

## RESEARCH ARTICLE

# Broiler chickens and early life programming: Microbiome transplant-induced cecal community dynamics and phenotypic effects

Gustavo A. Ramírez<sup>1,2</sup>, Ella Richardson<sup>1</sup>, Jory Clark<sup>1</sup>, Jitendra Keshri<sup>1</sup>, Yvonne Drechsler<sup>1</sup>, Mark E. Berrang<sup>3</sup>, Richard J. Meinersmann<sup>3</sup>, Nelson A. Cox<sup>3</sup>, Brian B. Oakley<sup>1\*</sup>

**1** College of Veterinary Medicine, Western University of Health Sciences, Pomona, CA, United States of America, **2** Department of Marine Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States of America, **3** USDA Agricultural Research Service, National Poultry Center, Athens, GA, United States of America

\* [boakley@westernu.edu](mailto:boakley@westernu.edu)



## OPEN ACCESS

**Citation:** Ramírez GA, Richardson E, Clark J, Keshri J, Drechsler Y, Berrang ME, et al. (2020) Broiler chickens and early life programming: Microbiome transplant-induced cecal community dynamics and phenotypic effects. PLoS ONE 15(11): e0242108. <https://doi.org/10.1371/journal.pone.0242108>

**Editor:** Michael H. Kogut, USDA-Agricultural Research Service, UNITED STATES

**Received:** August 30, 2020

**Accepted:** October 26, 2020

**Published:** November 13, 2020

**Copyright:** © 2020 Ramírez et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All sequence data generated by this work has been deposited in the NCBI Short Read Archive with PRJNA663615 as the BioProject ID and under the following accession numbers SRX9130516 - SRX9130648.

**Funding:** Support was provided by the U.S. Poultry & Egg Association, the Western University of Health Sciences College of Veterinary Medicine, and the Office of the Vice-President for Research and Biotechnology. Additional funding was provided by USDA NIFA grants (Accession

## Abstract

The concept of successional trajectories describes how small differences in initial community composition can magnify through time and lead to significant differences in mature communities. For many animals, the types and sources of early-life exposures to microbes have been shown to have significant and long-lasting effects on the community structure and/or function of the microbiome. In modern commercial poultry production, chicks are reared as a single age cohort and do not directly encounter adult birds. This scenario is likely to initiate a trajectory of microbial community development that is significantly different than non-industrial settings where chicks are exposed to a much broader range of environmental and fecal inocula; however, the comparative effects of these two scenarios on microbiome development and function remain largely unknown. In this work, we performed serial transfers of cecal material through multiple generations of birds to first determine if serial transfers exploiting the ceca *in vivo*, rather than the external environment or artificial incubations, can produce a stable microbial community. Subsequently, we compared microbiome development between chicks receiving this passaged, *i.e.* host-selected, cecal material orally, versus an environmental inoculum, to test the hypothesis that the first exposure of newly hatched chicks to microbes determines early GI microbiome structure and may have longer-lasting effects on bird health and development. Cecal microbiome dynamics and bird weights were tracked for a two-week period, with half of the birds in each treatment group exposed to a pathogen challenge at 7 days of age. We report that: i) a relatively stable community was derived after a single passage of transplanted cecal material, ii) this cecal inoculum significantly but ephemerally altered community structure relative to the environmental inoculum and PBS controls, and iii) either microbiome transplant administered at day-of-hatch appeared to have some protective effects against pathogen challenge relative to uninoculated controls. Differentially abundant taxa identified across treatment types may inform future studies aimed at identifying strains associated with beneficial phenotypes.

numbers 1015210 and 1011327) to Western University of Health Sciences College of Veterinary Medicine and BBO.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Since the middle of the last century, antimicrobial growth promoters (AGPs), in-feed antibiotics at sub-therapeutic concentrations, have been commonly used in commercial broiler chicken farming to improve efficiency of production [1–3]. Despite the proven effectiveness of AGPs, presumed to result from modulations of the gastrointestinal (GI) microbiota and their interactions with the host [4–8], concerns about antibiotic overuse and shifting consumer preferences have led to new regulatory guidelines and industry practices removing AGPs from feed in the E.U. and the U.S [9–12]. The search for effective and commercially viable alternatives to antibiotics is therefore an important research priority [13, 14]. Promising alternatives to AGPs include the modulation of the chicken GI microbiome with prebiotics such as starches in the diet, antimicrobials such as phytochemicals or bacteriophages, and mono- or mixed-culture probiotics, as reviewed elsewhere e.g. [15–17]. While many of these alternatives to antibiotics have shown some efficacy compared to controls, re-capturing the performance benefits of AGPs remains an elusive goal. A better understanding of specific bacterial strains associated with desirable phenotypes could help identify effective probiotic alternatives to AGPs and improve understanding of their modes of action.

One promising approach to better understand how specific GI bacterial taxa may influence growth performance and pathogen tolerance in poultry is the use of microbiome transplants (MTs). Targeted modulation of the GI microbiome, particularly during early development, may alter successional trajectories of the GI microbiota and significantly influence phenotypes as the host matures [18]. Work in mammalian models highlights the importance of early-life exposures to microbes in, for example, cesarean-section vs. vaginal birth [19] or breast-fed vs formula fed infants [20]. Additionally, fecal transplants have been shown to affect host energy balance and weight gain [21, 22]. In chickens, transplantation of GI material from healthy adult birds to newly hatched chicks has been demonstrated to increase microbiome richness and diversity and improve tolerance against enteric pathogens such as *Salmonella* [23–26]. MTs may affect host health via the competitive exclusion of potential pathogens, lowering community production of growth suppression metabolites, and/or improving host energy metabolism [15, 27]. By inducing desirable phenotypes such as changes in body weight-gain or pathogen tolerance, MTs can be used to infer which bacterial strains, consortia, or metabolic pathways may contribute to host phenotype.

In the natural environment, chickens are exposed to a wide diversity of microbes early in life from environmental sources and excreta from multi-age cohorts of birds. In contrast, chicks in typical commercial broiler production systems do not encounter adult birds and are reared as a single age cohort in relatively controlled conditions under modern biosecurity regimens in which the re-use of litter across multiple flocks may be the dominant source of vertical transmission of microbes from older generations to newly hatched chicks [28]. The establishment and population dynamics of the broiler chicken GI microbiome in commercial settings have been fairly well-documented previously [29–32] but how exposure to environmental versus host-derived microbial communities (e.g. MTs) shapes the microbiome remains poorly described.

Here, we report the microbial community changes that occur when MT source material is passaged through multiple generations of birds. Our objective was to determine if host selection through multiple serial passages could derive a stable microbial community. Additionally, we report the effects of this serially-passaged material on growth and pathogen resistance when administered to newly-hatched chicks. Specifically, we explore cecal microbiome dynamics of healthy broiler chicks, from hatching to 14 days post-hatch, administered one of three treatments: i) a community derived from serial passages of cecal contents through

multiple generations of chicks (CMT), ii) an environmental community obtained from commercial poultry litter (EMT), or iii) a phosphate buffer saline (PBS) control. At one week of age, approximately half of the chicks in each treatment group were pathogen challenged via the administration of a 0.2 ml oral gavage containing ca.  $10^9$  live cells of each of *Salmonella typhimurium* and *Campylobacter jejuni*. We report significant differential phenotypic effects elicited by specific MT treatments for weight gain and pathogen tolerance. Further, we identify shifts in the cecal microbiome at the community- and taxon-level and identify differentially abundant taxa across MT treatment types associated with observed phenotypes.

## Results

### Community dynamics of serially passaged CMT

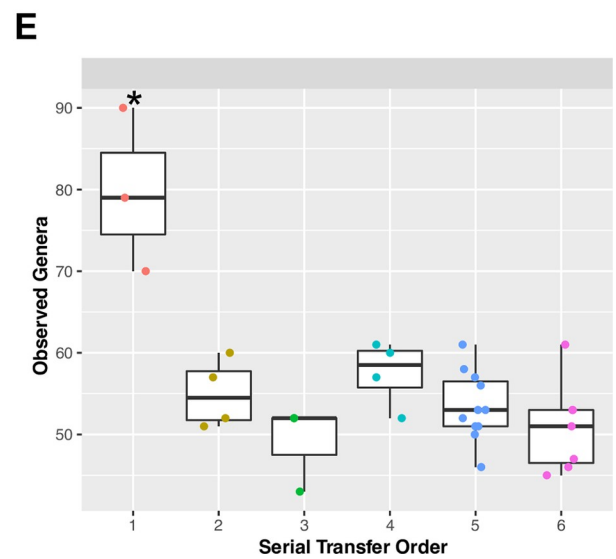
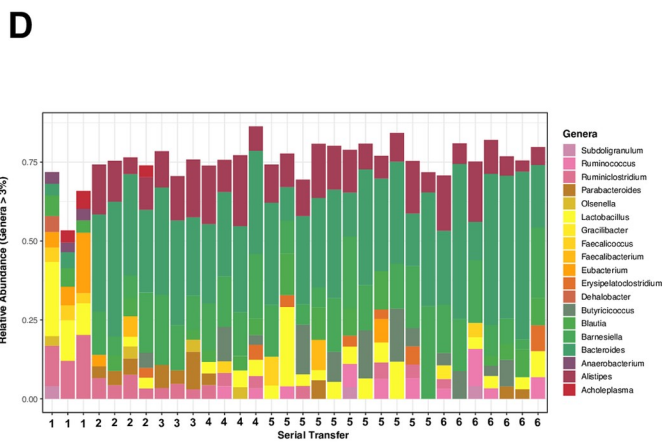
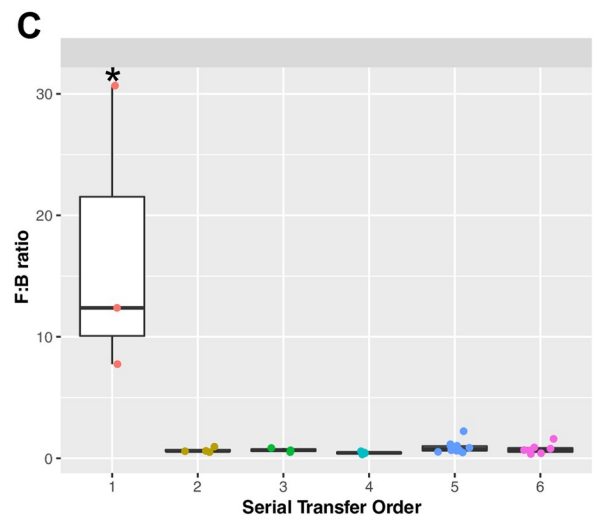
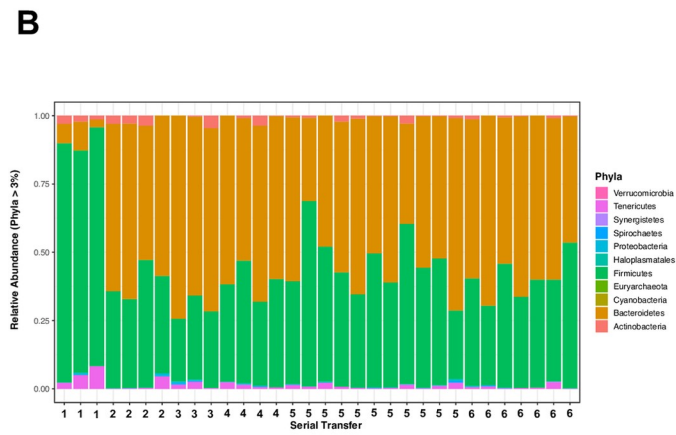
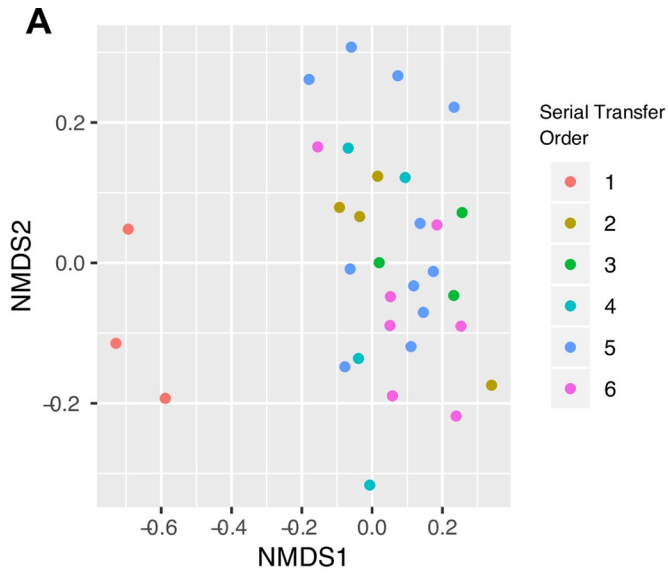
Community composition of the cecal microbiome transplants generally stabilized after a single passage (Fig 1A). Samples prior to the first serial passage were dominated (nearly 90% of all sequences) by the phylum Firmicutes, whereas, after one transfer, the phylum Bacteroidetes was dominant (Fig 1B). This shift in community composition at the phylum level after one transfer could be clearly seen in a stable Firmicutes to Bacteroidetes ratio after the first serial passage (Fig 1C). At the genus level, the community prior to the first serial passage was comprised primarily of *Lactobacillus*, *Eubacterium*, *Faecalicoccus*, and *Anaerobacterium*; whereas communities after one transfer were dominated by a few Bacteroidetes genera including *Alistipes*, *Barnesiella*, and *Blautia* (Fig 1D). Summaries of alpha diversity at the genus level showed significantly higher taxonomic richness prior to the first of the serial passages while all subsequent serial passages show lower and stable counts of observed genera (Fig 1E). Overall, despite some individual variability, frozen cecal material was significantly altered after the first passage and stable thereafter. This stable community derived from serial passages through young chicken ceca was subsequently used as the CMT inoculum in this study.

### Bacterial community composition of gavage inocula

We used a simple factorial design to assess the effects of day-of-hatch microbiome transplant type (*i.e.*: EMT, CMT, and PBS) on cecal microbiome dynamics and pathogen tolerance (Fig 2A). Community composition of the environmental and cecal-enrichment gavages (EMT and CMT treatments, respectively) differed dramatically (Fig 2B). Over 98% of the sequences recovered from the EMT gavage belong to the phylum Firmicutes, primarily the genus *Lactobacillus*. In contrast, at the phylum-level, the CMT gavage community was predominantly (>75%) comprised of the phylum Bacteroidetes with the remainder (< 25%) of sequences classified as Firmicutes. At the genus-level, the CMT gavage was more diverse than the EMT gavage with the Bacteroidetes genera *Alistipes*, *Bacteroides*, and *Barnesiella* representing approximately 75% of the CMT community (Fig 2B).

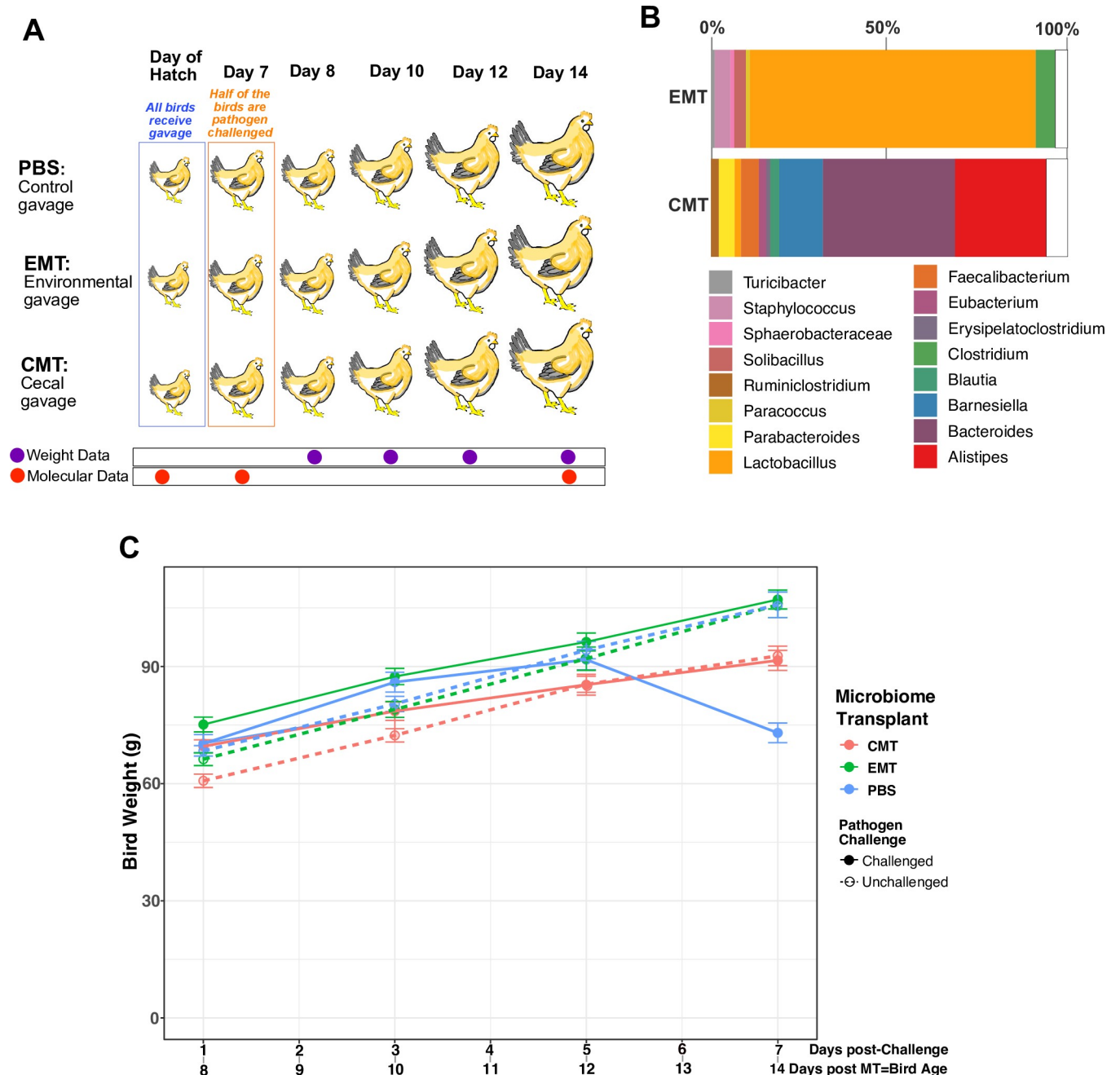
### Bird weight as a function of treatment group and pathogen challenge

Body weight differences across treatment groups were only significantly different at d14 post-hatching (Fig 2C, Table 1). In the non-challenged group at d14, weight distributions significantly differed as a function of the type of day-of-hatch MT received; EMT and PBS recipients were significantly heavier relative to birds that received a CMT. Interestingly, in the pathogen-challenged group at d14, significant differences were observed as a function of receiving either CMT or EMT at day-of-hatch relative to PBS controls. The PBS gavage (negative MT control) recipients lost approximately 20% of their average body weight between 12 and 14 days of age (5–7 days post-challenge) and at day 14 of age were significantly lighter than MT (EMT and



**Fig 1. Microbiome characterizations of serial transfer samples.** A) Ordination analysis color coded by serial transfer number. B) Phylum level community composition. C) Firmicutes:Bacteroidetes ratios for each serial passage. D) Genus level community composition. E) Number of observed genera as a function of serial transfer order (\*: significantly different means,  $p < 0.05$ ). Samples were rarefied to even depth of 850 sequences per sample.

<https://doi.org/10.1371/journal.pone.0242108.g001>



**Fig 2. Study design, community composition of MT source material, and effects on bird weights.** A) Schematic of the pooled cross-sectional study design for assessing the combined influence MT type (PBS, EMT, CMT) and pathogen challenge status (challenged vs. unchallenged). MT (via oral gavage) and pathogen challenge administration, both experimental variables, are time-stamped and depicted in blue and orange fonts, respectively. Longitudinal cross-sectional data collection for cecal molecular analyses and panel data collection for bird weight time series are depicted by red and orange purple, respectively. B) Bacterial community composition at the genus-level for gavages used to administer EMT and CMT in day-of-hatch chicks. C) Time series results for bird weight as a function of MT type and pathogen challenge status for birds age 8 through 14 days.

<https://doi.org/10.1371/journal.pone.0242108.g002>

**Table 1. Weight data replicates used to produce Fig 2C.**

	CMT	EMT	PBS
<b>Day 8</b>	n = 20 (11NC, 9C)	n = 15 (8NC, 7C)	n = 18 (10NC, 8C)
<b>Day 10</b>	n = 20 (11NC, 9C)	n = 15(8NC, 7C)	n = 18(10NC, 8C)
<b>Day 12</b>	n = 20 (11NC, 9C)	n = 15(8NC, 7C)	n = 18(10NC, 8C)
<b>Day 14</b>	n = 20 (11NC, 9C)	n = 15(8NC, 7C)	n = 18(10NC, 8C)

The same 53 birds had their weight in two-day intervals at the following post-microbiome transplant (bird age) dates and data was tabulated as a function of gavage type and pathogen challenge status (NC for not challenged and C for challenged).

<https://doi.org/10.1371/journal.pone.0242108.t001>

CMT) recipients. Also, at d14 of age, EMT recipients were significantly heavier than CMT recipients.

### Alpha-diversity

The number of observed taxa (genus- and 99% OTU-level) was lowest in 1-day old birds for all treatment groups (Fig 3A & 3B). However, significantly more taxa at the genus and 99% OTU levels were observed at d1 for birds administered a CMT relative to the EMT treatment or PBS controls (Fig 3A & 3B). From day 1 to day 7, significant increases in observed taxa occurred for all treatment groups (Fig 3). Subsequently, for birds that did not undergo a pathogen challenge, there were no significant differences in genus- or OTU-level richness between bird age 7 and 14 days (Fig 3A & 3B). For birds that were pathogen challenged at 7 days of age, a significant decrease in OTU-level richness at 14d relative to 7d was observed in the group that received a day-of-hatch CMT (Fig 3D). A day-of-hatch CMT administration generally resulted in higher OTU richness at d7 versus d14 for both the non-challenged and pathogen-challenged groups; however, these observations were only statistically significant in the challenged group (Fig 3B & 3D).

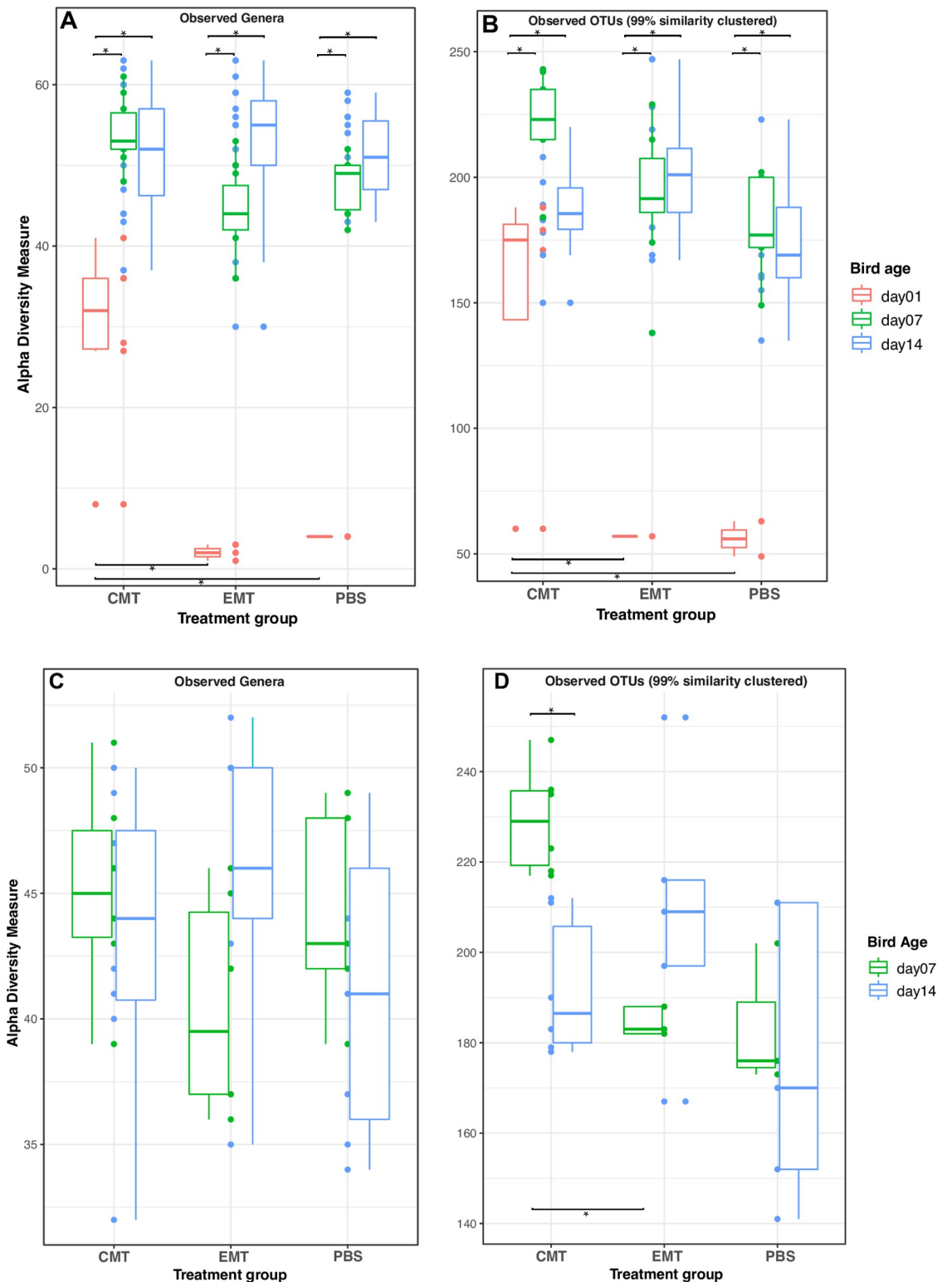
### Beta-diversity

Cecal communities of 1-day old birds (1d) showed few distinct patterns but CMT recipients generally clustered close to the CMT gavage itself along positive axis 1 and 2 values (Fig 4A). Cecal communities from EMT and PBS recipients and the EM gavage spread along the range of axis 2 but were largely confined to negative axis 1 values (Fig 4A). By 7 days of age (d7), cecal communities from birds that received a PBS gavage instead of a microbiome transplant were most similar to each other and generally clustered along negative axis 1 values (Fig 4B & 4D). Cecal communities of CMT or EMT recipients also clustered together and were more similar to the CMT than the EMT gavage community (Fig 4B & 4D). By 14 days of age (d14), community distinctions among treatments collapsed and no discernable patterns associated with MT type were observed (Fig 4C & 4E).

### Differentially abundant taxa in MTs relative to PBS controls in 7-day old chicks

**Unchallenged birds: EMT.** In the unchallenged group, a total of 9 OTU lineages, belonging to three genera within the phylum Bacteroidetes, exhibited significant differences in abundance in cecal communities from birds that received EMTs compared to PBS controls (Fig 5A). These OTUs were classified as members of the *Barnesiella*, *Parabacteroides*, and *Alistipes* genera (Fig 5A).





**Fig 3. Community richness by treatment group and time for unchallenged and challenged groups.** Only taxa with abundances greater than 5 in the dataset and samples with 1000 sequences are retained. All samples were rarefied to even depth A) Operational taxonomic units defined at the Genus-level (n = 1012 per sample) for non-pathogen challenged group. B) Operational taxonomic units defined at the 99% sequence similarity-level (n = 1044 per sample) for non-pathogen challenged group. C) Operational taxonomic units defined at the Genus-level (n = 1012 per sample) for pathogen challenged group. D) Operational taxonomic units

defined at the 99% sequence similarity-level ( $n = 1044$  per sample) for pathogen challenged group. Horizontal bars with asterisks denote significant differences between comparison pairs (student t-test,  $\alpha = 0.05$ ). Significant differences within MT groups and between MT groups are depicted at the top and bottom of the figure, respectively.

<https://doi.org/10.1371/journal.pone.0242108.g003>

**Unchallenged birds: CMT.** In the unchallenged group, a total of 24 OTU lineages, belonging to either the Firmicutes or Bacteroidetes exclusively, were significantly differentially abundant in cecal communities from birds that received CMT relative to PBS controls (Fig 5B). Specifically, 18 OTUs were significantly more abundant in CMT versus PBS treatments (Fig 5B). These OTUs were classified within the following genera: *Rikenella*, *Parabacteroides*, *Lactobacillus*, *Alistipes*, and *Barnesiella* (Fig 5B). Five OTUs classified as *Coprococcus*, *Barnesiella*, *Alistipes* and *Sporobacter* were significantly less abundant in CMT versus PBS treatments (Fig 5B). Interestingly, two genera, *Alistipes* and *Barnesiella*, had OTUs that were either significantly more and less abundant in cecal communities of CMT recipients relative to PBS controls (Fig 5B).

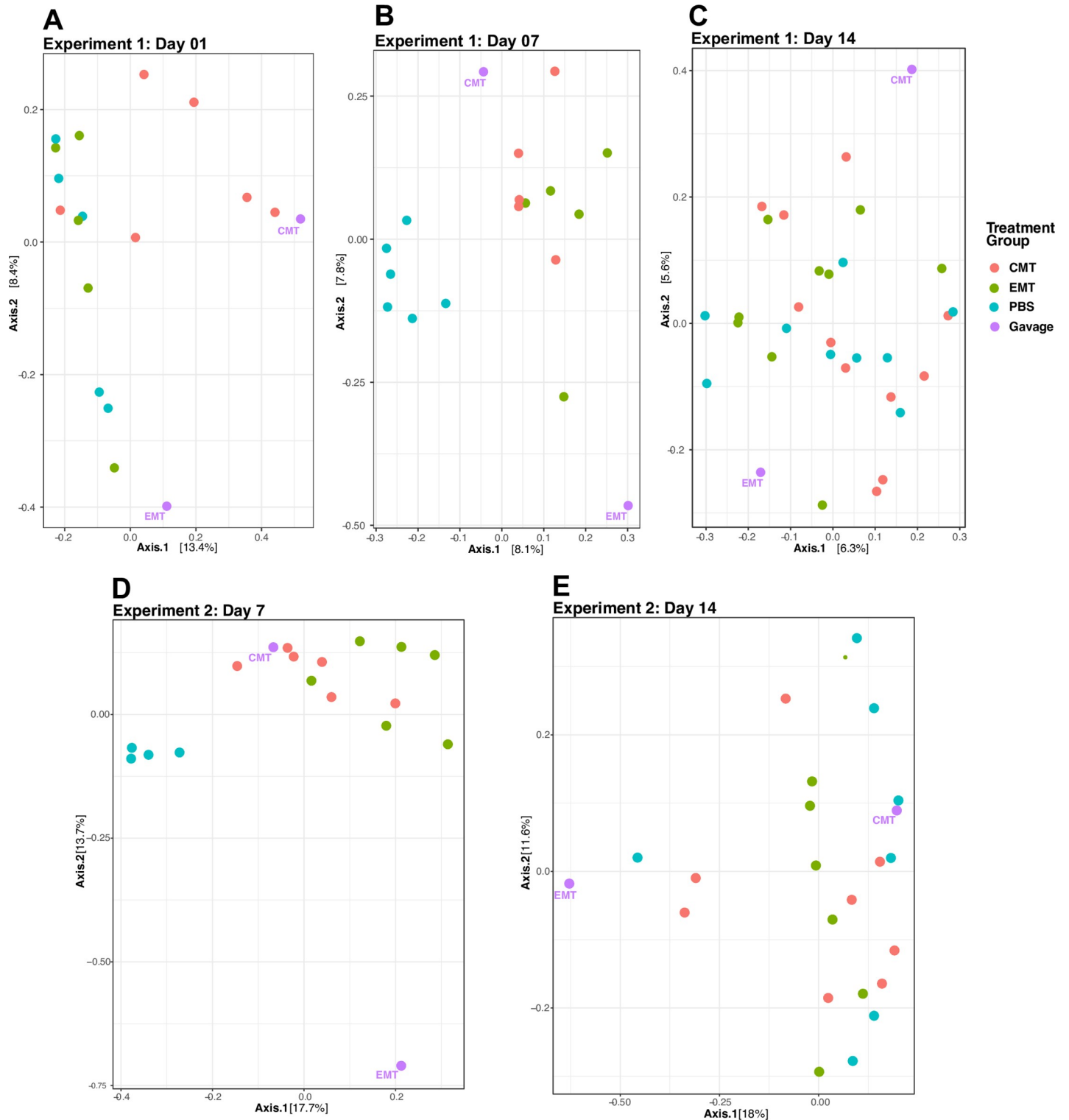
**Pathogen challenged birds: EMT.** In the challenged group, a total of 54 OTU lineages, belonging to either the Firmicutes or Bacteroidetes, exhibited significant differences in abundance in cecal communities from birds in the EMT group versus PBS controls (Fig 5C). Specifically, thirty-six and nineteen OTU lineages were significantly more and less abundant, respectively, in cecal communities from EMT recipients relative to PBS controls. All OTUs classified as *Lactobacillus*, *Butyricoccus*, *Bacillus*, and *Parabacteroides*, were significantly enriched in EMT relative to PBS controls. All OTUs classified as *Faecalitalea*, *Barnesiella*, *Odoribacter*, and *Faecalibacterium* were significantly less abundant in cecal communities from birds that received an EMT relative to PBS controls. Interestingly, three genera (*Alistipes*, *Barnesiella*, and *Bacteroides*) contained some OTUs that were significantly enriched and some that were significantly less abundant in cecal communities of EMT recipients relative to controls (Fig 5C).

**Pathogen challenged birds: CMT.** In the challenged group, a total of 90 OTU lineages, belonging to either the Firmicutes or Bacteroidetes, exhibited significant differences in abundance in cecal communities from birds that received a CMT compared to PBS controls (Fig 5D). 61 and 29 OTU lineages were significantly more abundant or less abundant, respectively, in cecal communities from CMT recipients relative to PBS controls. All OTUs classified as *Butyricoccus*, *Rikenella*, *Bacteroides*, *Parabacteroides*, and *Bacillus*, were significantly enriched in CMT relative to PBS controls. All OTUs classified as *Odoribacter*, *Blautia*, and *Faecalibacterium*, were significantly less abundant in CMT relative to PBS controls. Four genera (*Alistipes*, *Barnesiella*, *Ruminiclostridium*, and *Eubacterium*) contained some OTUs that were significantly enriched and some that were significantly less abundant in cecal communities of CMT recipients relative to PBS controls (Fig 5D).

### Taxa differentially abundant in both challenged and unchallenged groups

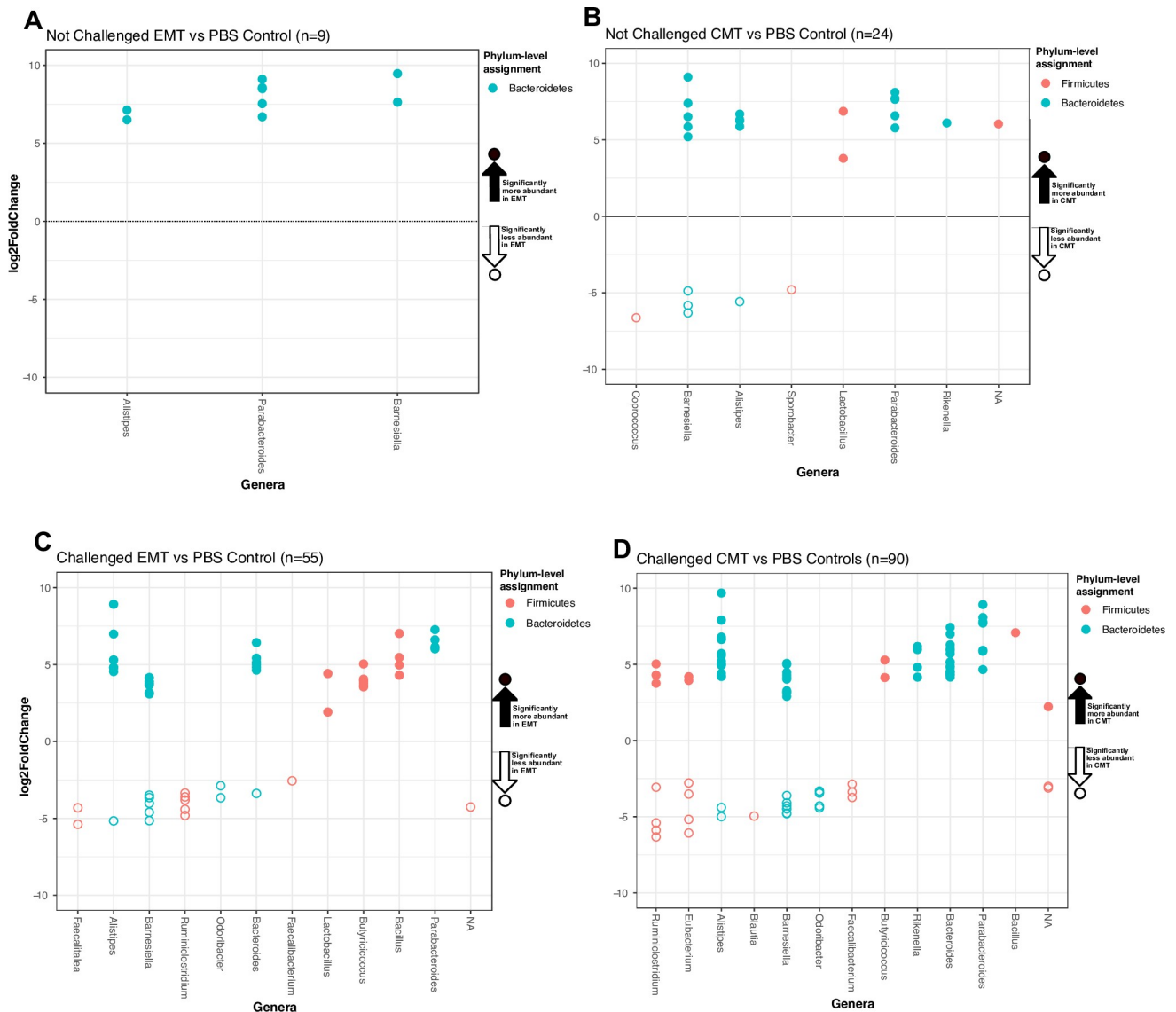
A total of 178 OTU lineages exhibited significant differences in relative abundance between birds that received a MT (EMT or CMT) versus PBS controls (Fig 6A). 125 and 13 of these OTUs were observed exclusively in challenged and unchallenged groups, respectively. Twenty differentially abundant OTUs, all classified as Bacteroidetes, were observed in both pathogen-challenged and unchallenged groups. Interestingly, these 20 OTUs exhibit similar trends in magnitude and fold change direction as a function of MT administration in both pathogen-challenged and unchallenged groups even though these were independent experimental cohorts (Fig 6B).





**Fig 4. Patterns of community composition by treatment group and time for unchallenged and challenged groups.** A-C) Ordinations plots depicting community composition for unchallenged bird group of each treatment group as a function of time (bird age in days). D & E) Ordinations plots depicting community composition for challenged birds of each treatment group as a function of time (bird age in days).

<https://doi.org/10.1371/journal.pone.0242108.g004>

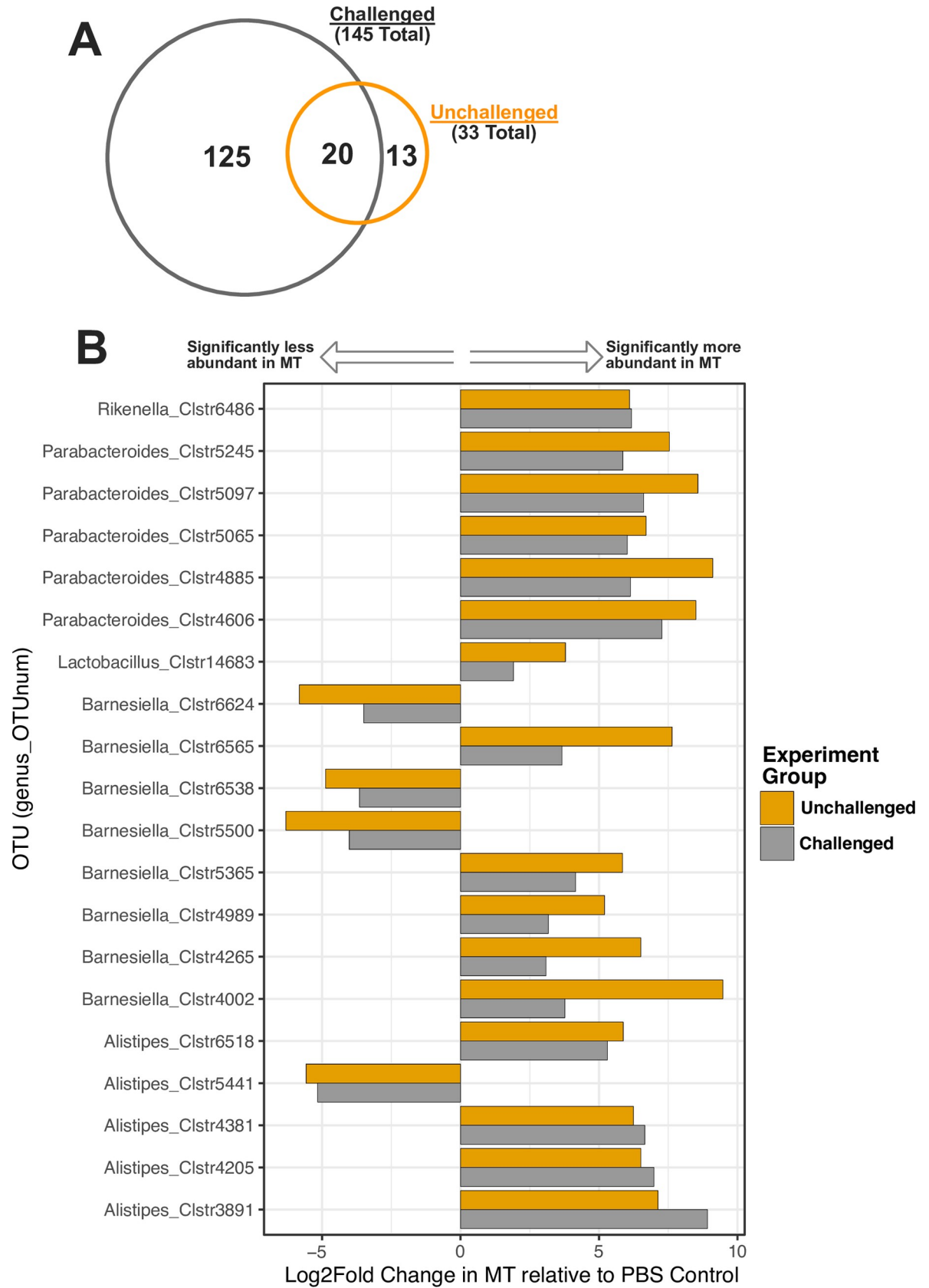


**Fig 5. Taxa exhibiting significant differences in abundance following MT treatments relative to PBS controls in cecal communities of 7-day old birds.** The x-axis shows taxonomic assignment at the genus-level for individual OTU depicted as circles. Circle color depicts phylum-level taxonomic assignments. The y-axis shows the differential Log<sub>2</sub>-fold abundance change for each taxon. Open circles represent OTUs that are significantly (Wald Test, alpha = 0.05) less abundant in MT data relative to PBS. Closed circles represent OTUs that are significantly (Wald Test, alpha = 0.05) more abundant in MT data relative to PBS. See Supplemental Materials for a comprehensive list of differentially abundant OTU IDs and fasta sequences. A) Not challenged group: Significant differences in EMT relative to controls. B) Not challenged group: Significant differences in CMT relative to controls. C) Pathogen challenged group: Significant differences in EMT relative to controls. D) Pathogen challenged group: Significant differences in CMT relative to controls.

<https://doi.org/10.1371/journal.pone.0242108.g005>

## Discussion

Applying the conceptual framework of successional trajectories [33], similar to the concept of “early life programming” [18], we hypothesized that exposure of newly hatched chicks to environmental microbes influences early GI microbiome structure and may have long-lasting effects on bird health and development. To test this hypothesis, we tracked cecal microbiome dynamics and body weight-based pathogen tolerance of broiler chicks that received complex microbiome transplants at day-of-hatch within their first two weeks of life. This experimental period was chosen based on previous results showing that two-week body weight is a robust



**Fig 6. Observed abundances of differentially abundant taxa present in both pathogen-challenged and unchallenged experiment groups.** A) Proportional Venn diagram depicting the number of taxa in the Challenged and Unchallenged groups identified as differentially abundant (challenged and unchallenged abundances summarized in gray and orange, respectively). B) Fold change magnitudes for all 28 differentially abundant taxa present in both pathogen-challenged and unchallenged experiment groups. The x-axis shows the differential Log<sub>2</sub>-fold abundance change for each OTU observed per experiment group (challenged and unchallenged abundances summarized in gray and orange, respectively). The y-axis shows taxonomic assignment at the genus-level for each OTU.

<https://doi.org/10.1371/journal.pone.0242108.g006>

predictor of slaughter weight in broilers [34] and due to logistical constraints of personnel and animal husbandry. We focused on cecal communities because of the high cell densities and relatively stable communities in the ceca [35–37]. Importantly, the ceca are a major site for bacterial fermentations and the production of short-chain fatty acids [SCFAs; [38, 39]]. SCFAs, including lactate, acetate, propionate, and butyrate, directly stimulate increases in absorptive surface area [27], suppress the growth of zoonotic pathogens [40], induce the expression of host-defense peptides [41], and modulate host epigenetic regulation [42]. To compare the effects of very different first microbial exposure starting points, we compared a stable inoculum derived from serial passages of cecal material to a complex environmental community derived from used poultry litter and sterile PBS controls. To assess if early microbial exposure influences tolerance to pathogenic infection, we performed this study on two bird panels, one that was pathogen challenged at 7d of age and one that was not pathogen challenged (Fig 2A).

### Microbiome dynamics through serial passages of cecal material

To obtain a transplant community inoculum selected by the cecal environment of broiler chicks, we serially transplanted cecal material from 14-day-old birds to newly hatched chicks. When the chicks reached 2 weeks of age, cecal contents were harvested and transplanted to a new batch of chicks. This serial passaging was repeated for five generations of chicks. We hypothesized that environmental filtering [43] would result in an overall reduction in community richness with each serial transfer of cecal material and eventually lead to a stable microbial cohort consistently sorted by host-mediated and environmental factors. In fact, a relatively stable inoculum was derived after just one passage (Fig 1). After the initial passage, the starting inoculum changed significantly in community diversity and composition from Firmicutes to Bacteroidetes dominance and remained relatively stable thereafter (Fig 1C–1E).

### Either CMTs or EMTs enhance tolerance to pathogen infection

We observed two significant effects of day-of-hatch MT on bird weight. First, in unchallenged birds, day-of-hatch EMT administration had no effect on weight while CMT administration led to significantly lower bird weight relative to controls (Fig 2C). In pathogen challenged birds, administration of either MT type resulted in higher bird weight relative to controls; however, birds administered the EMT gavage were significantly heavier than CMT recipients (Fig 2C). These observations lend credence to the notion that MT-elicited modulations of the GI flora, are both a consequence of host genetics and health status [44], and also a cause of changes in host phenotype. Because EMT rather than CMT administration resulted in increased weight gain, independent of pathogen challenge status, we concluded that gavage composition drives phenotypic outcomes and that EMT inoculation alone may be sufficient to produce desirable phenotypes. The EMT gavage was largely comprised of Firmicutes lineages assigned as *Lactobacillus* spp. while the CMT was primarily comprised of Bacteroidetes lineages within the *Alistipes*, *Bacteroides*, and *Barnesiella* genera. Notably, despite being sourced from used commercial poultry litter, the EMT composition (predominantly Firmicutes, Fig 2B) differs from previously reported communities of chicken feces [predominantly

Proteobacteria [26]]. Generally, a high prevalence of Firmicutes in the broiler GI tract is associated with beneficial immunomodulation [45, 46]. *Lactobacillus* spp. are common probiotics that have been shown to enhance energy metabolism [46], and inhibit colonization of *Campylobacter jejuni* in broilers [47]. Together, these factors may explain our observations that EMT treatment consistently resulted in higher bird weight relative to CMT. However, we note that the CMT gavage, comprised primarily of Bacteroidetes lineages, also resulted in increased weight gain relative to controls in pathogen challenged birds. This suggests that Firmicutes dominance (*Lactobacillus* spp., specifically) is not the sole determinant of the phenotypic effects elicited by both MT types in pathogen challenged birds. Although *Lactobacillus* strains are frequently used as probiotics, in some cases, *Lactobacillus* has also been associated with poor growth performance [48].

Notably, in our cecal samples, we did not see a significant number of sequences assigned to the lineages used for the pathogen challenge in our data [*i.e.*: no sequences classified as *Salmonella* were observed and only three occurrences of a low abundance phylotype classified as *Campylobacter* sp. in 7d old pathogen challenged birds were detected: one and two observations from single birds in the CMT and EMT cohorts, respectively (data not shown)]. These results suggest that these pathogens did not effectively colonize the ceca, perhaps due to unfavorable conditions encountered during passage through the GI tract, competitive exclusion within the cecum itself, or other interactions. *Salmonella* spp., are also difficult to properly resolve with 16S rRNA gene sequences [49].

Overall, enhanced tolerance to pathogen infection during early development (< 2 weeks of age) appears to be a global benefit conferred by administration of day-of-hatch MT (EMT and CMT) in broilers.

### MT-induced bacteriome dynamics

Early life microbiome status plays a critical role in establishing immune functions in murine [50] and chicken models [44]. We report rapid increases in community richness between 1d and 7d independent of MT type administered at day-of-hatch and pathogen-challenge status, however, richness generally remained stable between 7d and 14d. This corroborates previous work suggesting the rapid (within a week post-hatching) establishment of taxonomically rich GI communities [51]. Interestingly, pathogen-challenged birds at 7d had significantly more diverse cecal communities if a CMT gavage was administered at day-of-hatch (Fig 3D), however, no additional effects of either MT treatment on bacterial community richness were observed. Enrichment of *Lactobacillus* spp. and a concurrent drop in alpha-diversity have been reported in chicken ceca of birds receiving Virginamycin as a prophylactic AGP [4]; here, MT administration generally led to higher observed community richness relative to controls, however, these observations were not statistically significant (Fig 3). Ordination analyses of 7d cecal communities show compositional differences between birds that received MTs relative to controls in both pathogen-challenged and non-challenged birds (Fig 4B & 4D). Given that differences in bird weight as a function of administered MT were observed at 14d, the microbial community clustering at 7d, where both CMT and EMT communities are similar to each other and dissimilar to controls, is particularly intriguing. Both MT types altered the cecal microbiome relative to controls prior to the observed phenotypic differences. These short-lived patterns in cecal bacteriome structure completely dissipated by 14d (Fig 4E) but may have had longer lasting effects on bird phenotype since both CMT and EMT recipients exhibit weight trajectories that were unaffected by pathogen challenge (Fig 2C). Overall, we show that ephemeral GI microbial community states specifically elicited by MT administration early in a bird's life may result in longer-lasting phenotypes. The mechanisms underlying this

observation may involve immunological programming or metabolic redundancy amongst different communities in birds that received a day-of-hatch MT relative to controls. These and other potential mechanistic explanations for our observations, testable with standard tools of immunology and metagenomics, are worthy of further investigation.

### Differentially abundant lineages

To better understand the potential mechanisms of action of MTs, we identified taxa that were significantly differentially abundant between MTs and control communities at 7d (Fig 5).

In non-pathogen challenged birds, significantly higher abundances of 9 lineages belonging to the *Barnesiella*, *Parabacteroides*, and *Alistipes* genera were observed in the EMT treatments relative to controls at 7d (Fig 5A). The differential abundance of these taxa at 7d did not result in significant differences in bird weight at 14d (Fig 2C). Conversely, day-of-hatch CMT administration did result in lower bird weights at 14d relative to controls (Fig 2C), and thus taxa that differed significantly between the CMT and control communities at 7d (Fig 5A), may represent specific lineages implicated in longer term phenotypic outcomes. At 7d, taxa significantly less abundant in CMT communities relative to controls were *Coprococcus*, *Barnesiella*, *Alistipes*, and *Sporobacter* spp. while *Lactobacillus*, *Parabacteroides*, and *Rikenella* spp. OTUs were significantly more abundant relative to controls (Fig 5B). Other studies have reported *Coprococcus* spp., a butyrate-producing genera [52], enriched in chicken ceca in response to AGP treatment [6]. A depletion of *Coprococcus* at 7d in the CMT treatment may lead to lower production of SCFAs which are well-described as key microbially-produced metabolites mediating host GI tract health, resulting in lower bird weight by 14d in our study. *Lactobacillus* spp. have been implicated in improved feed conversion ratios [53] and reduced mortality [54] in broilers and are thus generally considered beneficial probiotics [55]. Despite the relative enrichment of *Lactobacillus* spp., birds in the CMT group ultimately experienced less weight gain relative to controls. Remarkably, the 9 lineages that were significantly more abundant in the 7d cecal communities of EMT recipients were also significantly more abundant in CMT recipient communities, even though the EMT and CMT treatments were derived and administered independently. These taxa may represent a core transplant microbiome, perhaps part of a consortium. Based on performance outcomes, the differentially abundant lineages in the CMT comparison, a total of 18 OTUs, should be considered potential performance-related phylotypes. In contrast, the subset of 9 lineages differentially abundant in the EMT comparison were not associated with any significant phenotypic differences. Together, these observations highlight specific OTU lineages that are differentially abundant across MTs and controls at critical points in early cecal community establishment and may provide clues to disentangle the complex links between broiler microbiome modulation and desirable phenotypes.

In pathogen challenged birds, day-of-hatch administration of a CMT or EMT gavage resulted in significantly higher bird weight relative to controls at 14d (Fig 2C). Taxa that were differentially abundant in both the CMT and EMT treatments at 7d compared to controls include: i) increases in OTUs assigned to the *Bacillus*, *Parabacteroides*, and *Butyricoccus* genera, ii) depletion of OTUs assigned to the *Odoribacter*, and *Faecalibacterium* genera, iii) and increases and decreases in OTUs within the genera *Barnesiella* and *Alistipes* (Fig 5C & 5D). Both *Bacillus* and *Butyricoccus* spp. are currently used as probiotics that have been shown to reduce heat stress-associated inflammatory responses [56] and confer protection against necrotic enteritis [57], respectively, in broiler chickens. Interestingly, despite being a common lineage recovered from chicken feces, here *Parabacteroides* spp. is significantly enriched along with *Bacillus* and *Butyricoccus* spp., suggesting its potential as a possible probiotic. *Faecalibacterium* spp. have been repeatedly associated with positive health outcomes in humans [58,



59] and have also been inversely correlated with expression of pro-inflammatory cytokines in broiler chickens [45]. *Odoribacter* spp. decreases in cecal communities have been associated with butyric acid supplementation in chicken diets [60]. Together, these observations suggest that increases in abundance and/or activity of butyrate-producing taxa, such as *Faecalibacterium* and *Butyricoccus* spp., may in fact dictate community dynamics and host-microbiome activities by generating fermentative metabolites and perhaps influence phenotypes later in life. Interestingly, we observed multiple genera (*Alistipes*, *Barnesiella*, *Bacteroides*, *Ruminoclostridium*, and *Eubacterium*) with OTUs that were both positively and negatively associated with experimental treatment and phenotype, reinforcing existing dogma that ‘strains matter’, *i.e.* specific bacterial strains can elicit significantly different phenotypes. We note that in pathogen challenged birds, day-of-hatch MT administration yielded significantly higher bird weights relative to controls, however, the highest weight gains were observed in EMT recipients (Fig 1C). Two OTU lineages of *Lactobacillus* spp. were significantly more abundant in the EMT recipients at 7d relative to controls. Butyrate producers are known to cross-feed with lactic acid produced by *Lactobacillus* spp. [61] and the significant co-enrichment of *Lactobacillus* and, for example, *Butyricoccus* spp. in the 7d cecal community of EMT recipients relative to controls, not observed in CMT recipients, suggests that the observed benefits of MT administration may result from enhanced cecal SCFA production.

## Conclusions

To advance our knowledge of microbiome-induced modulation of host health outcomes, microbiome transplants are potentially powerful tools that can identify specific taxa differentially represented between treatments and phenotypes. Here we used MTs to better understand microbiome establishment from diverse inocula and to identify specific strains associated with pathogen tolerance. Our results show that i) a relatively stable community was derived after a single passage of transplanted cecal material, ii) this cecal inoculum significantly but ephemerally altered community structure relative to the environmental inoculum and PBS controls, and iii) either microbiome transplant administered at day-of-hatch appeared to have some protective effects against pathogen challenge relative to uninoculated controls. We identify lineages that significantly differ in abundance in cecal contents from birds treated with MTs at day-of-hatch relative to controls that may drive observed phenotypic effects. These results suggest that early-life exposure to a complex microbial community, including via environmental exposure to used poultry litter may provide an effective inoculum that could protect against pathogens and identifies specific taxa that may be responsible for this effect. Further mechanistic studies to better understand these phenomena are warranted.

## Materials and methods

### Microbiome transplant source materials

The CMT source material was developed as follows: Frozen cecal material pooled from 10 healthy 6 week-old commercial broiler chickens was reconstituted by diluting 3:1 (w:v) in PBS and 0.2 mL administered via oral gavage to ten day-of-hatch chicks hatched from SPF eggs from VALO BioMedia (Adel, IA, USA). When these chicks reached 2 weeks of age, their cecal contents were similarly prepared and administered to the next set of ten chicks. This serial passaging was repeated for five sets of chicks, with 10 chicks belonging to each group for a total of 50 birds. Chicks in each cohort were housed together. Cecal contents from each bird were sequenced as described below. The cecal contents from the final 10 birds were suspended 3:1 in PBS, pooled, and used immediately as the CMT inoculum. The EMT source material was

generated from built up litter collected from an organic commercial poultry operation mixed 3:1 (w:v) in PBS and also provided as an oral gavage of 0.2 mL.

## Experimental design

To determine the effects of host-derived versus environmental microbiome transplants (MT) on cecal microbiome dynamics and pathogen tolerance in commercial broiler chicks, we designed a simple factorial experiment with SPF chicks as described above receiving either cecal microbiome transplants (CMT;  $n = 20$ ), environmental microbiome transplants (EMT;  $n = 15$ ), or PBS ( $n = 18$ ) control at day-of-hatch (Fig 2A, Table 2). The CMT and EMT inocula were derived and administered as described above and the PBS control was also provided as an oral gavage of 0.2 mL. At 7d post-hatch, half of the birds in each treatment group received a pathogen challenge via oral gavage and the other half remained as controls (Fig 2A). Birds were co-housed until pathogen challenge when they were separated by challenge group. A subset of birds from each treatment group were euthanized and cecal contents removed at the following time points: day-of-hatch, day 7, and day 14 (Fig 1A). For the pathogen challenge, birds in each treatment group were inoculated via oral gavage of 0.2 mL of live *Salmonella enterica subsp enterica serovar Typhimurium* (ATCC 14028) and a *Campylobacter jejuni* strain previously isolated from commercial broiler chickens by our laboratory at an approximate total load per bird of  $10^9$  cells for each bacterium. Individual bird weights were recorded as a function of MT type and challenge group (Fig 1B). All birds were provided *ad libitum* access to food and water.

This experiment was approved by the Western University of Health Sciences Institutional Animal care and Use Committee, Protocol R15IACUC021.

## DNA extraction and sequencing

DNA was extracted from ~100 mg of cecal contents using the MoBio UltraClean Soil DNA extraction kit (Qiagen, Carlsbad, CA) following the manufacturer's protocol. Extracts concentration and quality was checked via spectrophotometry (NanoDrop Products, Wilmington, DE, USA). Amplicons for the V4-V5 hypervariable regions of the 16S rRNA gene were generated via PCR using the 519F (5' -CAG CMG CCG CGG TAA TWC-3') and 926R (5' -CCG TCA ATT CCT TTR AGG TT-3') primers following the barcoding scheme of [62] as detailed elsewhere [45, 63]. Amplicons were paired-end sequenced on an Illumina MiSeq platform, using a 2x250bp v2 kit, following the manufacturer's protocol.

## Sequence data availability

All sequence data generated by this work has been deposited in the NCBI Short Read Archive with PRJNA663615 as the BioProject ID and under the following accession numbers SRX9130516—SRX9130648.

**Table 2. Molecular sequencing replicates.**

	CMT	EMT	PBS
<b>Day 1</b>	n = 6	n = 5	n = 5
<b>Day 7</b>	n = 11 (5NC, 6 C)	n = 11 (5NC, 6 C)	n = 10 (6NC, 4C)
<b>Day 14</b>	n = 19 (11NC, 8C)	n = 16 (9NC, 7C)	n = 16 (9NC, 7C)

Each replicate represents a cecal community from a euthanized bird. For days 7 and 14, total replicates are subdivided into not challenged (NC) and Challenged (C) groups.

<https://doi.org/10.1371/journal.pone.0242108.t002>

## Sequence analysis

Custom PERL and Unix shell scripts were used to implement portions of the QIIME [64] and Mothur [65] sequence analyses packages, as described previously [45, 63, 66]. In brief, sequences were trimmed with trimmomatic [67], subsequently merged with Flash [68], and quality-trimmed (Phred quality threshold of 25) using fastq\_quality\_trimmer [69]. Chimera detection was performed with usearch [70] using a type strain database assembled from the SILVA v128 database [71]. Taxonomic assignments were performed with usearch against the SILVA database v128 and by the RDP naïve Bayesian classifier against the RDP database [72]. Sequences were clustered into Operational Taxonomic Units (OTUs) at the RDP genus-level and at 99% sequence similarity with usearch [70].

## Statistical analyses and data summaries

Community analyses were performed in RStudio version 0.98.1091 [73] using the vegan [74] and phyloseq [75] R-packages. Briefly, observed community richness was separately assessed for rarefied Genus-level ( $n = 1012$  per sample) and 99% similarity clustered ( $n = 1044$  per sample) OTU datasets. Bray-Curtis distances were calculated from the rarefied 99% similarity OTU dataset and used for Principal Coordinate Analyses (PCoA). Differential abundance analyses were performed on abundant taxa (minimum  $n < 100$  total reads per OTU) with DESeq2 [76] using unrarefied experimental subsets, as suggested elsewhere [77].

## Acknowledgments

The authors thank George M. Montoya and Bruce S. Seal for helpful comments on the manuscript.

## Author Contributions

**Conceptualization:** Yvonne Drechsler, Mark E. Berrang, Richard J. Meinersmann, Nelson A. Cox, Brian B. Oakley.

**Data curation:** Gustavo A. Ramírez, Brian B. Oakley.

**Formal analysis:** Gustavo A. Ramírez, Ella Richardson, Jory Clark, Jitendra Keshri, Richard J. Meinersmann, Brian B. Oakley.

**Funding acquisition:** Brian B. Oakley.

**Investigation:** Gustavo A. Ramírez, Ella Richardson, Jory Clark, Jitendra Keshri, Yvonne Drechsler, Mark E. Berrang, Nelson A. Cox, Brian B. Oakley.

**Project administration:** Ella Richardson, Brian B. Oakley.

**Supervision:** Brian B. Oakley.

**Validation:** Brian B. Oakley.

**Writing – original draft:** Gustavo A. Ramírez.

**Writing – review & editing:** Gustavo A. Ramírez, Brian B. Oakley.

## References

1. Landers T, Cohen B, Wittum T, Larson E. A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep.* 2012; 127:4–22. <https://doi.org/10.1177/003335491212700103> PMID: 22298919

2. McEwen SA, Fedorka-Cray PJ. Antimicrobial use and resistance in animals. *Clin Infect Dis*. 2002; 34: S93–S106. <https://doi.org/10.1086/340246> WOS:000175621100006. PMID: 11988879
3. Sarmah AK, Meyer MT, Boxall ABA. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*. 2006; 65(5):725–59. <https://doi.org/10.1016/j.chemosphere.2006.03.026> WOS:000242330200001. PMID: 16677683
4. Costa MC, Bessegatto JA, Alfieri AA, Weese JS, Filho JA, Oba A. Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. *PLoS One*. 2017; 12(2): e0171642. <https://doi.org/10.1371/journal.pone.0171642> PMID: 28222110; PubMed Central PMCID: PMC5319738.
5. Gadde UD, Oh S, Lillehoj HS, Lillehoj EP. Antibiotic growth promoters virginiamycin and bacitracin methylene disalicylate alter the chicken intestinal metabolome. *Sci Rep*. 2018; 8(1):3592. <https://doi.org/10.1038/s41598-018-22004-6> PMID: 29483631; PubMed Central PMCID: PMC5827016.
6. Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ, Johnson TJ. Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One*. 2011; 6(11): e27949. <https://doi.org/10.1371/journal.pone.0027949> PMID: 22114729; PubMed Central PMCID: PMC3218064.
7. Niewold TA. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry Science*. 2007; 86(4):605–9. <https://doi.org/10.1093/ps/86.4.605> WOS:000245120100002. PMID: 17369528
8. Gaskins HR, Collier CT, Anderson DB. Antibiotics as growth promotants: Mode of action. *Animal Biotechnology*. 2002; 13(1):29–42. <https://doi.org/10.1081/ABIO-120005768> WOS:000177595300004. PMID: 12212942
9. Aidara-Kane A, Angulo FJ, Conly JM, Minato Y, Silbergeld EK, McEwen SA, et al. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. *Antimicrob Resist Infect Control*. 2018;7. <https://doi.org/10.1186/s13756-017-0294-9> WOS:000423128900001. PMID: 29375825
10. Batte MT, Hooker NH, Haab TC, Beaverson J. Putting their money where their mouths are: Consumer willingness to pay for multi-ingredient, processed organic food products. *Food Policy*. 2007; 32(2):145–59. <https://doi.org/10.1016/j.foodpol.2006.05.003> WOS:000244351700001.
11. Veterinary Feed Directive, RIN: 0910-AG95. Sect. 21 CFR 514 21 CFR 558 (2015).
12. Van Loo E, Caputo V, Nayga RM, Meullenet JF, Crandall PG, Ricke SC. Effect of Organic Poultry Purchase Frequency on Consumer Attitudes Toward Organic Poultry Meat. *Journal of Food Science*. 2010; 75(7):S384–S97. <https://doi.org/10.1111/j.1750-3841.2010.01775.x> WOS:000282179200014. PMID: 21535573
13. Allen HK, Levine UY, Looft T, Bandrick M, Casey TA. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol*. 2013; 21(3):114–9. <https://doi.org/10.1016/j.tim.2012.11.001> WOS:000316715200002. PMID: 23473629
14. Seal BS, Lillehoj HS, Donovan DM, Gay CG. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. *Anim Health Res Rev*. 2013; 14(1):78–87. Epub 2013/05/25. <https://doi.org/10.1017/s1466252313000030> PMID: 23702321.
15. Yadav S, Jha R. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. *J Anim Sci Biotechnol*. 2019; 10:2. <https://doi.org/10.1186/s40104-018-0310-9> PMID: 30651986; PubMed Central PMCID: PMC6332572.
16. Clavijo V, Flórez M. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. *Poult Sci*. 2018; 97(3):1006–21. <https://doi.org/10.3382/ps/pey359> PMID: 29253263
17. Huyghebaert G, Ducatelle R, Van Immerseel F. An update on alternatives to antimicrobial growth promoters for broilers. *Vet J*. 2011; 187(2):182–8. <https://doi.org/10.1016/j.tvjl.2010.03.003> PMID: 20382054.
18. Rubio LA. Possibilities of early life programming in broiler chickens via intestinal microbiota modulation. *Poult Sci*. 2019; 98(2):695–706. <https://doi.org/10.3382/ps/pey416> PMID: 30247675.
19. Neu J, Rushing J. Cesarean versus vaginal delivery: long-term infant outcomes and the hygiene hypothesis. *Clin Perinatol*. 2011; 38(2):321–31. <https://doi.org/10.1016/j.clp.2011.03.008> PMID: 21645799; PubMed Central PMCID: PMC3110651.
20. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahohy J, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev*. 2017; 81(e00036-17). <https://doi.org/10.1128/MMBR>

21. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science*. 2013; 341(6150):1241214. <https://doi.org/10.1126/science.1241214> PMID: 24009397; PubMed Central PMCID: PMC3829625.
22. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. 2006; 444(7122):1027–31. <https://doi.org/10.1038/nature05414> PMID: 17183312.
23. Corrier DE, Nisbet DJ, Scanlan CM, Hollister AG, Deloach JR. Control of *Salmonella typhimurium* colonization in broiler chicks with a continuous-flow characterized mixed culture of cecal bacteria. *Poult Sci*. 1995; 74(6):916–24. Epub 1995/06/01. <https://doi.org/10.3382/ps.0740916> PMID: 7644420.
24. Stavric S, Gleeson TM, Blanchfield B, Pivnick H. Competitive Exclusion of *Salmonella* from Newly Hatched Chicks by Mixtures of Pure Bacterial Cultures Isolated from Fecal and Cecal Contents of Adult Birds. *J Food Prot*. 1985; 48(9):778–82. Epub 1985/09/01. <https://doi.org/10.4315/0362-028X-48.9.778> PMID: 30939688.
25. Rantala M, Nurmi E. Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. *Br Poult Sci*. 1973; 14(6):627–30. Epub 1973/11/01. <https://doi.org/10.1080/00071667308416073> PMID: 4759990.
26. Siegerstetter SC, Petri RM, Magowan E, Lawlor PG, Zebeli Q, O'Connell NE, et al. Fecal Microbiota Transplant from Highly Feed-Efficient Donors Shows Little Effect on Age-Related Changes in Feed-Efficiency-Associated Fecal Microbiota from Chickens. *Appl Environ Microbiol*. 2018; 84(2). <https://doi.org/10.1128/AEM.02330-17> PMID: 29101192; PubMed Central PMCID: PMC5752867.
27. Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: History and mode of action. *Poultry science*. 2005; 84:634–43. <https://doi.org/10.1093/ps/84.4.634> PMID: 15844822
28. Liljebjelke K, Hofacre C, Lui T, White D, Ayers S, Young S, et al. Vertical and Horizontal and horizontal transmission of *Salmonella* within integrated broiler production system. *ntal Transmission of Salmonella Within Integrated Broiler Production System*. *Foodborne Pathogens and Disease*. 2005; 2(1):90–102. <https://doi.org/10.1089/fpd.2005.2.90> PMID: 15992303
29. Lu J, Idris U, Harmon B, Hofacre C, Maurer JJ, Lee MD. Diversity and Succession of the Intestinal Bacterial Community of the Maturing Broiler Chicken. *Applied and Environmental Microbiology*. 2003; 69(11):6816–24. <https://doi.org/10.1128/aem.69.11.6816-6824.2003> PMID: 14602645
30. Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, et al. The chicken gastrointestinal microbiome. *FEMS Microbiol Lett*. 2014; 360(2):100–12. <https://doi.org/10.1111/1574-6968.12608> PMID: 25263745.
31. Kers JG, Velkers FC, Fischer EAJ, Hermes GDA, Stegeman JA, Smidt H. Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. *Frontiers in Microbiology*. 2018; 9:14. <https://doi.org/10.3389/fmicb.2018.00014> WOS:000425294100001. PMID: 29445359
32. Oakley BB, Buhr RJ, Ritz CW, Kiepper BH, Berrang ME, Seal BS, et al. Successional changes in the chicken cecal microbiome during 42 days of growth are independent of organic acid feed additives. *Bmc Veterinary Research*. 2014; 10:8. <https://doi.org/10.1186/1746-6148-10-8> WOS:000347025800001. PMID: 24401205
33. Fastie C. Causes and Ecosystem Consequences of Multiple Pathways of Primary Succession at Glacier Bay, Alaska. *Ecology*. 1995; 76(6):1899–916. doi: <https://doi.org/10.2307/1940722>.
34. Mendeş M, Akkartal E. Regression tree analysisi for predicting slaughter weight in broilers. *Ital J Anim Sci* 2009;8.
35. Choi JH, Kim GB, Cha CJ. Spatial heterogeneity and stability of bacterial community in the gastrointestinal tracts of broiler chickens. *Poultry Science*. 2014; 93(8):1942–50. <https://doi.org/10.3382/ps.2014-03974> WOS:000340041100009. PMID: 24931967
36. Shapiro SK, Sarles WB. MICROORGANISMS IN THE INTESTINAL TRACT OF NORMAL CHICKENS. *Journal of Bacteriology*. 1949; 58(4):531–44. <https://doi.org/10.1128/JB.58.4.531-544.1949> WOS: A1949UK05400016. PMID: 16561816
37. Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ. Extensive microbial and functional diversity within the chicken cecal microbiome. *PLoS One*. 2014; 9(3):e91941. <https://doi.org/10.1371/journal.pone.0091941> PMID: 24657972; PubMed Central PMCID: PMC3962364.
38. Dunkley KD, Dunkley CS, Njongmeta NL, Callaway TR, Hume ME. Comparisons of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. *Poultry science*. 2007; 86:801. <https://doi.org/10.1093/ps/86.5.801> PMID: 17435012
39. Van der Wielen PWJJ, Biesterveld S, Notermans S, Hofstra H, Urlings BAP, VanKnapen F. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. *Appl Environ Microbiol*. 2000; 66(6):2536–40. <https://doi.org/10.1128/aem.66.6.2536-2540.2000> PMID: 10831435



40. Namkung H, Yu H, Gong J, Leeson S. Antimicrobial activity of butyrate glycerides toward *Salmonella Typhimurium* and *Clostridium perfringens*. *Poult Sci*. 2011; 90(10):2217–22. <https://doi.org/10.3382/ps.2011-01498> PMID: 21934003.
41. Sunkara LT, Achanta M, Schreiber NB, Bommineni YR, Dai G, Jiang W, et al. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. *PLoS One*. 2011; 6(11):e27225. <https://doi.org/10.1371/journal.pone.0027225> PMID: 22073293; PubMed Central PMCID: PMC3208584.
42. Canani RB, Costanzo MD, Leone L, Bedogni G, Brambilla P, Cianfarani S, et al. Epigenetic mechanisms elicited by nutrition in early life. *Nutr Res Rev*. 2011; 24(2):198–205. <https://doi.org/10.1017/S0954422411000102> PMID: 22008232.
43. Szekely AJ, Langenheder S. The importance of species sorting differs between habitat generalists and specialists in bacterial communities. *FEMS Microbiol Ecol*. 2014; 87(1):102–12. <https://doi.org/10.1111/1574-6941.12195> PMID: 23991811.
44. Schokker D, Veninga G, Vastenhouw SA, Bossers A, de Bree FM, Kaal-Lansbergen LM, et al. Early life microbial colonization of the gut and intestinal development differ between genetically divergent broiler lines. *BMC Genomics*. 2015; 16:418. <https://doi.org/10.1186/s12864-015-1646-6> PMID: 26017153; PubMed Central PMCID: PMC4446945.
45. Oakley BB, Kogut MH. Spatial and Temporal Changes in the Broiler Chicken Cecal and Fecal Microbiomes and Correlations of Bacterial Taxa with Cytokine Gene Expression. *Front Vet Sci*. 2016; 3:11. <https://doi.org/10.3389/fvets.2016.00011> PMID: 26925404; PubMed Central PMCID: PMC4759570.
46. LeBlanc J, Milani C, Savoy de Giori G, Sesma F, Van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr Opin Biotechnol*. 2013; 24:160–8. <https://doi.org/10.1016/j.copbio.2012.08.005> PMID: 22940212
47. Neal-McKinney JM, Lu X, Duong T, Larson CL, Call DR, Shah DH, et al. Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. *PLoS One*. 2012; 7(9):e43928. <https://doi.org/10.1371/journal.pone.0043928> PMID: 22962594; PubMed Central PMCID: PMC3433458.
48. Diaz-Sanchez S, Perrotta AR, Rockafellow I, Alm EJ, Okimoto R, Hawken R, et al. Using fecal microbiota as biomarkers for predictions of performance in the selective breeding process of pedigree broiler breeders. *PLoS One*. 2019; 14(5):e0216080. Epub 2019/05/08. <https://doi.org/10.1371/journal.pone.0216080> PMID: 31063485; PubMed Central PMCID: PMC6504170 interests to declare: Funding for this study was provided by Cobb-Vantress, Inc. Authors RH and RO are commercially affiliated with the funder. The funder provided support in the form of salaries for SDS, and RH and RO, employed by the funder had active roles in the study as stated in the authors' contributions. This does not alter our adherence to all PLOS ONE policies on sharing data and materials.
49. Malorny B, Hoorfar J, Bunge C, Helmuth R. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl Environ Microbiol*. 2003; 69(1):290–6. Epub 2003/01/07. <https://doi.org/10.1128/aem.69.1.290-296.2003> PMID: 12514007; PubMed Central PMCID: PMC152403.
50. Cahenzli J, Koller Y, Wyss M, Geuking MB, McCoy KD. Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe*. 2013; 14(5):559–70. <https://doi.org/10.1016/j.chom.2013.10.004> PMID: 24237701; PubMed Central PMCID: PMC4049278.
51. Apajalahti J, Kettunen A, Graham H. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal*. 2004; 60:223–32. <https://doi.org/10.1079n;VPS200415>
52. Pryde S, Duncan S, Hold G, Stewart C, Flint H. The microbiology of butyrate formation in the human colon. *FEMS Microbiol Lett*. 2002; 217:133–9. <https://doi.org/10.1111/j.1574-6968.2002.tb11467.x> PMID: 12480096
53. Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R, et al. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Appl Environ Microbiol*. 2011; 77(17):5868–78. <https://doi.org/10.1128/AEM.00165-11> PMID: 21742925; PubMed Central PMCID: PMC3165380.
54. Timmerman H, Veldman A, Van den Elsen E, Rombouts F, Beynen A. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. *Poult Sci*. 2006; 85:1383–8.
55. Bai SP, Wu AM, Ding XM, Lei Y, Bai J, Zhang KY, et al. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. *Poult Sci*. 2013; 92(3):663–70. <https://doi.org/10.3382/ps.2012-02813> PMID: 23436517.
56. Wang WC, Yan FF, Hu JY, Amen OA, Cheng HW. Supplementation of *Bacillus subtilis*-based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens. *J Anim Sci*. 2018;



- 96(5):1654–66. <https://doi.org/10.1093/jas/sky092> PMID: 29528406; PubMed Central PMCID: PMC6140875.
57. Eeckhaut V, Wang J, Van Parys A, Haesebrouck F, Joossens M, Falony G, et al. The Probiotic *Butyrivibrio* *faecalis* Reduces Feed Conversion and Protects from Potentially Harmful Intestinal Microorganisms and Necrotic Enteritis in Broilers. *Front Microbiol.* 2016; 7:1416. <https://doi.org/10.3389/fmicb.2016.01416> PMID: 27708624; PubMed Central PMCID: PMC5030265.
  58. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A.* 2008; 105(43):16731–6. <https://doi.org/10.1073/pnas.0804812105> PMID: 18936492; PubMed Central PMCID: PMC2575488.
  59. Miquel S, Martin R, Rossi O, Bermudez-Humaran LG, Chatel JM, Sokol H, et al. *Faecalibacterium prausnitzii* and human intestinal health. *Curr Opin Microbiol.* 2013; 16(3):255–61. <https://doi.org/10.1016/j.mib.2013.06.003> PMID: 23831042.
  60. Bortoluzzi C, Pedroso AA, Mallo JJ, Puyalto M, Kim WK, Applegate TJ. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. *Poult Sci.* 2017; 96(11):3981–93. <https://doi.org/10.3382/ps/pex218> PMID: 29050425.
  61. De Maesschalck C, Eeckhaut V, Maertens L, De Lange L, Marchal L, Nezer C, et al. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. *Appl Environ Microbiol.* 2015; 81(17):5880–8. <https://doi.org/10.1128/AEM.01616-15> PMID: 26092452; PubMed Central PMCID: PMC4551243.
  62. Faircloth BC, Glenn TC. Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. *PLoS One.* 2012; 7(8):e42543. <https://doi.org/10.1371/journal.pone.0042543> PMID: 22900027; PubMed Central PMCID: PMC3416851.
  63. Oakley BB, Morales CA, Line J, Berrang ME, Meinersmann RJ, Tillman GE, et al. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PLoS One.* 2013; 8(2):e57190. <https://doi.org/10.1371/journal.pone.0057190> PMID: 23468931; PubMed Central PMCID: PMC3584146.
  64. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 2012; 6(8):1621–4. <https://doi.org/10.1038/ismej.2012.8> PMID: 22402401; PubMed Central PMCID: PMC3400413.
  65. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* 2009; 75(23):7537–41. <https://doi.org/10.1128/AEM.01541-09> PMID: 19801464; PubMed Central PMCID: PMC2786419.
  66. Oakley BB, Morales CA, Line JE, Seal BS, Hiatt KL. Application of high-throughput sequencing to measure the performance of commonly used selective cultivation methods for the foodborne pathogen *Campylobacter*. *FEMS Microbiol Ecol.* 2012; 79(2):327–36. <https://doi.org/10.1111/j.1574-6941.2011.01219.x> PMID: 22092388.
  67. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014; 30(15):2114–20. <https://doi.org/10.1093/bioinformatics/btu170> PMID: 24695404; PubMed Central PMCID: PMC4103590.
  68. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics.* 2011; 27(21):2957–63. <https://doi.org/10.1093/bioinformatics/btr507> PMID: 21903629; PubMed Central PMCID: PMC3198573.
  69. Blankenberg D, Gordon A, Von Kuster G, Coraor N, Taylor J, Nekrutenko A, et al. Manipulation of FASTQ data with Galaxy. *Bioinformatics.* 2010; 26(14):1783–5. <https://doi.org/10.1093/bioinformatics/btq281> PMID: 20562416; PubMed Central PMCID: PMC2894519.
  70. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 2011; 27(16):2194–200. <https://doi.org/10.1093/bioinformatics/btr381> PMID: 21700674; PubMed Central PMCID: PMC3150044.
  71. Yarza P, Ludwig W, Euzéby J, Amann R, Schleifer KH, Glöckner FO, et al. Update of the All-Species Living Tree Project based on 16S and 23S rRNA sequence analyses. *Syst Appl Microbiol.* 2010; 33(6):291–9. <https://doi.org/10.1016/j.syapm.2010.08.001> PMID: 20817437.
  72. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 2014; 42(Database issue):D633–42. <https://doi.org/10.1093/nar/gkt1244> PMID: 24288368; PubMed Central PMCID: PMC3965039.
  73. Racine JS. RStudio: A Platform-Independent IDE for R and Sweave. *Journal of Applied Econometrics.* 2012; 27(1):167–72. <https://doi.org/10.1002/jae.1278>

74. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. *vegan*: Community Ecology Package. R Package Version 2.2–1. Available online at: <http://CRANR-projectorg/package=vegan>. 2015.
75. McMurdie PJ, Holmes S. *phyloseq*: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013; 8(4):e61217. <https://doi.org/10.1371/journal.pone.0061217> PMID: [23630581](https://pubmed.ncbi.nlm.nih.gov/23630581/); PubMed Central PMCID: PMC3632530.
76. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014; 15(12):550. <https://doi.org/10.1186/s13059-014-0550-8> PMID: [25516281](https://pubmed.ncbi.nlm.nih.gov/25516281/); PubMed Central PMCID: PMC4302049.
77. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput Biol*. 2014; 10(4):4. <https://doi.org/10.1371/> PMID: [29219400](https://pubmed.ncbi.nlm.nih.gov/29219400/)