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# Utilization of Pyrosequencing to Monitor the Microbiome Dynamics of Probiotic Treated Poultry (*Gallus gallus domesticus*) during Downstream Poultry Processing

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## Abstract

Antibiotic growth promoters that have been historically employed to control pathogens and increase the rate of animal development for human consumption are currently banned in many countries. Probiotics have been proposed as an alternative to control pathogenic bacteria. Traditional culture methods typically used to monitor probiotic effects on pathogens possess significant limitations such as a lack in sensitivity to detect fastidious and non-culturable bacteria, and are both time consuming and costly. Here, we tested next generation pyrosequencing technology as a streamline and economical method to monitor the effects of a probiotic on microbial communities in juvenile poultry (*Gallus gallus domesticus*) after exposure to several microbiological challenges and litter conditions. Seven days and repeated again at 39 days following hatching, chicks were challenged with either *Salmonella enterica* serovar Enteritidis, *Campylobacter jejuni*, or no bacteria in the presence of, or without a probiotic (*i.e.*, *Bacillus subtilis*) added to the feed. Three days following each of two challenges (*i.e.*, days 10 and 42, respectively) the microbiome distributions of the poultry caecum were characterized based on 16S rDNA analysis. Generated PCR products were analyzed by automated identification of the samples after pooling, multiplexing and sequencing. A bioinformatics pipeline was then employed to identify microbial distributions at the phylum and genus level for the treatments. In conclusion, our results demonstrated that pyrosequencing technology is a rapid, efficient and cost-effective method to monitor the effects of probiotics on the microbiome of poultry propagated in an agricultural setting.

## Keywords

Poultry Production, Probiotics, Pyrosequencing, Mutiplex Identifier (MID),

Microbiota

## 1. Introduction

Since 1995, the Poultry industry has become one of the globes' largest and fastest growing segments of animal food production [1]. The United States is one of the largest producers of poultry with the broiler sector playing a major role [2]. Crowded poultry housing conditions are stressful to the birds leading to an elevated disease potential. Additionally, packed houses cause deterioration of environmental conditions providing a situation that is conducive to the spread of disease and thus increase the possibility of transmission to humans [3]. Outbreaks of campylobacteriosis and salmonellosis infections due to the consumption of contaminated poultry or derived products have occurred in human populations throughout the world and are thus a major concern [4] [5] [6] [7] [8].

Antimicrobial growth promoters (AGPs) consist of antibiotics that are added to the feed of animals to enhance their growth rate and production performance [9]. Unfortunately, the large quantities of AGPs that have been used in poultry production provided a source for development of antibiotic resistant bacteria [10]. For example, *Campylobacter* was found to be increasingly resistant to antibiotics such as fluoroquinolones and macrolides that are used as antimicrobials for the treatment of campylobacteriosis [11]. Additionally, the development of resistance to antibiotics by *Salmonella* has also been reported [12]. Hence, there is an increased necessity not only to minimize AGP use but also to develop novel non-antibiotic-based alternative treatments. Probiotics are being considered to fill this gap with utilization in certain farms instead of antibiotics [13] [14]. The most common probiotic additives used in the broiler industry include *Aspergillus, Bacillus, Bifidiobacterium, Candida, Lactobacillus and Sterptomyces* [15] [16] [17].

Effects of the implementation of probiotics on the poultry microbiome typically employ classical culture and classification methods. Notably, traditional culture methods are energy intensive and time consuming practices consisting of isolations that do not account for the presence of fastidious growing or non-culturable bacteria [18]. Further, the costs of selective media along with reagents for carbon utilization and enzyme production testing for classification of numerous bacterial isolates are high. In contrast, cultureless examination of the microbiome of host tissue specimens using pyrosequencing of 16S rDNA is a method that directly detects bacterial communities and provides a means for an added metagenomics research approach [19]. Pyrosequencing using Roche next-generation sequencing (NGS) 454 technology, in particular, is comparatively thorough for 16S rRNA gene analysis because of the relatively long sequence reads obtained (ca. 500 bp) compared to other high throughput sequencing technologies that average 100 bp. In addition to the length of reads, NGS tech-

nologies provide more nucleotides to characterize from a given DNA sample when compared with conventional approaches [20].

Here, we compared microbe populations present in chick cecum following feeding probiotics consisting of *Bacillus subtilis* with those fed a normal diet (feed without probiotics). Additionally, litter microbiota present before and after composting the litter were analysed. Bacterial populations were classified based on 16S rDNA sequencing analysis. An understanding of the development of the normal bacterial community provided a method to detect disruption in the flora and determine the effects of food animal management changes. The success and precise assessment of the bacterial information using high throughput pyrosequencing demonstrated in this study may allow for timely manipulation of the intestinal flora with the intention of enhancing intestinal health and feed conversion ratios.

## 2. Materials and Methods

### 2.1. Probiotic Experiment

**Poultry Rearing**. A total of 450 male broilers (*Gallus domesticus*) were obtained from the Cobb-Vantress hatchery, Inc (Timpson, Texas, USA) immediately after hatching (*i.e.* zero days of age). At the hatchery, birds were vaccinated for Marek's disease, Newcastle's disease and bronchitis using standard methods [21]. The broilers were divided among 28 floor pens (1.2 m 1.2 m) at the Stephen F. Austin State University (SFASU) Science Research Center, Nacogdoches, TX, USA with 15 birds per pen on fresh litter. The facility is environmentally controlled with negative pressure rooms (*i.e.* air expelled from the room). To provide the birds a relatively stable thermal environment, ventilation and heat ranged from 32˚C daily to 21˚C nightly. All birds received the same basal diet formulated according to the Nutrient Requirements of Chickens [22]. Clean water and feed were provided ad *libitum* throughout the study via Lubing Feather Soft Nipple Drinkers and then Tube Feeders (QC Supply, Schuyler, NE, USA).

**Preparation of Inocula for Challenges**. From glycerol stocks, 500 µL a of Salmonella enterica poultry isolate was added to 30 mL of DifcoTM Rappaport-Vassiliadis R10 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) broth amended with novobiocin at 25 µg/mL (Sigma, St. Louis, MO) and nalidixic acid at 20 µg/mL (Sigma, St. Louis, MO), and incubated for 16 - 18 h in an Innova 4300 shaker (New Brunswick, Edfield, CT, USA) at 37˚C and 250 RPM. A Campylobacter jejuni poultry isolate was propagated by adding 500 µL of a frozen glycerol stock to 30 mL of Bolton broth base (Sigma-Aldrich, St. Louis, MO) amended with novobiocin at 25 µg/mL (Sigma-Aldrich, St. Louis, MO) and nalidixic acid (20 µg/mL, Sigma-Aldrich, St. Louis, MO) and incubated at 42˚C for 16 - 18 h without any agitation in the presence of 10.0% carbon dioxide, 4.9% oxygen and 8% nitrogen obtained as a compressed gas (Gibson Laboratories, Lexington, KY, USA).

Following the incubation, 1 ml of the S. *enterica* culture was inoculated into 100 mL of fresh R10 broth contained in a 500 mL flask and incubated at 37˚C,

250 RPM in Innova 4300 shaker incubator. Similarly, 1 ml of *C. jejuni* was inoculated in a 50 mL conical tube containing Bolton broth and incubated at 42˚C after the passage of compressed gas. Optical density of the culture was checked periodically until attaining an absorbance 0.45 at a wavelength of 625 nm. Once the cultures entered log phase, the bacteria were pelleted by centrifugation at 12,000 × g for 5 min. The supernatant was discarded and the cells were washed with sterile Phosphate Buffer Saline (PBS; 130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.3) twice and then suspended in 40 mL of PBS for inoculations.

Final concentrations administered in the challenges were 3 × 109 colony forming units (CFU)/mL for *S.enterica* and 1 × 109 CFU/mL for *C.jejuni.* Birds were infected by oral gavage with S.enterica or C. jejuni in 0.2 mL of physiological saline 0.85% w/v on the 7th and 39th day of age as designated in Table 1. Control groups (Treatments 5 and 6) were provided PBS.

**Caecum Samples**. Previous studies by Barnes *et al.* (1972), and Wei *et al.* (2013) showed that a diverse microbiota was found primarily in the caecum. Therefore, this study focused on the ceca microbiome [23] [24]. Three days following each challenge, caecum samples (n = 56) were harvested (*i.e.* at the 10th day and 42nd). The cecal sacs were removed from two randomly selected birds per pen on the day of harvest. The caecum contents of both chicks from a pen were pooled for molecular analysis.

**Genomic DNA Isolation**. Caecum contents were aseptically scraped into sterile 50 mL tubes containing 10 mL of sterile PBS and mixed by vortexing for 3 min. Debris was removed by centrifugation at 700 × g for 1 min. The supernatant was collected and centrifuged at 12,000 × g for 5 min to pellet bacteria that was then suspended in 2 mL PBS and centrifuged at 12,000 × g for 5 min. The PBS wash was repeated and the pellet was finally suspended in 2 ml PBS. Glycerol stocks were prepared by drawing 500 µL of the washed cells and flash freezing in liquid nitrogen immediately after the addition of 1 mL glycerol. The remaining cells were stored at −20˚C for DNA extraction.

The bacterial genomic DNA was isolated using a Wizard Genomic DNA purification Kit (Promega Corporation, Madison, USA) as per the manufacturer's protocol. The DNA purity was checked spectrophotometrically using a Varian Cary 50 UV—Vis spectrophotometer equipped with a Hellma microcell tray for microliter sample volumes (Hellma Analytics, Mullheim, Germany).

## 2.2. Litter Compost Experiment

Bird Rearing Facility. Between batches of birds the litter was composted to measure changes in the distribution of pathogenic bacteria. The broiler housing facility at the SFASU Poultry Center is temperature controlled with four tunnel-ventilations and a solid sidewall. The length and width of each house measured 152 m × 13 m and was stocked with 27,900 newly hatched broiler chicks at a stocking density of 0.23 m2/bird. The two facilities used were designated as House 1 and House 2 each of which was partitioned into two with a composted

Table 1. Poultry experiment with probiotic treatments. Effect of the Probiotic (*Bacillus subtilis*) on chick cecum microbial flora was determined after challenging birds with Salmonella enterica (S.e) serovar Enteritidis, *Campylobacter* jejuni (C.j), and appropriate controls. The experiment consisted of six treatments: Treatment 1 (S.e challenged and no Probiotic), Treatment 2 (S.e challenged + Probiotic), Treatment 3 (C.j challenged and no Probiotic), Treatment 4 (C.j challenged + Probiotic), Treatment 5 (no challenge + Probiotic) and Treatment 6 (no challenge and no probiotic).

| Treatment | Challenge & Organism Used | Probiotic added |
|---|---|---|
| **1** | Yes (S.e) | No |
| **1** | Yes (S.e) | No |
| **1** | Yes (S.e) | No |
| **1** | Yes (S.e) | No |
| **2** | Yes (S.e) | Yes |
| **2** | Yes (S.e) | Yes |
| **2** | Yes (S.e) | Yes |
| **2** | Yes (S.e) | Yes |
| **2** | Yes (S.e) | Yes |
| **3** | Yes (C.j) | No |
| **3** | Yes (C.j) | No |
| **3** | Yes (C.j) | No |
| **3** | Yes (C.j) | No |
| **3** | Yes (C.j) | No |
| **4** | Yes (C.j) | Yes |
| **4** | Yes (C.j) | Yes |
| **4** | Yes (C.j) | Yes |
| **4** | Yes (C.j) | Yes |
| **4** | Yes (C.j) | Yes |
| **5** | None | Yes |
| **5** | None | Yes |
| **5** | None | Yes |
| **5** | None | Yes |
| **6** | None | No |
| **6** | None | No |
| **6** | None | No |
| **6** | None | No |

and non-composted litter section. Each flock was reared for 49 days to an average market weight of 2.4 kg/bird.

**In-House Windrow Composting**. Prior to the beginning of the study, both houses were depopulated. Recycled litter was used in order to have a higher population of microorganisms than new pine wood shavings. The shavings had been used as bedding for five previous flocks. Immediately after flock removal, the litter in one house was turned into two windrow composting piles that ran the length of the house using a hydraulic blade turned at a 45 degree angle. Litter windrows were left unturned for 7 days to allow composting. After 7 days, the litter was spread across the house and leveled prior to chick placement. This procedure was repeated at the completion of each consecutive flock.

Litter Samplings. Each house (House 1 and House 2) was divided into four 38 m sections lengthwise. Using a 30 cm soil collection tube (Acorn Naturalists, Tustin, CA USA), six litter samples were collected per 38 m sections from each house. Samples were then pooled and homogenized to make four composted and four non-composted samples in sterile bags and stored at −20˚C. The bacterial DNA from 2.5 g poultry litter was isolated using the ZR Soil microbe DNA midiprep kit (Zymoresearch, Irvine, USA) as per the manufacturer's protocol.

## 2.3. Next-Generation Sequencing

**16S rDNA Synthesis**. Extracted DNA from the poultry probiotic experiment and litter compost studies were used as templates to amplify 16S rDNA sequences using the polymerase chain reaction (PCR) in a MyCycler (BioRad Laboratories, Inc., USA). Reactions were performed in a 50 µL total volume with GoTaq Green Master Mix from Promega Corp. (Madison, USA). The forward primer 27F (5'–AGAGTTTGATCMTGGCTCAG–3') is a 16S ribosomal DNA specific universal primer for prokaryotes that was previously employed by Lane *et al.* [25]. The universal reverse primer for prokaryotes called 519R (5'–GWATTACCGCGGCKGCTG–3') was used by Turner *et al.* [26].

All the primers including Multiplex Identifiers (MIDs) listed in Table 2 were purchased from Sigma Genosys (a division of Sigma Aldrich). Figure 1 provides an illustration of the strategy used to combine the bacterial 16S ribosomal DNA primer set with the MIDs. The PCR conditions were the following: 97˚C for 5 minutes; 40 cycles of 60˚C for 1 minute, 72˚C for 1 minute 20 seconds and 95˚C for 30 seconds; followed by a 72˚C for 5 minutes and hold at 4˚C. The PCR amplified product was analyzed using agarose gel electrophoresis.

Pyrosequencing Application. After quantification of DNA, equal amounts of purified PCR products were pooled for Roche emPCR amplification that was performed as per the manufacturer's protocol (454 Roche Life Sciences,

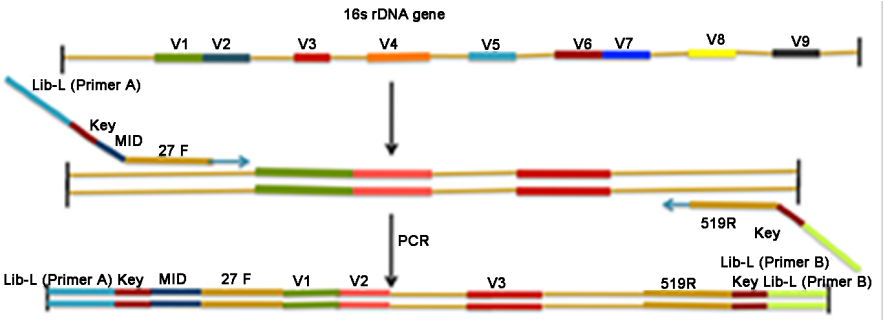



Figure 1. Schematic depiction of PCR primers used to amplify a 16S rDNA gene segment. The 16S rDNA gene includes a conserved region (thin line) and nine hypervariable regions (V1-V9). The primers were designed to target conserved regions and amplify variable sections with added Multiplex Identifiers (MIDs). The MID's were tagged to adaptors for sequencing using Roche 454 Lib-L Primer A: 5'-CCATCTCATCCCTGTCTCCGAC-3', Lib-L Primer B: 5'-CCTATCCCCTGTGTGCCTTGGCAGTC-3', Sequencing key-TCAG, Universal Primer 27F: 5'-GRGTTTGATCMTGGCTAG, and the Universal Primer 519R: 5'-GTNTTACNGCGGCKGCTG-3'.

**Table 2.** Degenerate primers used for bacterial 16S rDNA amplification. Primers were designed with Roche 454 Lib–L forward primer (Primer A) at the 5' end, the sequencing key in the middle, and with a Multiplex Identifier (MID) and universal primer at the 3' end. The reverse primer with Roche 454 Lib-L (Primer B) and universal primer were positioned at the 3' end.

| Oligo Name | Sequence (5'-3') |
|---|---|
| **MID-1** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTAGRGTTTGATCMTGGCTCAG |
| **MID-2** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAAGRGTTTGATCMTGGCTCAG |
| **MID-3** | CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACGCGCTCAGRGTTTGATCMTGGCTCAG |
| **MID-4** | CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGAGRGTTTGATCMTGGCTCAG |
| **MID-5** | CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGAGRGTTTGATCMTGGCTCAG |
| **MID-6** | CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGAGRGTTTGATCMTGGCTCAG |
| **MID-7** | CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTAAGRGTTTGATCMTGGCTCAG |
| **MID-8** | CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCANGRGTTTGATCMTGGCTCAG |
| **MID-10** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGAGRGTTTGATCMTGGCTCAG |
| **MID-11** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTAGRGTTTGATCMTGGCTCAG |
| **MID-13** | CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGAGRGTTTGATCMTGGCTCAG |
| **MID-14** | CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACAGRGTTTGATCMTGGCTCAG |
| **MID-15** | CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTAAGRGTTTGATCMTGGCTCAG |
| **MID-16** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAAGRGTTTGATCMTGGCTCAG |
| **MID-17** | CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACAGRGTTTGATCMTGGCTCAG |
| **MID-18** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTACGTAGCAGRGTTTGATCMTGGCTCAG |
| **MID-19** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCAGRGTTTGATCMTGGCTCAG |
| **MID-20** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGAGRGTTTGATCMTGGCTCAG |
| **MID-21** | CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGAGRGTTTGATCMTGGCTCAG |
| **MID-22** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGAGTATGAGRGTTTGATCMTGGCTCAG |
| **MID-23** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGAGRGTTTGATCMTGGCTCAG |
| **MID-24** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGAGRGTTTGATCMTGGCTCAG |
| **MID-25** | CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTCGCTCGAGRGTTTGATCMTGGCTCAG |
| **MID-26** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACATACGCGTAGRGTTTGATCMTGGCTCAG |
| **MID-27** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGAGTATAGRGTTTGATCMTGGCTCAG |
| **MID-28** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTAGRGTTTGATCMTGGCTCAG |
| **MID-29** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTAGRGTTTGATCMTGGCTCAG |
| **MID-30** | CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTAGRGTTTGATCMTGGCTCAG |
| **519R** | CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGTNTTACNGCGGGCKGCTG |

Indianapolis, IN, USA). This method was followed for 10th day, 42nd day and litter samples separately. A Roche GS Junior System was used for pyrosequencing. The GS De Novo Assembler (Roche) was used to trim and group the data (*i.e.*, adapter, linker and primer sequences) based on MID as well as generate

consensus sequences of the DNA libraries. The assembled contigs were used in a metagenomic analysis with the CAMERA database [27] [28].

**Statistical Analysis**. Based upon the CAMERA BLASTn derived matches, the sequences were classified at the appropriate taxonomic levels based on Data Analysis Methodology offered by the Research and Testing Laboratory, Lubbock, TX, USA (http://rtlgenomics.com/). Additionally, RDP Naïve Bayesian rDNA classifier version 2.5 was used to organize the data into taxonomy groups with a bootstrap cutoff of 80% (https://rdp.cme.msu.edu/). Two-Way ANOVA was used with GraphPad Prism version 6.0. (https://www.graphpad.com/scientific-software/prism/), in order to further analyze the data and calculate the variance to observe the effect of treatments on the chick's microbiome. A p-value <0.05 was considered statistically significant. The SAS based program JMP Genomics Version 5.1 was employed to organize the distribution of identified bacteria [29].

## 3. Results

### 3.1. Probiotic Poultry Experiment

Results of the 454 sequencing experiments performed on the samples collected on day 10 and 42 showed that the primers designed with the MIDs successfully amplified specific 16S rDNA regions of multiple bacteria. Pyrosequencing generated 19.8 Mbp with average reads of 389 bp and 43.7 Mbp with average reads of 342 bp for the pooled chick caecum samples from the 10$^{th}$ and 42$^{nd}$ day collection periods, respectively.

The CAMERA BLASTn analysis using the collective data produced over 20,000 significant matches for all of the treatment samples (**Table 3**). The Two-Way ANOVA analysis using both the 10$^{th}$ and 42$^{nd}$ day samples from treatments

**Table 3.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from the caeca of chicks that had been provided a probiotic and challenged with either *Salmonella enterica* (S.e) serovar Enteriditis or *Campylobacter jejuni* (C.j*)*, and appropriate controls. The experiment consisted of six treatments: Treatment 1 (S.e challenged and no Probiotic), Treatment 2 (S.e challenged + probiotic), Treatment 3 (C.j challenged and no Probiotic), Treatment 4 (C.j challenged + Probiotic), Treatment 5 (no challenge + Probiotic) and Treatment 6 (no challenge and no probiotic). Genomic DNA was extracted from poultry caeca at the 10$^{th}$ and 42$^{nd}$ day following each Treatment.

| Treatment | Unique Bacterial Classified | | | Taxonomic Bacterial Groups | | |
|---|---|---|---|---|---|---|
| | 10 Day | 42 Day | Total | 10 Day | 42 Day | Total |
| **1** | 609 | 3444 | 4053 | 132 | 409 | 541 |
| **2** | 1097 | 1902 | 2999 | 249 | 307 | 556 |
| **3** | 1699 | 1974 | 3673 | 361 | 300 | 661 |
| **4** | 34 | 1631 | 1665 | 5 | 226 | 231 |
| **5** | 1468 | 2871 | 4339 | 332 | 390 | 722 |
| **6** | 2016 | 2626 | 4642 | 345 | 394 | 739 |

that included exposure to *S. enterica* or *C. jejuni* along with the probiotic revealed a difference in the number of genera with respect to collection period. However, the probiotic treatment did not provide statistical evidence for a reduction in pathogens detected ($p = 0.1751$). Overall, Firmicutes were predominant in both days sampled from the six phyla identified (Figure 2(a)). Further, increased levels of beneficial genera such as *Blautia*, *Eubacteria*, *Faecalibacteria*, among others were detected from the 10th to the 42nd sample collections (Figure 2(b)). *Clostridia* that includes both pathogenic and non-pathogenic species were unaffected by treatment with or without the probiotic. *Bacillus* spp. were detected in all of the treatment samples (Figure 2(d)). A total of 6923 and 14,448 bacterial strains were identified for the 10th and 42nd day samples, respectively. Figures 3-8 illustrate the distributions of the identified bacteria that comprised >1.5% of the population. However, the number of genera decreased as time increased irrespective of the treatment with or without the probiotic for both beneficial (Figure 2(b)) and pathogenic bacteria (Figure 2(c)). As *Bacillus* spp. are ubiquitous, expectantly they were detected in the entire sample analyzed (Figure 2(d)).

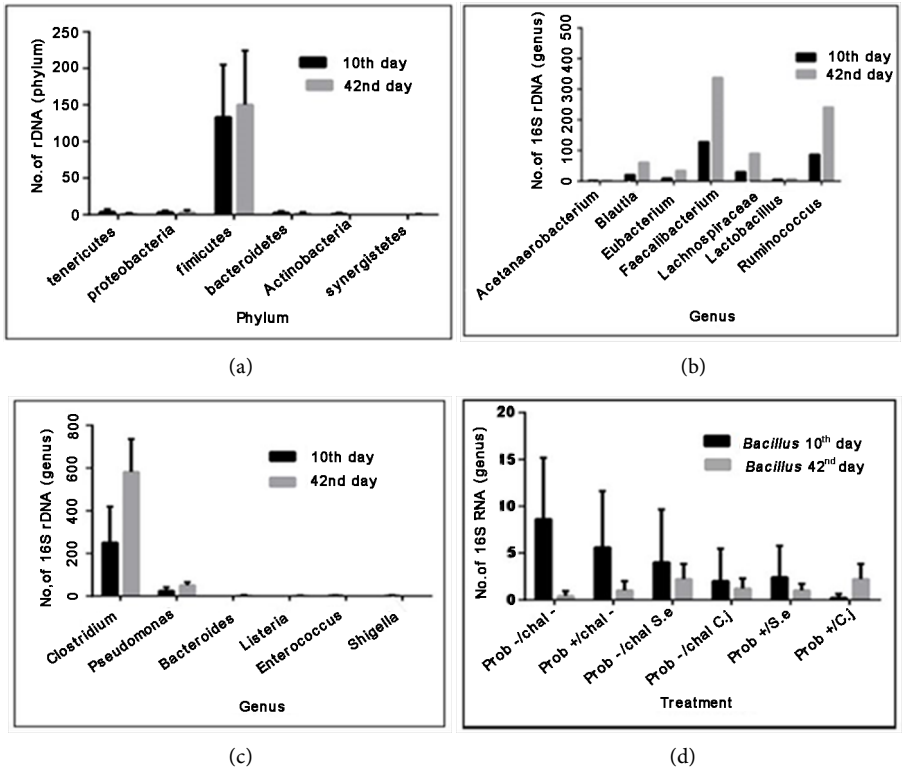



Figure 2. Two-way ANOVA providing the distribution of significant ($p < 0.05$) phyla and genera based on 16S rDNA sequence analysis. Genomic DNA was extracted from poultry caeca at the 10th and 42nd day following a challenge ($Chal^+$) or no challenge ($Chal^-$) with a bacterial pathogen (*Salmonella enterica*—S.e or *Campylobacter jejuni*—C.j), and/or probiotic (*Bacillus subtilis*) administration. Figure (a) illustrates the phylum distribution. Figure (b) illustrates beneficial bacterial genera identified. Figure (c) illustrates pathogenic genera representatives. Figure (d) illustrates the detection of *Bacillus* spp. without administration of the probiotic ($Prob^-$) or following the probiotic treatment ($Prob^+$).

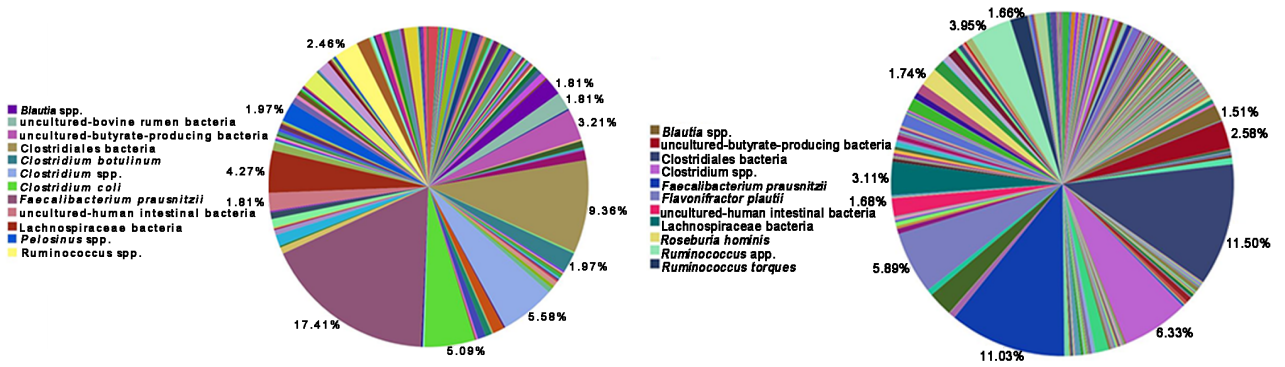



**Figure 3.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from caeca of chicks that had been challenged with *Salmonella enterica* and not administered a probiotic. Genomic DNA was extracted from poultry caeca at the 10th (Pie (a)) and 42nd (Pie (b)) day following the treatment.

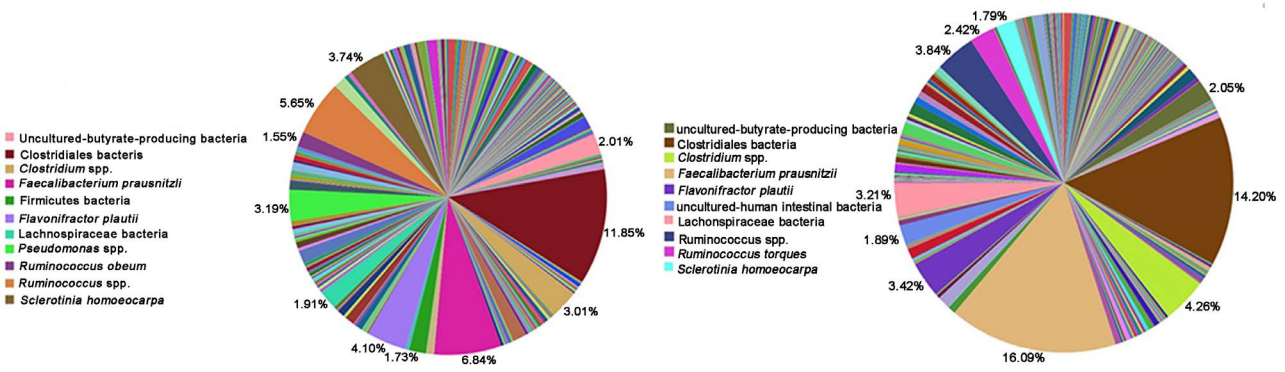



**Figure 4.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from caeca of chicks that had been challenged with *Salmonella enterica* and administered a probiotic. Genomic DNA was extracted from poultry caeca at the 10th (Pie (a)) and 42nd (Pie (b)) day following the treatment.

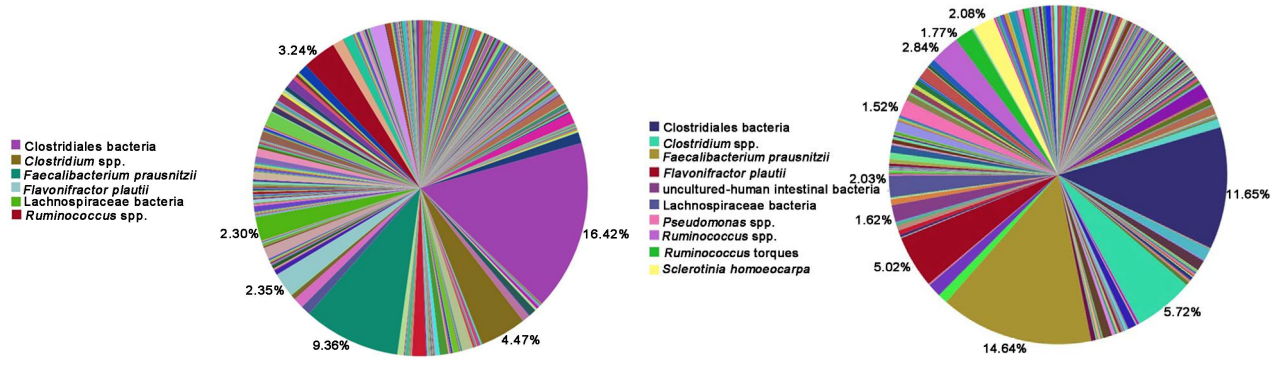
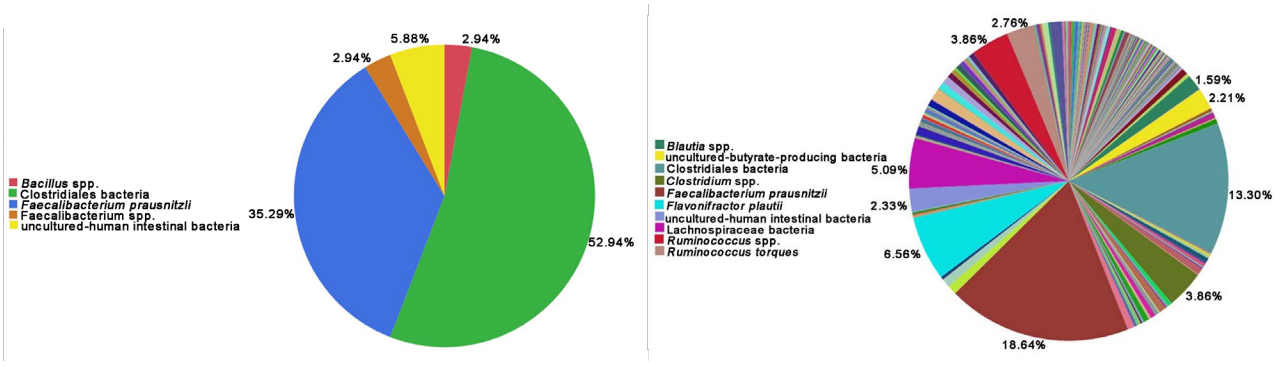



**Figure 5.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from caeca of chicks that had been challenged with *Campylobacter jejuni* and not administered a probiotic. Genomic DNA was extracted from poultry caeca at the 10th (Pie (a)) and 42nd (Pie (b)) day following the treatment.

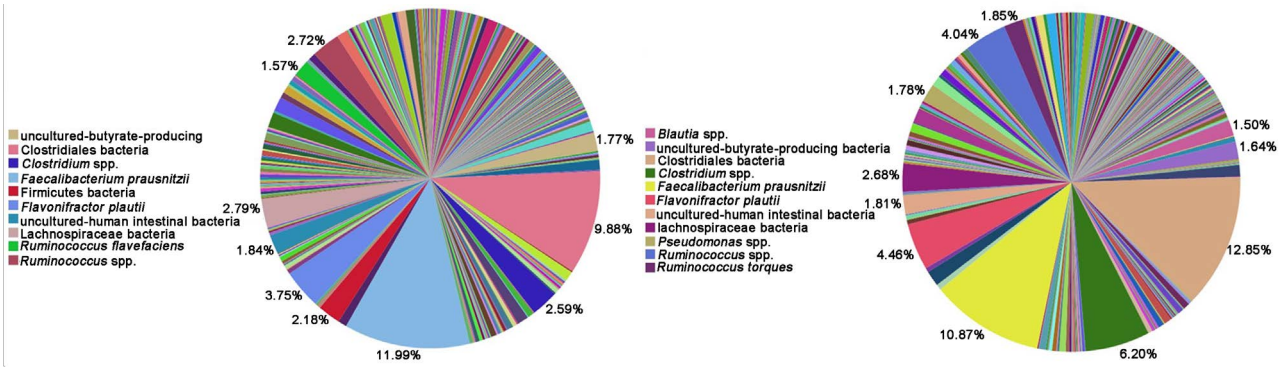
## 3.2. Litter Compost Analysis

Pyrosequencing data generated 4.4 Mbp with an average read length of 412 bp for the pooled DNA extracted from the litter samples. The RDP database



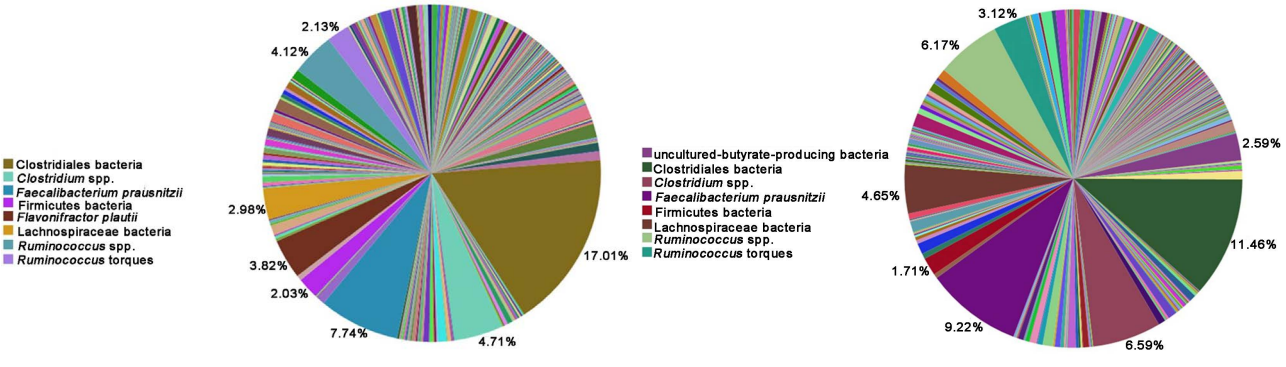

(a) (b)

**Figure 6.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from caeca of chicks that had been challenged *Campylobacter jejuni* and administered a probiotic. Genomic DNA was extracted from poultry caeca at the 10th (Pie (a)) and 42nd (Pie (b)) day following the treatment.




(a) (b)

**Figure 7.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from caeca of chicks that had not been challenged with a bacterial pathogen and administered a probiotic. Genomic DNA was extracted from poultry caeca at the 10th (Pie (a)) and 42nd (Pie (b)) day following the treatment.




(a) (b)

**Figure 8.** Distribution of bacteria identified based on 16S ribosomal DNA sequence analysis from caeca of chicks that had not been challenged with a bacterial pathogen and not administered a probiotic. Genomic DNA was extracted from poultry caeca at the 10th (Pie (a)) and 42nd (Pie (b)) day following the mock a challenge with a bacterial pathogen and no probiotic administration.

classification identified six phyla from the broiler litter with a majority consisting of Firmicutes and Actinobacteria at the two housing units (Table 4). Staphylococcus and *Salinicoccus* were the predominant Firmicutes genera detected

irrespective of the treatments (Figure 9). Comparisons of genera from the phylum Actinobacteria showed that *Bracybacterium* was predominant in broiler litter irrespective of the treatments (Figure 10).

## 4. Discussion

The current study was intended to determine the proof of concept that next-generation sequencing technology could be applied to rapidly and efficiently

Table 4. Two poultry rearing facilities partitioned to house poultry pens (House 1 – H1) and House 2 - H2) that were bedded with either non-composted or composted wood shavings to assess prokaryotic composition differences between the litter. Extractions of DNA from litter samples were used to detect bacterial phyla composition based on 16S rDNA bacterial sequence analysis by employing the Ribosomal Database Project—Naive Bayesian rDNA classifier version 2.5 (https://rdp.cme.msu.edu/).

| Phylum | Non-Compost H1 | Compost H1 | Non-Compost H2 | Compost H2 |
|---|---|---|---|---|
| **Actinobacteria** | 4807 | 4380 | 38 | 62 |
| **Bacteroidetes** | 24 | 103 | 7 | 0 |
| **Cyanobacteria** | 1 | 5 | 0 | 0 |
| **Firmicutes** | 6914 | 8013 | 125 | 120 |
| **Proteobacteria** | 10 | 24 | 2 | 2 |
| **Tenericutes** | 4 | 3 | 0 | 0 |

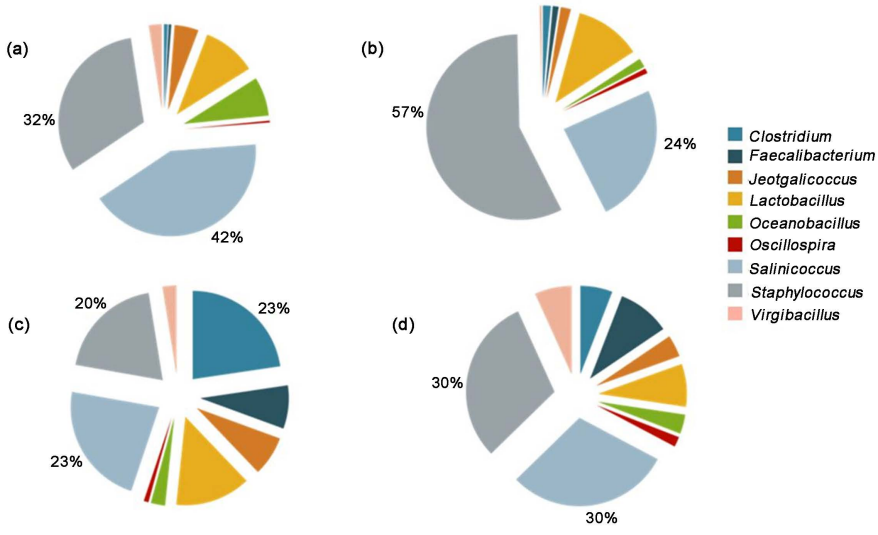



Figure 9. Distribution of genera from the phylum Actinobacteria detected in composted and non-composted poultry litter. Extractions of DNA from litter samples were used to determine bacterial phyla composition based on 16S rDNA bacterial sequence analysis by employing the Ribosomal Database Project (RDP)—Naive Bayesian rDNA classifier version 2.5 (https://rdp.cme.msu.edu/). Classifications from the RDP analysis of the sequences had an 80% cutoff. Charts (a) and (b) illustrate the 1st set of non-composted and composted litter genera. Charts (c) and (d) illustrate the 2nd set non-composted and composted litter genera.

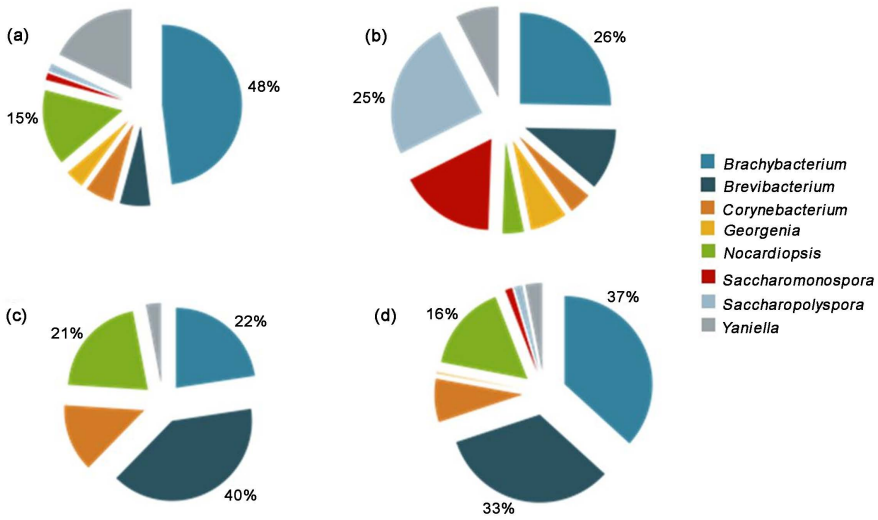



**Figure 10.** Distribution of genera from the phylum Firmicutes detected in composted and non-composted poultry litter. Extractions of DNA from litter samples were used to determine bacterial phyla composition based on 16S rDNA bacterial sequence analysis by employing the Ribosomal Database Project (RDP)—Naive Bayesian rDNA classifier version 2.5 (https://rdp.cme.msu.edu/). Classifications from the RDP analysis of the sequences had an 80% cutoff. Charts (a) and (b) illustrate the 1st set of non-composted and composted litter genera. Charts (c) and (d) illustrate the 2nd set non-composted and composted litter genera.

assess the cecal microbiome of poultry treated with a probiotic. The MIDs provided a manner to pool samples for cost savings and sequencing efficiency yet differentiate between the sample sources. Effects of probiotic administration on the chick microbiome, specifically on two known pathogenic bacteria *S. enterica* and *C. jejuni* were the basis of the measurements. Additionally, the effect of windrow composting on microbial populations in reused poultry litter was also examined. Pyrosequencing results of pooled samples that reduced cost and processing time showed that the chick microbiome and poultry litter consists of numerous varieties of bacteria.

In accordance with previous work [30] [31], Firmicutes were the predominant phylum identified. Representatives of Lachnospiraceae and Ruminococcaceae families were also detected as reported by Danzeisen *et al.* [32]. At genus level, the overall number of 16S rDNA sequence matches decreased from the first to second time interval sampled.

The probiotic employed in this study was found to not significantly reduce putative pathogen levels in the microbiome of the chick caecum. Though the chicks were challenged with *S. enterica* and *C. jejuni*, no significant difference was observed in their populations at day 10 or day 42 with or without the probiotic. A possible explanation could be that study was conducted in an academic setting in contrast to an actual poultry production facility [32].

Here, as in general poultry industry protocol, we used recycled wood shavings as bedding material with a comparable ventilation system. Other work has con-

sidered possible alternatives to litter to examine the diversity of microbial communities. For example, Kim *et al.* [33], working on pigs showed that animal-to-animal variation could be negligible in genetically similar production animals where environment plays a critical role in determining animal gut microbiome. Kasier *et al.* [34] reported that colonization of *S. enterica* occurred on day seven post challenge. Interestingly, Wisner *et al.* [35], showed that the Salmonella was cleared after 3 and 4 days of post challenge which suggests that testing the feces could have helped in finding specific inoculated bacteria.

The poultry litter microbiota analysis showed an increased bacterial diversity in the poultry litter at the genus and species level after windrow composting (440 genera and 1700 species). Interestingly at the phylum level, (**Table 4**) reduced levels of Firmicutes, Proteobacteria and Tenericutes that constitute the majority of known pathogenic bacteria were measured. By the use of the RDP database, Firmicutes were the predominant phylum identified in reused litter irrespective of the treatments. Cressman *et al.* [36], showed that the pathogenic bacteria such as Campylobacter, Salmonella, Listeria and Yersinia species were not detectable by using traditional PCR platform screens. In contrast, the pyrosequencing method detected these pathogenic genera. Chlortetracycline-resistant bacteria and tylosin-resistant bacteria were also observed in reused poultry litter that might enter birds' gut that fed on this litter thus causing resistance to antibiotics. Staphylococcus and Clostridia were found to be the predominant genera in reused litter.

## 5. Conclusions

The results of this study showed that pyrosequencing is both a sensitive and powerful tool to study the microbiome of chicks after treatments in poultry management aimed to minimize downstream contamination. Also, the study showed that multiple samples can be sequenced simultaneously using MID's thus, demonstrating that next-generation sequencing is an economical platform in combination with freely available bioinformatic database tools for chick microbiome analysis. Additionally, the computational analysis provided a mechanism to identify novel and uncommon genera. There were over 400 genera and 800 species identified from the different treatments. Results from this study suggested that neither of the treatments (probiotic administration or in-house windrow composting) had caused significant reductions of pathogenic bacteria.

This is the first study to employ next-generation sequencing technology to analyze the effects of a specific probiotic administrated on the microbiome of broiler chicks. Results of the study indicated that neither addition of this particular probiotic nor administration of the composting schemes provided a decrease in pathogen presence. Nevertheless, the study helped in identifying drawbacks of probiotic and litter treatments that could be modified in future studies for optimum pathogen control. Results from this work demonstrated that high throughput next-generation pyrosequencing technology is a cost effective method to streamline the effectiveness of potential biological strategies to minimize the occurrence of animal pathogens in poultry.

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