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

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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.
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, Faecalibacillus, 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.

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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.

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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.
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*, *Butyricicoccus*, *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 *Butyricicoccus*, *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 5A). 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).

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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 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 Log2-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.

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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
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 passing 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

![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 Log2-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)
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 *Butyricicoccus* 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 *Butyricicoccus* 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 *Butyricicoccus* spp., suggesting its potential as a possible probiotic. *Faecalibacterium* spp. have been repeatedly associated with positive health outcomes in humans [58,
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 *Butyricicoccus* 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*, *Ruminiclostridium*, 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, *Butyricicoccus* 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 pas-saging 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   |
|--------|-------|-------|-------|
| Day 1  | n = 6 | n = 5 | n = 5 |
| Day 7  | n = 11 (5NC, 6 C) | n = 11 (5NC, 6 C) | n = 10 (6NC, 4C) |
| Day 14 | 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.

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Sequence analysis

Custom PERL and Unix shell scripts were used to implement portions of the QIIME [64] and Mothur [65] sequence analysis 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 na"ive 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].

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