobial activity againest pathogens colonized in diarrhea calves. Meanwhile, other mitigation strategies, including diet manipulation, farm management that will enhance the gut microbiota diversity and increase the abundance of identified benefit commensals can also be applied to relieve diarrhea on the farm. We plan to transfer our research findings to application in the near future to reduce the economic cost caused by diarrhea.

Knowledge Gained:

With the support of the SARE grant, we have extensively understood the gut microbiota between disease-resistant Brahman cattle and fast-growing Angus cattle across different growth stages, which provide informative evidence showing how animal breeding influences animal growth and immunity throughout modulating the gut microbiota. Furthermore, we characterize the microbiota structure between healthy and diarrheic calves according to their fecal morphology and identify specific bacteria that are potential for combat diarrhea.

Information Products