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ARTICLE

Mining chicken ileal microbiota for immunomodulatory microorganisms

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The gut microbiota makes important contributions to host immune system development and resistance to pathogen infections, especially during early life. However, studies addressing the immunomodulatory functions of gut microbial individuals or populations are limited. In this study, we explore the systemic impact of the ileal microbiota on immune cell development and function of chickens and identify the members of the microbiota involved in immune system modulation. We initially used a time-series design with six time points to prove that ileal microbiota at different succession stages is intimately connected to immune cell maturation. Antibiotics perturbed the microbiota succession and negatively affected immune development, whereas early exposure to the ileal commensal microbiota from more mature birds promoted immune cell development and facilitated pathogen elimination after *Salmonella* Typhimurium infection, illustrating that early colonization of gut microbiota is an important driver of immune development. Five bacterial strains, *Blautia coccoides*, *Bacteroides xylanisolvens*, *Fournierella* sp002159185, *Romboutsia lituseburensis*, and *Megamonas funiformis*, which are closely related to the immune system development of broiler chickens, were then screened out and validated for their immunomodulatory properties. Our results provide insight into poultry immune system–microbiota interactions and also establish a foundation for targeted immunological interventions aiming to combat infectious diseases and promote poultry health and production.

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INTRODUCTION

Chicken consumption is growing in popularity worldwide, with a preference for chicken over red meat [1, 2]. Concurrent with an increase in consumption is the increasing production efficiency and the fast intensification of poultry industry. As a result of high-intensity stocking and crowded conditions, the rates of communicable diseases in poultry are significantly increasing. Especially in the early stage of life, when the immune system is not fully developed [3, 4], chicks are susceptible to various viral, bacterial, and fungal infections that cause great economic losses to the poultry industry. Antibiotics have been a key method for ensuring effective poultry production for a long time, but they are no longer allowed in the production of food animals in many nations due to rising worries about antibiotic resistance [5]. Therefore, timely alternative approaches should be considered to establish early life immunity against diseases.

After birth, the gut ecosystem undergoes a dramatic transition from an essentially sterile state to extremely dense colonization, culminating in the establishment of an adult-like microbial community [6, 7]. Early life microbial succession occurs concomitantly with development, expansion, and education of the local immune system [8]. Numerous human and mouse studies demonstrate that the intestinal microbiota makes essential contributions to the development of the host immune system and the resistance to pathogen infections [9–11], particularly during early life. Germ-free mice have poorly developed innate and adaptive immune systems [12, 13], but their immune systems could be reinstated after bacterial reconstitution of mice gut ecosystem [14]. Several bacterial species that possess immunoregulatory properties have been identified. For example, segmented filamentous bacteria can promote the development of Th17 cells [15]. *Clostridium* clusters XIVa and IV [16] and *Bifidobacterium bifidum* [17] can also induce colonic T-regulatory cell (T-reg) differentiation. Although minimal research has been performed on cross-talk between chicken commensal bacteria and the immune system, preliminary studies in germ-free chickens have emphasized the key importance of microbial exposure for the appropriate development and maturation of the immune system [18]. Previous studies have also demonstrated that disrupting the microbiota with antibiotic therapy could change the intestinal inflammatory response and the host immune status of chickens [19, 20]. These studies emphasize that gut symbionts have important consequences for shaping the chicken immune system and can lead to readily discernible effects on infection prevention. It was revealed that oral colonization of newly hatched chickens with cecal extracts from birds of different ages promotes resistance to *Salmonella enteritidis* [21]. Transferring fecal microbiota from high-weight adult chickens to newborn chicks can lead to remodeling of the jejunal microbiota, reduction in inflammatory responses, and improvement in chicken growth performance [22]. These results are encouraging, but it is not feasible to provide complex, undefinable stool material on a wide scale to maintain or

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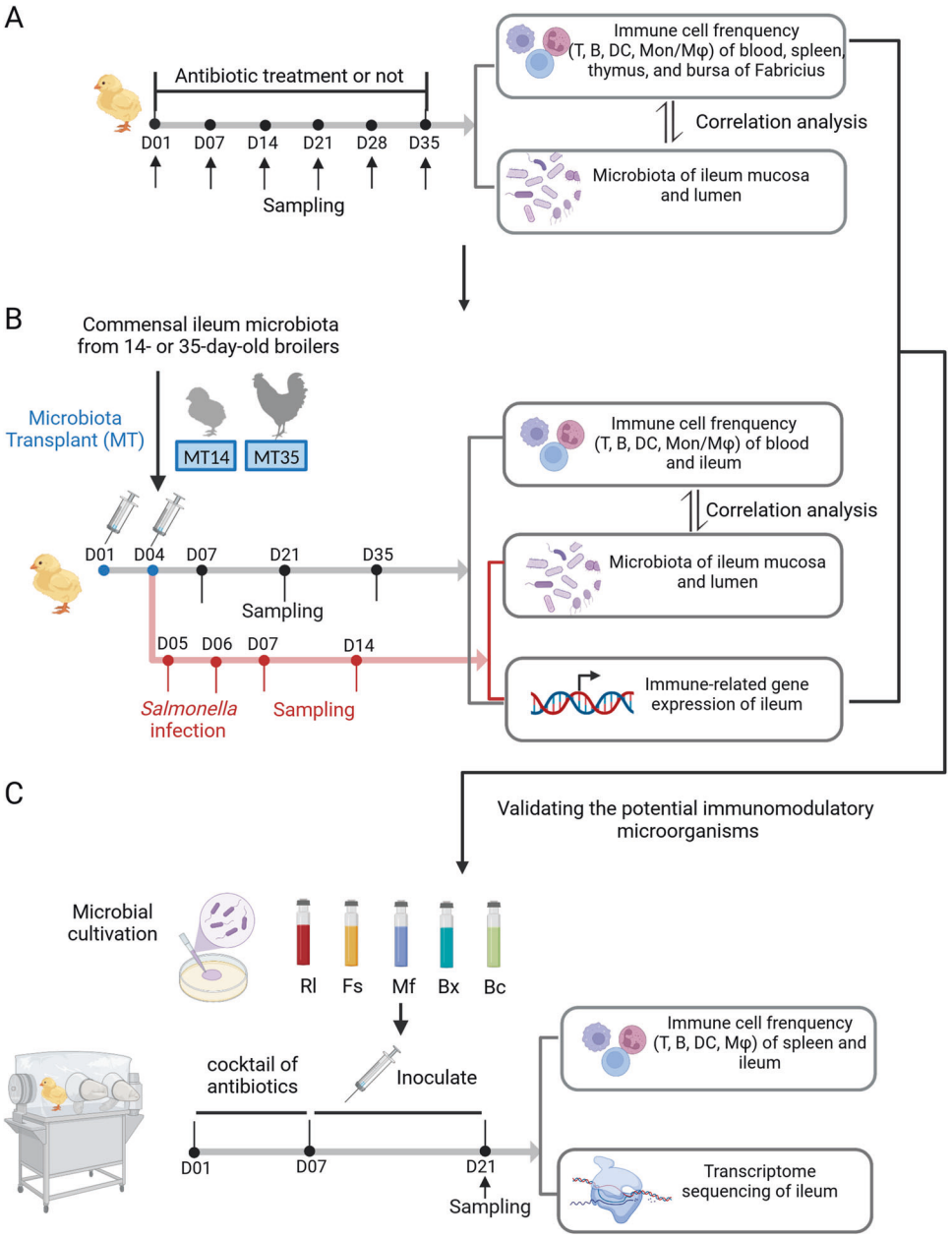


Fig. 1 Overview of experiment design. A Analyzing the patterns of microbial colonization, immunological development, and potential connections between them in broiler chickens with or without the antibiotic treatment. **B** Investigating the impact of commensal ileum microbiota from 14- or 35-day-old broiler chickens on immunological development and resistance to *Salmonella* infection. **C** Validating the potential the immunomodulatory gut microorganisms acquired from broiler intestine and then inoculated into broiler chickens with antibiotics treatment. Rl Romboutsia lituseburensis, Fs Fournierella sp002159185, Mf Megamonas funiformis, Bx Bacteroides xylanisolvens, Bc Blautia coccoides.

enhance chicken health. Therefore, more research is required to obtain a full understanding of chicken symbiotic microbes, particularly anaerobic bacteria.

Numerous studies have reported that immune cells abundantly exist in the ileum compared with other intestinal segments [23, 24]. Therefore, the ileum microbiota could play a fundamental role in the formation of a well-developed immune system. This study focused on the luminal and mucosal microbiota in ileum of broiler chickens and explored the systemic instruction of the ileum microbial populations and individuals on the development and function of immune cells. First, we focused on the dynamics of the ileal microbiota composition in relation to the immune development of chickens, after which we sought to alter the gut microbial colonization by long-term antibiotic exposure and short-term microbiota transplantation to investigate the immunological changes accordingly. Subsequently, potential strains that are closely related to the immune system development of broiler chickens were screened out and their immunomodulatory effects were further verified in low bacterial load chicks (Fig. 1).

RESULTS

Temporal and spatial development of ileum microbiota in broiler chickens after birth

Numerous studies have shown that the chicken gut microbiota undergoes rapid changes during the early stages of development

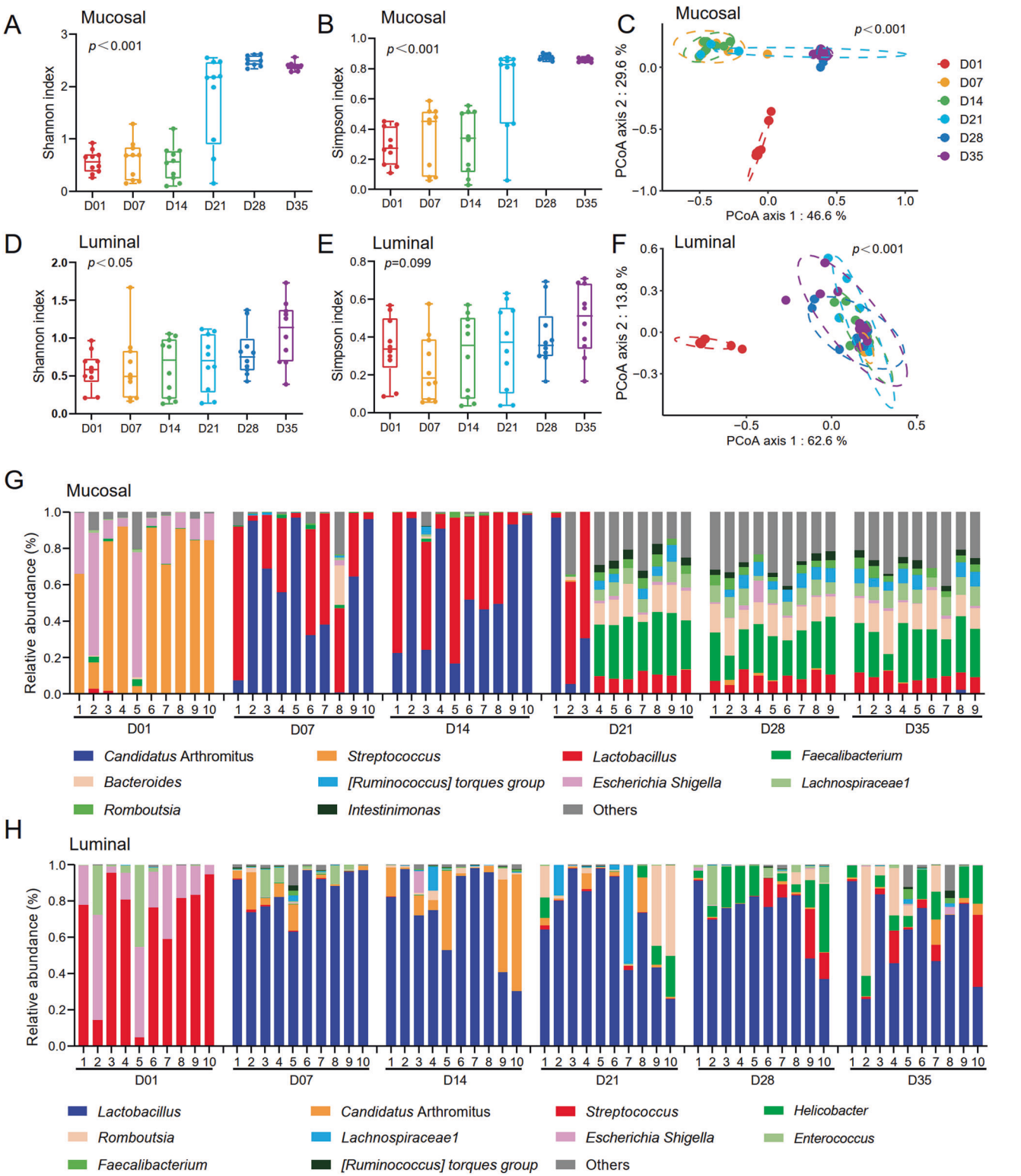


Fig. 2 Temporal and spatial development of ileum microbiota in broiler chickens. Shannon index (**A**) and Simpson index (**B**) of ileum mucosal microbiota. **C** Microbiota clustering based on a principle coordinate analysis (PcoA) of ileum mucosal microbiota. Shannon index (**D**) and Simpson index (**E**) of ileum luminal microbiota. **F** Microbiota clustering based on PcoA of ileum luminal microbiota. Relative abundance of top 10 bacterial genera in ileal mucosa (**G**) and lumen (**H**). $n = 9$–10/day.

and is gradually colonized by bacteria over time [25–27]. In order to obtain generic insight into the ileum mucosal and luminal microbiota succession, the microbiome was assessed based on 16S rRNA gene sequencing. We compared the overall ileum microbial diversity and community structural changes of six different ages (1, 7, 14, 21, 28, and 35 days). The Shannon and Simpson indices (Fig. 2A, B, respectively) of ileum mucosal microbiota were found to gradually increase in conjunction with

age since birth ($p < 0.001$). A principal coordinate analysis (PcoA) of ileum mucosal microbiota (Fig. 2C) highlighted that six bacterial communities formed three distinct clusters (permutation multivariate analysis of variance [PERMANOVA], $R^2 = 0.06$; $p < 0.001$), indicating that the bacterial community structure could be divided into three successional stages. At 1 day of age, the chicken's ileum mucosal bacterial community formed the first successional stage, which was mainly composed of *Streptococcus* and *Escherichia/Shigella*. The second successional stage was formed at 7 and 14 days of age. In this stage, the abundance of *Candidatus* Arthromitus and *Lactobacillus* increased rapidly after which they constituted the dominant bacteria of ileum mucosa. At 21 days of age, the microbial community seems to be situated in a transitional phase before entering the third successional stage. After 21 days of age, the third successional stage appeared, and the abundance of *Candidatus* Arthromitus and *Lactobacillus* decreased sharply, while the abundance of *Faecalibacterium*, *Bacteroides*, *Lachnospiaceae1*, and *Ruminococcus* increased thus forming a more diverse and stable bacterial community structure (Fig. 2G). Similarly, the Shannon index of ileal lumen microflora also increased with the age of the chicken (Fig. 2D, $p < 0.05$). PcoA analysis showed that the ileal lumen bacterial community structure of newborn broiler chickens was significantly different from that of post-growth broiler chickens (Fig. 2F, PERMANOVA, $R^2 = 0.06$; $p < 0.001$). Similar to the mucosal microbiome, the ileal lumen microbiome can also be divided into three stages of succession; *Streptococcus* and *Escherichia/Shigella* were the primary microorganisms in the ileal lumen of newborn chicks; the abundance of *Candidatus* Arthromitus in the ileal lumen increased at 7 and 14 days of age. However, unlike the mucosal microbiome, *Lactobacillus* remained the most abundant bacteria in the ileal lumen along with broiler chicken growth, and after 21 days of age, the abundance of *Helicobacter*, *Romboutsia*, *Streptococcus*, and *Lachnospiaceae1* gradually increased and formed a stable microflora structure similar to mucosal microflora (Fig. 2H). These results indicate that the changes in ileum mucosal and luminal microbial community are age-dependent, but the species and abundance of dominant bacteria in ileal mucosa and lumen at the different stages are not exactly the same even though a similar successional pattern between ileal mucosal and lumen microflora could be observed.

Sequential waves of immune cell expansion after birth

We then analyzed the development of immune cells in the blood, spleen, thymus, and Bursa of Fabricius from chickens at six time points using flow cytometry. Gating strategy is shown in Fig. S1.

We evaluated the frequency of immune cells in the central immune organs at first. The thymus is a primary organ for T cells developing in broiler chickens, where myeloid progenitors lacking $CD4^+$ and $CD8^+$ co-receptor expression undergo T-cell receptor rearrangement to generate $CD4^+CD8^+$ double-positive (DP) thymocytes. DP cells undergo selection to generate $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes that eventually export to the periphery as naive T-cells. Here, we quantified the proportion of T-lymphocytes in the thymus at different ages. In the first 14 days following birth, the percentage of DP thymocytes dramatically decreased, while the SP thymocytes peaked at 7 to 14 days (Fig. S2A), showing that the first 14 days after birth are a crucial time for the T-cell development in the thymus. The Bursa of Fabricius provides a unique microenvironment for lymphoid stem cells to proliferate, differentiate, and acquire the characteristics of mature and immunocompetent B-cells. Throughout the trial period, the percentage of B-cells in the bursa decreased (Fig. S2B), which was likely caused by the migration of many mature B-cells into peripheral tissues, including the blood and spleen as development progressed.

The developmental state of immune cells in peripheral immune organs was then determined. The proportions of blood monocytes, dendritic cells (DCs), B-cells, total T-cells, and $CD8^+$ T-cells grew fast in the first 14 days and then reached a plateau or decreased (Fig. 3A). The proportions of blood $CD4^+$ T-cells, spleen $CD4^+$ T-cells, and B-cells steadily grew to their maximum levels during the first 21 days (Fig. 3B). The ratios of spleen total T-cells and $CD8^+$ T-cells peaked at 28 days and then stabilized (Fig. 3C).

Collectively, these findings revealed that the critical windows in the immune cell development were between 14 and 28 days, which corresponded to the most crucial period for the gradual establishment of a mature and stable microbiota in the ileal mucosa and lumen (Fig. 2G, H).

The dynamic interplay between the gut microbiota and immune cell development

We performed a correlation analysis to obtain a deeper understanding of the role of microorganisms in the immunological development of broiler chickens. The age-dependent impact was evident when the interaction between parameters of the immune system and mucosal microbiota were assessed bidirectionally (Fig. 4A). Ileal mucosal microbes are divided into three major clusters based on their association with immune cells. The three microbe clusters were enriched at distinct ages and belonged to the three stages of microbial succession mentioned above, demonstrating that each microbial succession stage is important for the development of the immune system. We found that the abundance of *Candidatus* Arthromitus and *Lactobacillus* as the dominating bacterium in the second succession stage (7–14 days after birth) positively correlated with the proportion of B-cells, DCs, and $CD8^+$ T-cells in the blood, which all peaked within the first 14 days of life. The abundance of bacteria, such as *Faecalibacterium*, *Bacteroides*, *Lachnospiraceae*, *Megamonas*, *Romboutsia*, and others, which are enriched in the third succession stage (21–35 days of life), showed a strong positive correlation with the proportion of spleen $CD3^+$, $CD8^+$, and $CD4^+$ T-cells and a strong negative correlation with the proportion of Bursa B-cells. When compared with the analysis of ileal mucosa, the interaction between luminal microbiota and immune parameters displayed a weaker time dependence. We note that a strong positive correlation between the proportion of DN and SP thymocytes and the abundance of certain luminal bacteria, such as *Bacteroides*, *Blautia*, and *Parabacteroides* (Fig. 4B). These findings suggest that some specific microbes might play a pivotal role in the development of these immune cells at a certain stage.

Antibiotic-induced temporal and spatial changes in ileal microbiota with associated alterations in immune cell development and homeostasis

To explore the role of ileal microbial community succession on host immune system development, we attempted to interfere with gut microbial colonization of broiler chickens using chlortetracycline, a broad-spectrum antibiotic that is used as a growth promoter in livestock feed. The analysis of bacterial diversity and richness showed the antibiotic treatment disrupted the structure of both the mucosal and luminal microbiota in the ileum (Figs. 5A, B and S3A–D); moreover, the microbiome maturity curve reveals that the antibiotic treatment impeded microbiota development, especially that of the mucosal microbiota (Fig. 5C). These data reveal a dramatic shift in community structure that was found to occur quickly after antibiotic initiation followed by a more gradual alteration in community structure over time. Concurrently, flow cytometric analysis illustrates that antibiotic treatment inhibited proliferation of total T-lymphocytes for a longer period of time and $CD8^+$ T-lymphocytes only during the first week after hatching as represented by blood and spleen levels (Fig. 5D, E; $p < 0.05$). Antibiotic treatment also led to a significant reduction in the proportion of total B-cells in peripheral blood at 14 days of age (Fig. 5D; $p < 0.05$). Collectively, these data confirm that intestinal bacterial dysbiosis could affect the

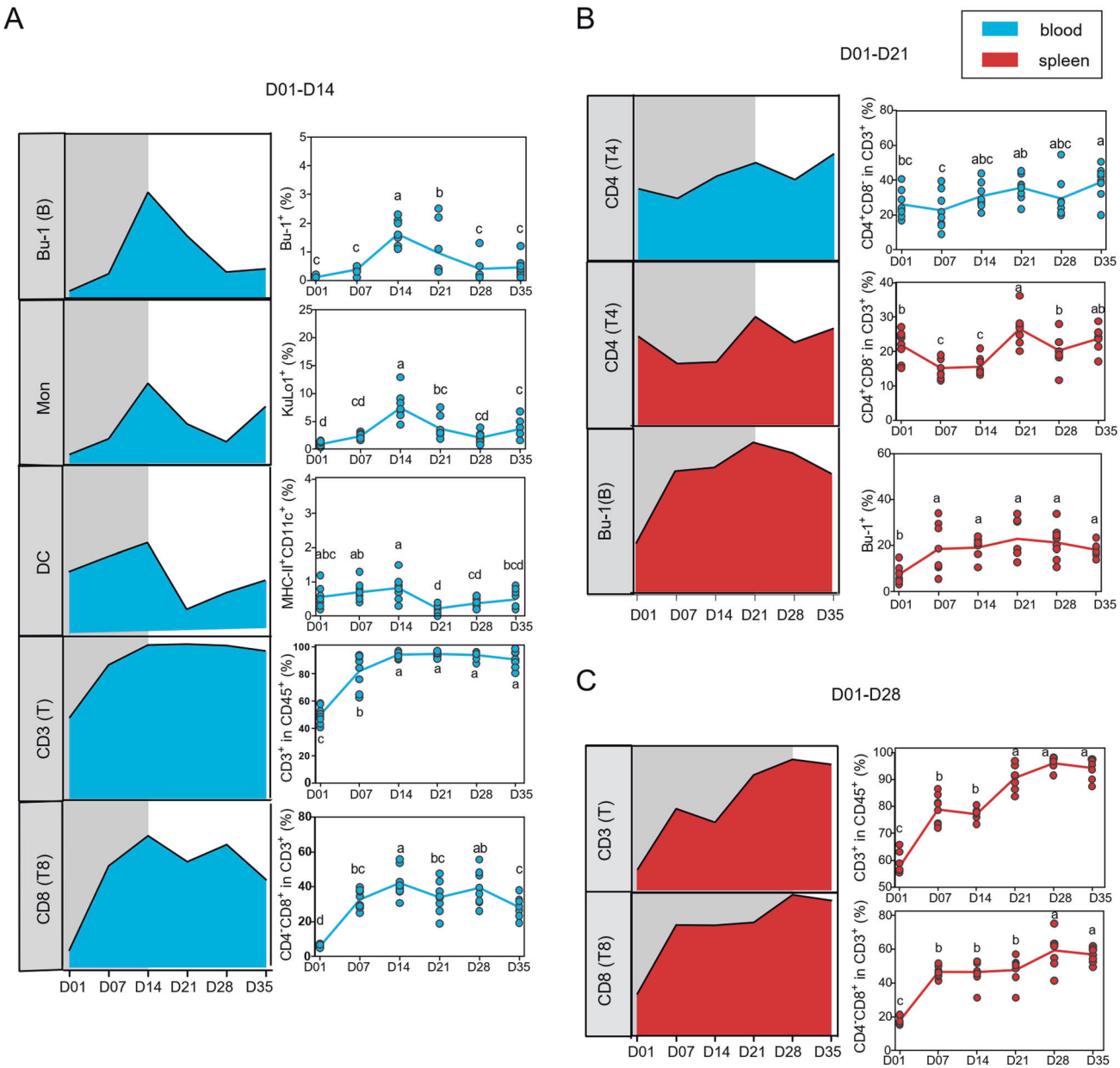


Fig. 3 Sequential waves of immune cell expansion after birth. Three distinct patterns of cell development, with periods of rapid development occurring on days 1–14 (**A**), 1–21 (**B**), and 1–28 (**C**), respectively. Data with different superscript letters are significantly different ($p < 0.05$). $n = 6$–8/day.

maturation and differentiation of the immune cells and support the role of microbial signals in the maintenance of immune homeostasis.

Early exposure to the ileal commensal microbiota accelerates gut microbiota maturation

To further determine the role of microbiota in different succession stages in immune education, we colonized newborn chicks with ileal microbiota from the chicken donors of different days of age to evaluate their effects on the ileal microbiota composition and the development of innate and adaptive immune cells. Based on the results presented above, 14- and 35-day-old chickens were chosen to represent the second and third stages of microbiota succession, respectively. The ileal mucosa and lumen contents from these two days of age donor birds were collected and then prepared as the inoculum as previously described [28]. The alpha and beta diversities of the two inocula were significantly different from each other (Fig. 6A, B; $p < 0.001$), indicating there was a significant distinction in microbiota composition between these two inocula. We next investigated the effect of ileal microbiota transplantation (MT) from 14-(MT14) and 35- (MT35) -day-old broiler chickens on the dynamic changes of bacterial community structures in the lumen and mucosa of the ileal of chickens. Although no significant differences in PCoA plots after MT treatment (Fig. S4A, B) were noted, the luminal Shannon diversity index in the MT14 and MT35 groups significantly increased at 7 days of age (Fig. 6C; $p < 0.05$), and subsequently the mucosal Shannon diversity index increased significantly at 21 days of age (Fig. 6D; $p < 0.05$), indicating that the microbiota transplantation promoted the development and maturation of the intestinal microbiota. The Venn diagram showed that the majority of ASVs were unique at all sampled

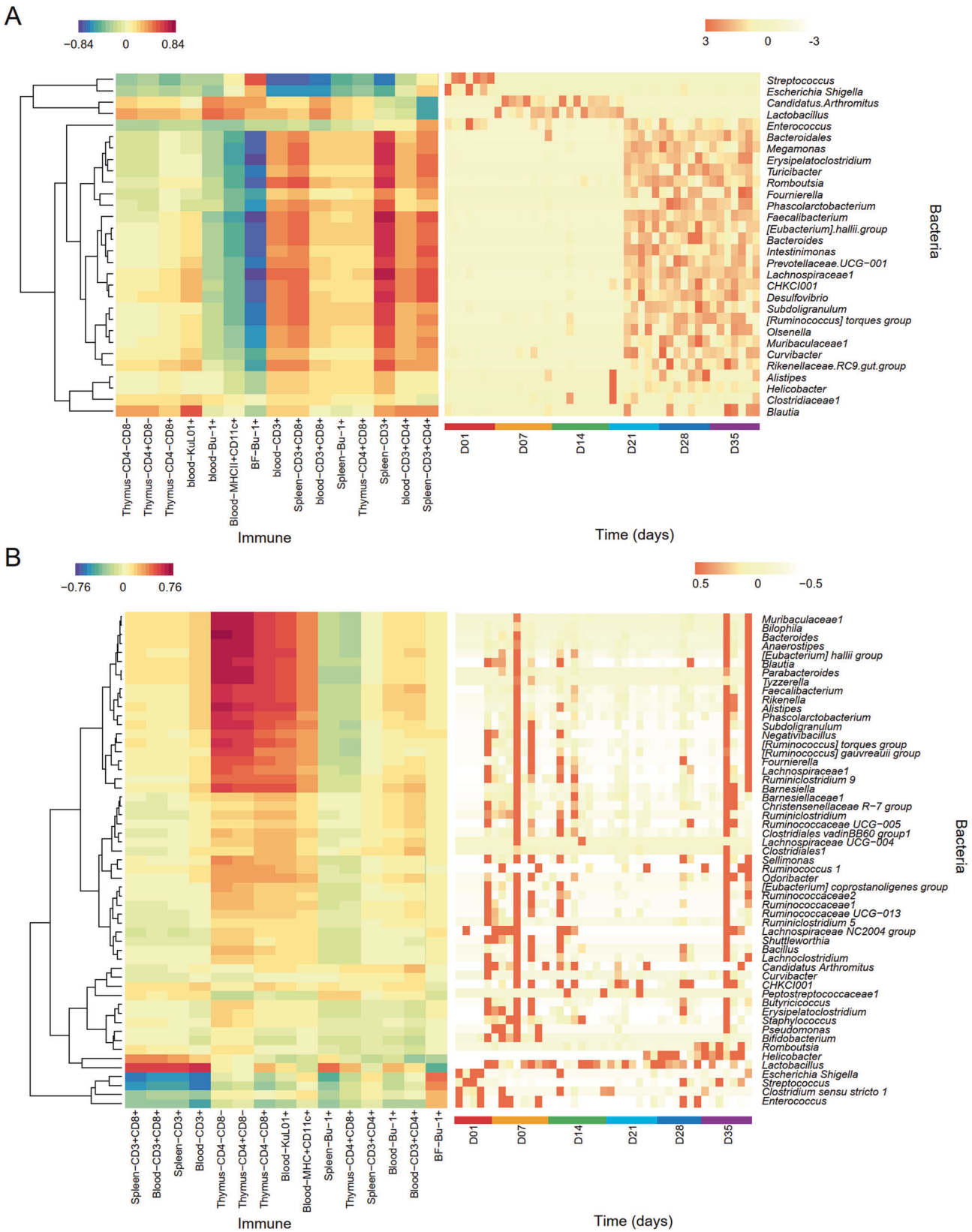


Fig. 4 Interaction between bacteria and the proportion of immune cells. A The regularized canonical correlation analysis (RCCA) between the abundance of ileal mucosal bacteria and the frequency of immune cells (on left). The change of bacterial abundance in the ileal mucosa at different ages (on right). **B** The RCCA correlation analysis between the abundance of ileal luminal bacteria and the frequency of immune cells (on left). The change of bacterial abundance in the ileal luminal at different ages (on right).

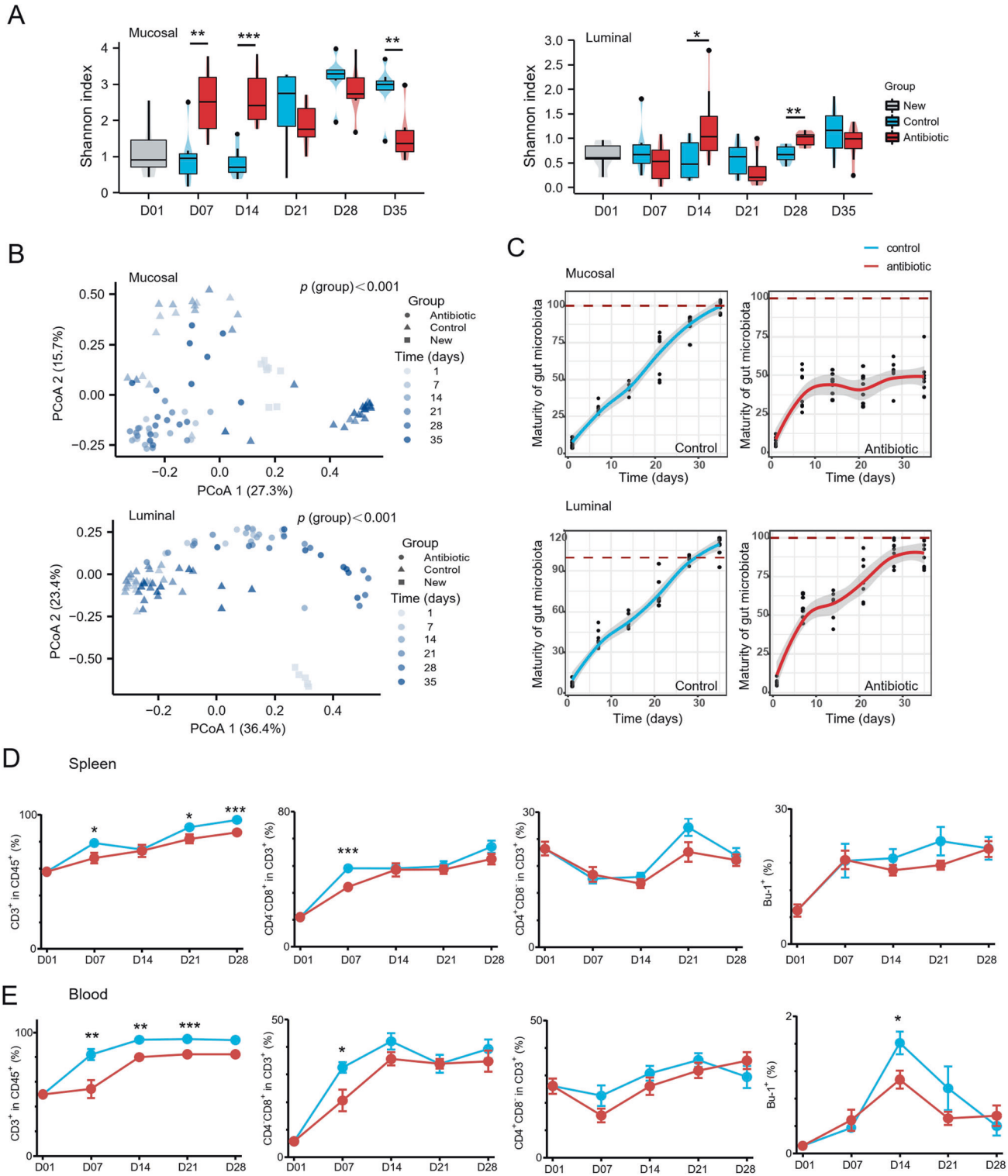


Fig. 5 The antibiotic chlortetracycline interferes with the development of ileum microbiota and immune cells. A Effects of antibiotic on Shannon index of ileum microbiota. **B** Microbiota clustering based on PcoA of ileum microbiota. **C** Ileum microbiota maturity curve of control group and antibiotic group. Proportion of B-, T-cells and their subsets in spleen (**D**) and in peripheral blood (**E**). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $n = 6$–8/day.

time points in lumen and mucus, and only a small number of ASVs were shared among the three groups (Fig. S4C, D), revealing that the initial exposure to different bacterial communities could lead to distinctive influences on the microbiota composition of chickens.

Early exposure to the ileal commensal microbiota influences immune cell composition of chickens

We further sought to investigate the effects of early exposure to the ileal commensal microbiota on immune homeostasis and examined the proportions of immune cell types from its innate

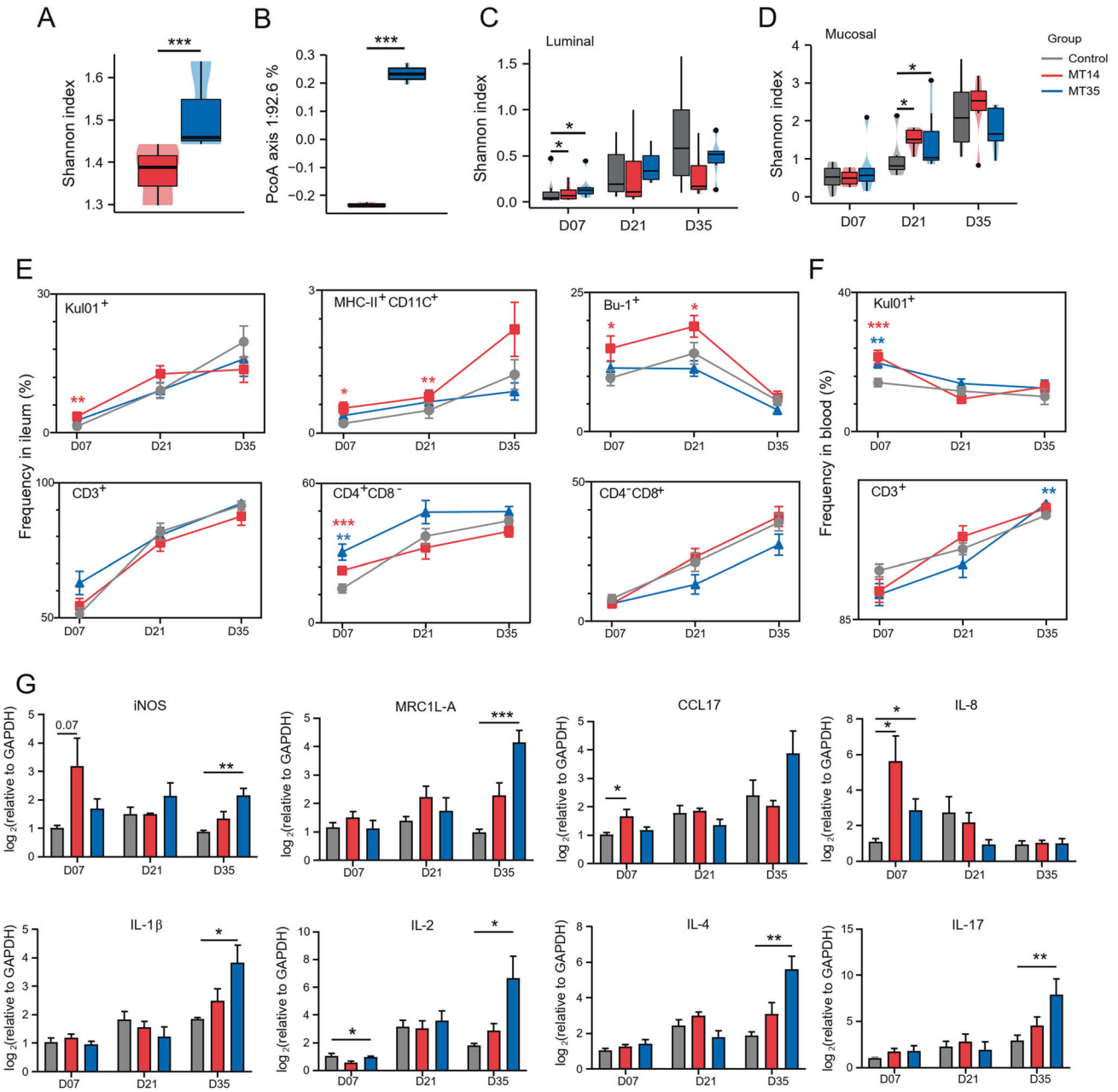


Fig. 6 Early exposure to ileal commensal microbiota from more mature individuals influences the immune cell composition of chickens. **A** Shannon index of MT14 and MT35 microbial suspension. **B** PcoA axis1 of MT14 and MT35 microbial suspension. Ileum mucosal (**C**) and luminal (**D**) microflora Shannon index of broiler chickens at different ages after microbiota transplantation. Microbiota transplantation modulates the proportion of immune cells in ileal lamina propria (**E**) and peripheral blood (**F**) at different ages. **G** Immune-related gene expression in ileum. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $n = 5$–7/day. MT14 and MT35 represent microbiota transplant from 14- and 35-day-old broiler chickens, respectively.

and adaptive arms (Figs. 6E, F and S5) and the expressions of related cytokines (Fig. 6G). After treatment with MT14, the proportions of B-cells and DCs in the ileum of 7- and 21-day-old chickens significantly increased (Fig. 6E; $p < 0.05$), which was consistent with the preceding strong correlation between the proportion of B- and DCs and the abundance of *Candidatus* Arthromitus and *Lactobacillus*, the dominating bacteria in the second succession stage (Fig. 4A). These results demonstrate that microorganisms in the second succession stage could promote the development of B- and DCs. MT14 treatment also caused a significant increase in the proportions of ileum macrophages (Fig. 6E) and peripheral blood mononuclear cells (Fig. 6F) and accordingly caused significant up-regulation of ileum macrophage polarization-related cytokine gene expression, such as CC ligand 17 and interleukin 8 (CCL17 and IL-8, respectively; $p < 0.05$) and an increasing trend in inductively nitric oxide synthase (iNOS) mRNA expression ($p = 0.07$) in 7-day-old chickens (Fig. 6G), which suggests that MT14 strongly facilitates macrophage development and polarization. These results underscore the significance of ileal commensal microbiota from 14-day-old chickens (in the second succession phase) in the growth and activation of innate immune cells, such as macrophages and DCs. In the MT35 group, significant increases in the proportions of $CD4^+$ T-cells in the ileum (Fig. 6E; $p < 0.05$) and mononuclear cells in peripheral blood (Fig. 6F; $p < 0.05$) occurred in 7-day-old chickens, indicating that MT35 contributes to the mononuclear cells and $CD4^+$-T cells

during the early growth stage of chicks. MT35 also led to up-regulation of the proportions of $CD3^+$ T-cells in the blood and ileum gene expression, such as iNOS, mammalian mannose receptor (MRC1L-A), IL-1β, -2, -4, and -17 in 35-day-old birds (Fig. 6G; $p < 0.05$), which indicates the immunoregulatory actions of commensal microbiota from adult chickens (in the third succession phase) were more likely to be reflected in the later growth stage of birds.

We then analyzed the correlation between the ileum microbiota composition and the frequency of immune cells. The strongest association with the abundance of ileal mucosal microbiota were the ileum macrophage cell, $CD3^+$ and $CD8^+$ T- and blood $CD3^+$ T-lymphocyte, which positively correlated with *Helicobacter*, *Megamonas*, *Lactobacillus*, *Succinivibrio*, *Phascolarctobacterium*, *Prevotella, Mitsuokella*, and others (Fig. S6A). When compared with the ileal mucosa, lower correlation coefficients between luminal microbiota and immune parameters were found, indicating a much tighter relationship between mucosal microbiota and host immune maturation (Fig. S6A, B).

Early exposure to the ileal commensal microbiota regulates the immune milieu against *Salmonella* infection

Young broiler chickens up to 1 week of age are especially susceptible to *Salmonella* infection [29–31], which might be attributed to an immature gut immune system at the time of hatching [32] and the gut microbiota with a protective effect is not fully developed [33]. The present results confirm that commensal microbiota transplantation can facilitate gut microbiota and immune system development. Thus, we investigated whether the matured flora patterns could help the young chickens defend against the invasion of pathogenic bacteria. *S*. Typhimurium was administered orally to 5-day-old broiler chicks that were colonized with the commensal microbiota from 14- (PMT14) or 35-day-old chicken (PMT35) at 1 and 4 days of age. The burden of *Salmonella* in PMT14 and PMT35 significantly decreased on day 7 post-infection (Fig. 7A; $p < 0.05$), suggesting that microbiota transplantation is effective in fighting *Salmonella* colonization.

In the acute stage of infection, initial recognition of *Salmonella* by epithelial cells and resident leukocytes results in chemokine and cytokine signals [34–36] caused an increase in mucin secretion and thickened physical barrier against the invasion of this pathogen [37]. Consistently, *Salmonella* infection produced up-regulated ileal gene expression of pro-inflammatory cytokines (TNF-α, IL-2), anti-inflammatory cytokine, IL-4, the tight junction complex (zona occludens [ZO-1], Occludin) and mucin 2 (MUC-2) on day 1 post-infection (Fig. 7C, E; $p < 0.05$). In PMT14 group, the gene expression of IL-4 and −8, iNOS, and ZO-1 increased significantly ($p < 0.05$), and the expression of IL-17 tended to increase along with a decrease in expression of tumor necrosis alpha (TNF-α) at day 1 post-infection (Fig. 7C, E; $p < 0.05$). In the PMT35 group, the gene expression of Claudin-1, IL-8, and the contents of sIgA in ileum mucosal increased significantly ($p < 0.05$), and the gene expression of IL-4 tended to increase at day 1 post-infection (Fig. 7B, C, E). Those dates illustrate that microbiota transplantation facilitated *Salmonella* clearance by thickening the physical barrier and eliciting a robust innate and humoral immune response. At day 7 post-infection, *Salmonella* infection up-regulated the ileal gene expression of iNOS, IL-8, IL-2, IL-4, MUC-2, ZO-1, and Claudin-1 (Fig. 7D, F; $p < 0.05$), which indicates that *Salmonella* infection elicited constant and vigorous immune and barrier protective responses. The expression of IL-8, MUC-2, and ZO-1 decreased in the PMT14 group, while the expression of IL-8, IL-4, MUC-2, ZO-1, and Claudin-1 decreased in the PMT35 group (Fig. 7D, F; $p < 0.05$). Down-regulation of cytokines and tight junction proteins indicate dissipation of inflammation so that normal function and homeostasis are restored in the body. In addition, we noted that the gene expression of IL-2 is markedly up-regulated by PMT35 (Fig. 7D, $p < 0.05$). Previous studies demonstrated that the increase in production of IL-2 at a later phase of the response may sustain T-reg cell activation [38], which could aid in the restoration of tissue homeostasis after *Salmonella* infection.

Although few changes in microbiota structure detected in microbiota-transplanted chickens without infection were found (Fig. S4A, B), the microbiota was altered following *Salmonella* infection at day 1 post-infection (Fig. 7G, H). Interestingly, the mucosal abundance of *Candidatus* Arthromitus (also known as segmented filamentous bacteria) in the ileum was reduced after *Salmonella* infection, but surged in microbiota-transplanted chickens (Fig. 7I). Previous studies showed that segmented filamentous bacteria could induce Th17 cells and sIgA production [39, 40]. It is likely that the up-regulated IL-17 gene expression in PMT14 and the increased ileum sIgA in PMT35 after microbiota transplantation are caused by the increased abundance of *Candidatus* Arthromitus. This finding suggests microbiota transplantation enhances immunity and protects intestinal barrier function after *Salmonella* infection by promoting the colonization of certain specific bacteria.

Immunologic changes in response to colonization with five gut bacterial species in broiler chickens

The present results demonstrate that microbes influence the development of immune cells through antibiotic treatment and commensal microbiota transplantation. However, the immunomodulatory effect of microbiota transplantation is highly variable due to the complex structure and diverse interactions of microbiota. Clues that the introduction of certain donor strains may play a decisive role in the immunomodulatory effects of microbiota transplantation can be found [41]. Therefore, we look forward to locating the beneficial bacteria that mainly play an immunomodulatory role from the microbiome. RCCA enabled us to preliminarily identify some potential immunomodulatory microorganisms (Figs. 4 and S6). Based on microbial culture-omics technology, we extracted certain microbiota members from chicken that are linked to the development of immune cells. We chose five isolates whose genomic maps are shown in Fig. S7 and included *Blautia coccoides* (Bc), *Bacteroides xylanisolvens* (Bx), *Fournierella* sp002159185 (Fs), *Romboutsia lituseburensis* (Rl), and *Megamonas funiformis* (Mf). Ecological distribution information showed that these five strains were widespread in the human gut and other animal guts and environments in addition to the chicken gut (Fig. S8), but only a few studies have investigated their immunomodulatory characteristics.

The five strains were inoculated into the young broiler chickens that had been pretreated with a cocktail of antibiotics and raised in isolators, after which the proportion of immune cells was measured 14 days after inoculation to assess their involvement in immune cell development and differentiation. Results show that none of the five strains had any adverse effects on the growth performance of broiler chickens and Rl, Fs, and Bc significantly increased the body weight of broiler chickens (Fig. S9A; $p < 0.05$). The corresponding fold changes (FCs) relative to control status were summarized in the heatmap, and the proportion of ileum immune cell types (compared to control) with a $|\log_2 FC| > 1$ was also shown (Fig. 8B). Immune cell populations reacted differently to several microbes with expansion and contraction. Bc and Mf exerted powerful immunomodulatory effects, being manifested in a marked increase of $CD8^+$ T-cells ($p < 0.001$), the decrease of macrophages and DCs (Fig. 8A; $p < 0.05$), and maximum extent of change relative to control in the ileum (as judged by the proportion of cell types modified by $|\log_2 FC| > 1$; Fig. 8B). Rl (a member of *Clostridia*) showed an intermediate induction of ileal T-reg cells (Fig. 8A; $p < 0.05$) in line with the previous reports in which T-reg cells were found to be strongly induced by several individual microbes, especially *Clostridia* strains [16, 17, 42]. In addition, we noted that stimulation of ileal immune cells altered

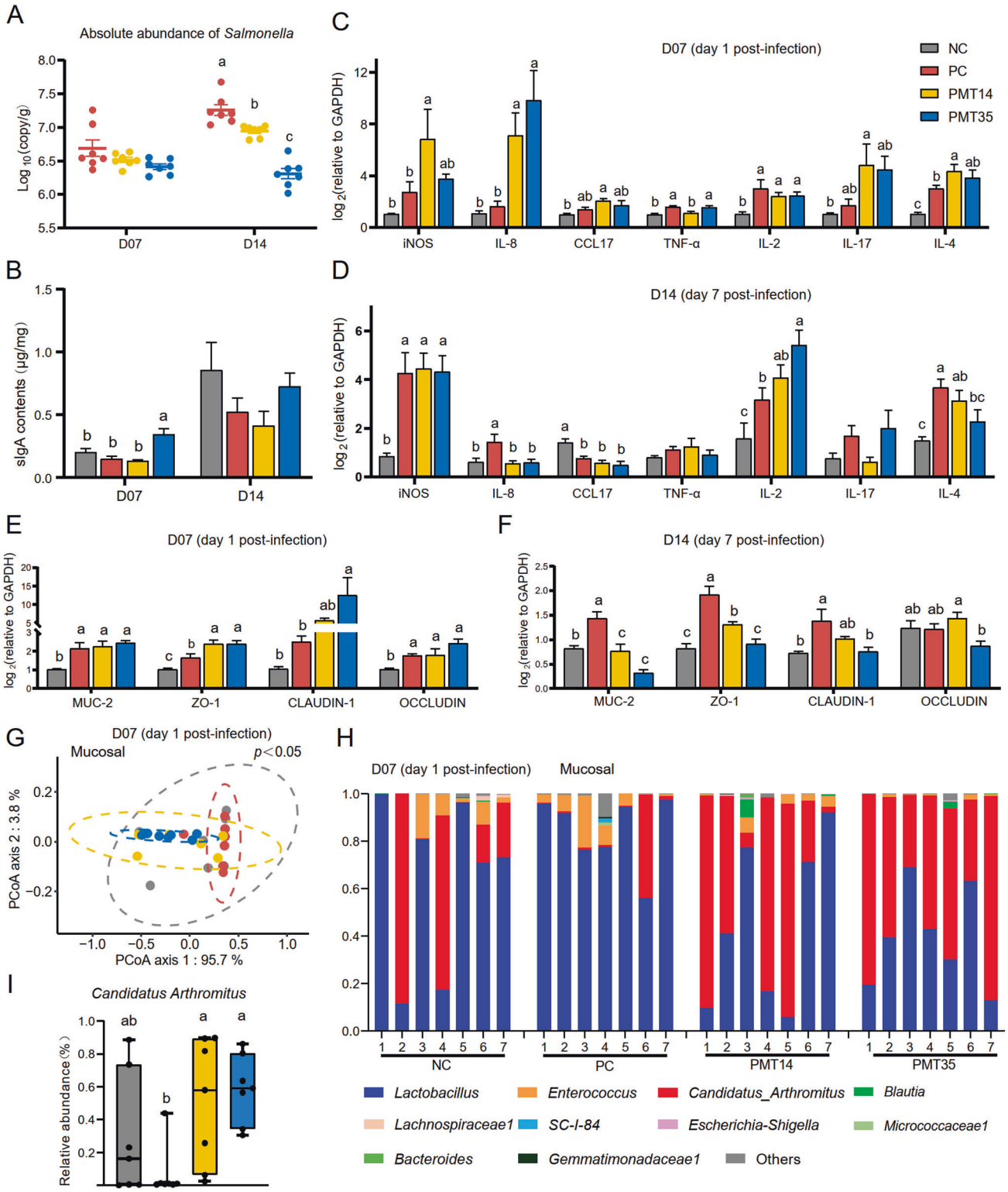
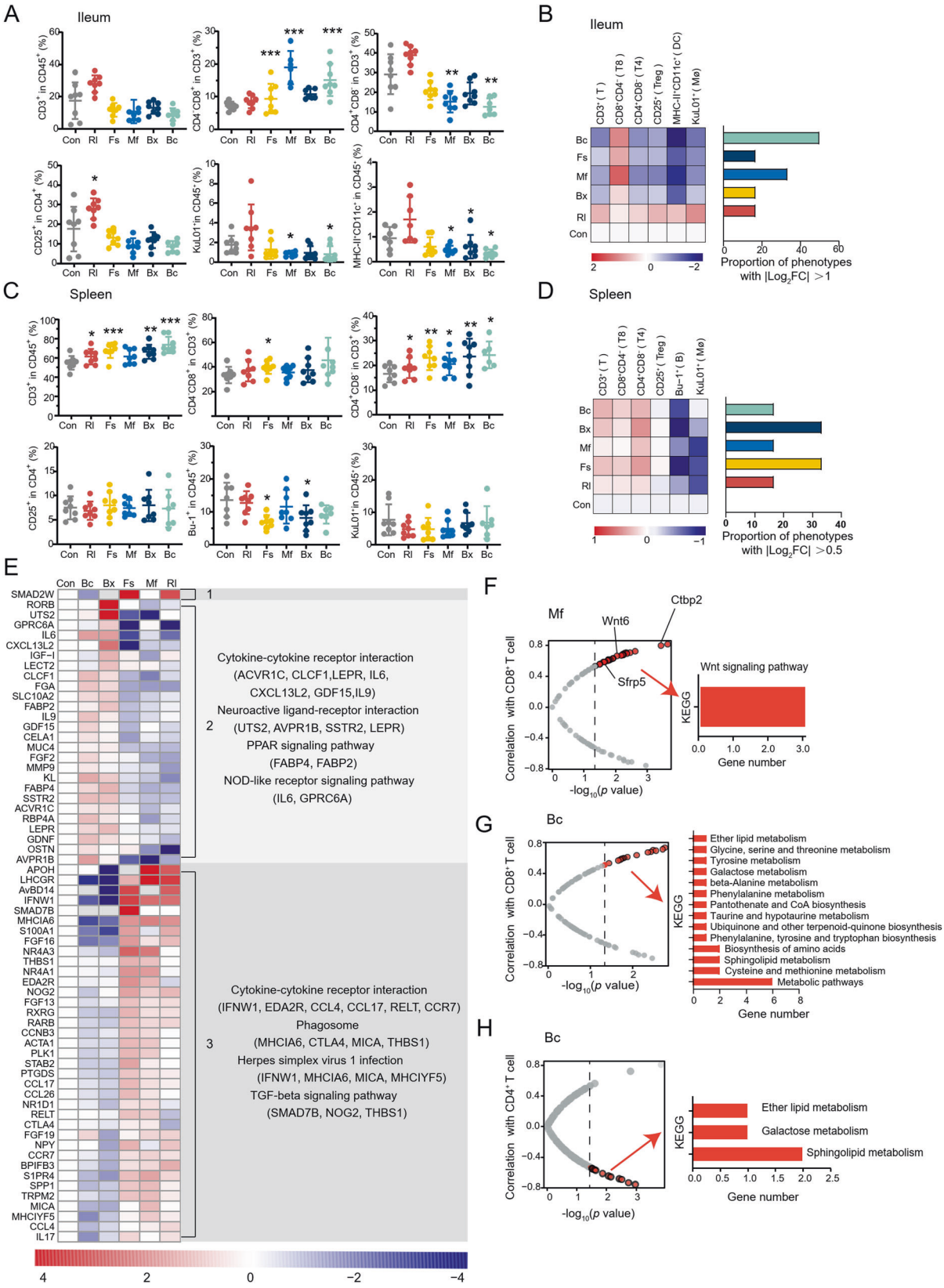


Fig. 7 Microbiota transplantation enhances the resistance to *Salmonella* Typhimurium infection of broiler chickens. A *Salmonella* loads in the ileal lumen on days 1 and 7 post-infection. **B** sIgA content in ileal mucosa on days 1 and 7 post-infection. Immune-related gene expression in ileum on day 1 (**C**) and 7 (**D**) post-infection. Gene expression related to physical intestinal barrier function in ileum on day 1 (**E**) and 7 (**F**) post-infection. **G** PcoA plot of ileum mucosal microbiota on day 1 post-infection. **H** Relative abundance of top 10 bacterial genera in ileal mucosa on day 1 post-infection. **I** Relative abundance of *Candidatus* Arthromitus in ileal mucosa on day 1 post-infection. The difference of relative abundance of species in different groups were tested using Wilcoxon rank sum test. Data with different superscript letters are significantly different ($p < 0.05$). $n = 6–7$/day. PMT14 and PMT35 represent infection of broiler chickens with *Salmonella* following transplanting of 14- and 35-day-old microbiota, respectively.



log FC by up to 2.1-fold, but stimulation of the spleen by microbes showed a maximum of 1.24-fold (Fig. 8D), indicating that microbiota can also modulate the peripheral organ immune responses although to a weaker extent than in the ileum. Among them, Bx and Fs exhibited the strongest influence on spleen immune cells, particularly on $CD4^+$ T- and B-cells (Fig. 8C, D).

To gain a deeper understanding of the host response to gut symbiont vaccination, we examined the transcriptional profiles

Fig. 8 Immunomodulatory properties of five bacterial strains. A Proportion of immune-related cells in the ileum of broiler chickens inoculated with different bacterial strains. **B** Heatmap displays the corresponding fold changes of immune-related cells in the ileum relative to control status. The bar graph displays the proportion of ileum immune cell types (compared to control) with a $|\log_2 FC| > 1$. **C** Proportion of immune-related cells in the spleen of broiler chickens inoculated with different bacterial strains. **D** Heatmap displays the corresponding fold changes of immune-related cells in spleen relative to control status. The bar graph displays the proportion of spleen immune cell types (compared to control) with a $|\log_2 FC| > 0.5$. **E** Heatmap of fold changes of transcripts related to the immune response differentially expressed in ileum of inoculated broiler chickens compared to control broiler chickens. Bacteria (columns) are clustered by hierarchical clustering; genes (rows) are clustered by K-means clustering. Enriched pathways were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Correlation was calculated between the expression value of differential expressed genes (DEG) and the proportions of $CD8^+$ T cells in ileum of broiler chickens inoculated with Mf (**F**) or Bc (**G**). **H** Correlation was calculated between the expression value of DEG from the ileum and the proportions of $CD4^+$ T-cell for broiler chickens in control and Bc treatment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 6$–8. Bc, Bx, Fs, Rl, Mf represent *B. coccoides*, *B. xylanisolvens*, *F.* sp002159185, *R. lituseburensis*, and *M. funiformis*, respectively.

induced by the five microbes. DEGs were identified and represented in the volcano (Fig. S9B). A Venn diagram displayed only six overlapping DEGs between the five datasets, which contained only six genes (Fig. S9C), indicating a larger fraction of genes might be microbe-specific. To further analyze the immune transcriptional responses, we considered a general approach by flagging DEGs related to the immune responses for at least one group. This strategy generated 64 transcripts (Fig. 8E). The expression of GPRC6A, a key gene in activation of the nucleotide-binding leucine-rich prying domain containing protein 3 inflammasome and initiate innate inflammatory responses [43], was significantly reduced in the Rl group, indicating that Rl could possess anti-inflammatory properties. Real-time PCR results indicated that Rl enhanced the mRNA expression of the anti-inflammatory factors, IL-10 and TGF-β (Fig. S9D; $p < 0.05$). These occurrences are consistent with an increased frequency of ileum T-reg cells, indicating that Rl may contribute to the promotion of immunological tolerance. We found Fs produced down-regulation of the expression of pro-inflammatory cytokines (CXCL13L2 and IL-6) but caused a notable increase in up-regulation of the expression of T-reg cell inducer (SMAD2), indicating a pivotal role in the maintenance of intestinal homeostasis of Fs. Major historic comparability (MHC) class I (MHC-I) molecules that were loaded with antigenic peptides to communicate with $CD8^+$ T-cells are the centerpieces of cross-presentation [44]. Up-regulation of MHC-I-A6 and MHC-I-YF5 gene expression in the Mf group enhanced antigen presentation, which contributes to activation of $CD8^+$ T-cells and might be responsible for the increased proportion of $CD8^+$ T-cells. It has been documented that macrophage showed strong transcriptional activation of osteopontin (SPP1) and led to up-regulation of gene expression of chemokine CCL4 [45, 46] after M1 polarization. We found Bc produced down-regulation of gene expression levels of CCL4 and SPP1, suggesting Bc could suppress the polarization of macrophages into M1 macrophages, which could be responsible for the diminished level of ileum macrophages. Gene expression of CCR7 and S1PR4 was down-regulated by Bx, which is required for the migration and differentiation of DCs [47, 48], consistent with the decrease in the proportion of DCs in the ileum. Interestingly, we noticed that none of the transcripts was uniformly induced by all inoculates. To further understand the properties of these differential transcripts related to the immune response, clustering was performed according to their expression profiles. Three co-regulated gene clusters were generated and cross-referenced with the KEGG pathway (Fig. 8E). Up-regulated genes induced by Bc and Bx were similar and predominantly enriched in neuroactive ligand-receptor interaction, peroxisome proliferator-activated receptor (PPAR) signaling pathway, and nucleotide-binding and oligomerization domain (NOD)-like receptor signaling pathway, and some of them are B-cell proliferation chemotaxis-related factors including CLCF1, IL-6, and CCXCL13L2. Up-regulated genes induced by Fs, Mf, and Rl were enriched in the phagosome, TGF-beta signaling pathway, and herpes simplex virus 1 infection pathway, indicating that these strains possess antiviral and anti-infectious properties.

For providing insights into the molecular mechanisms and/or physiological effects of microbes on regulating the proportion of immune cells, we identified the sets of genes whose expression was most correlated with immune cell frequencies in the ileum. Mf was found to possess potent capability to induce the development of $CD8^+$ T-cells and inhibit the formation of DCs, which is connected to the Wnt pathway (Fig. 8F) which can inhibit cytotoxic T-cells and promote DCs activities [49–51]. The expression of the Wnt pathway inhibitors, C-terminal-binding protein 2 and secreted frizzled-related protein 5 (CTBP2 and SFRP5, respectively) positively correlated with the proportion of $CD8^+$ T-cells, suggesting that Mf may increase the proliferation and differentiation of $CD8^+$ T-cells by blocking the Wnt pathway. We also noticed a significant negative correlation between SFRP5 and the proportion of DCs (Fig. S9E), suggesting that Mf may repress DCs by engrafting the Wnt pathway. It was previously found that *Clostridium butyricum* inhibits the development of intestinal tumors by inhibiting the Wnt/-catenin signaling pathway [52], indicating the potential value of microorganisms in achieving immune regulation by targeting the Wnt pathway. Bc also exhibited extraordinarily potent $CD8^+$ T-cell-promoting and $CD4^+$ T-cell-inhibiting activities but through a more distinct pathway than Mf. The most relevant transcripts for $CD8^+$ and $CD4^+$ T-cell frequencies were enriched in lipid or protein digestion and metabolic pathways (Fig. 8G, H), which supported a connection between Bc-mediated $CD8^+$ and $CD4^+$ T-cells and metabolic and absorptive processes in the host gut.

DISCUSSION

Pathogen infection has negative effects on the yield and quality of animal products, which has caused serious economic losses to the livestock industry. However, healthy immune-competent chickens can be able to combat infection through their effective immune response. Growing evidence has revealed the pivotal role of gut microbiota in the establishment of immune competence [9–11, 18]. A better understanding of the relationship between gut microbiota and immunity will help reduce the economic losses caused by pathogenic bacterial infections on broiler chickens. In the present study, we explored the systemic impact of the microbiota on immune cell development and function and identified the microbiota members with immunomodulatory roles. A time-series design with six-time points has shown that microbial development strongly correlates with the differentiation and function of immune cells. We then intervened in the normal microbial colonization by using antibiotic treatment and microbiota transplantation and found that antibiotic treatment perturbed microbiota succession and negatively affected immune homeostasis, whereas the early introduction of the commensal ileum microbiota from more mature birds promoted the development of the immune system, which facilitated pathogen elimination against *S.* Typhimurium infection. Furthermore, based on the correlation analyses between microbiota abundance and immune cell frequency, five immunomodulatory strains were

screened out, and their different immunomodulatory properties were proven by gavage.

Extensive studies have reported the undergoing successive transitions in microbiota composition before a mature community forms [25, 53]. In the present study, three succession patterns were outlined both in the ileal lumen and mucosal microbiota, but the greatest differences occurred between the composition of the mucus and lumen microbiota as reported previously [53, 54]. These variances may be the result of varied mucus densities, antimicrobial peptide levels, and oxygen concentrations [55, 56]. Coinciding with microbial colonization, the immune system also undergoes a rapid maturation process. Although numerous studies have examined how the immune system develops in broiler chickens [57–60], nearly all of them were completed more than 20 years ago, and its applicability to modern broiler chickens is unclear. With the rapid recent development of recombinant antibody technology, a multitude of new antibodies applied in chickens have been developed so that examination of postnatal changes in immune cells are more comprehensive and exact. In summary, B-cells in the Bursa begin to move to the periphery after birth, thymocytes grow rapidly during the first 14 postnatal days, and mass immune cells, such as lymphocytes, DCs, and macrophages in the peripheral immune organs, mature after 14 days. These results provide new insights into the immune development of modern broiler chickens and are a foundation for further studies.

In addition, our results display that the time nodes of immune development in broiler chickens overlap with those of intestinal microbial development. The correlation analysis between ileal microbiome and immune cells demonstrates that some of the dominated microbes in the succession stages appear to be strongly correlated with immune cell development, and the microbiota of mucus and lumen had different relationships with immune parameters. For example, the abundance of mucosal microbes, such as *Candidatus* Arthromitus and *Lactobacillus*, had strong positive correlation with DCs and $CD8^+$ T-lymphocytes. This finding is most likely due to the location of the mucosal microbiota, which is in close proximity to the intestinal epithelium and could interact directly with the host immune system [61, 62]. Meanwhile, luminal microbes, such as *Bacteroides*, *Blautia*, and *Parabacteroides* were strongly associated with the development of thymus T-cells, consistent with the previous studies [63], indicating the important role of commensal microbiota in modulating T-cell signaling during thymocyte development, which may be caused by the luminal bacterial metabolites [64] and the direct antigen-presentation via DCs [65]. These results highlight the synchronous succession between host immunity and intestinal microbial community composition [66]. However, we must be conscious of the fact that correlation does not imply causation and that many of the changes we observed may really be caused by changes in the bird's developmental process rather than by the microbiome.

The antibiotic interventional strategy allowed us to formulate a deeper understanding of microbiota-induced immunoregulation. Following antibiotic treatment, considerable changes occurred in the luminal and mucosa-associated bacterial communities, including modifications in microbial diversity and microbiota structure as well as a delay in the maturation of the microbiota, which is in accordance with previous studies [67–69]. We found that the proliferation of blood and spleen-derived $CD8^+$ and $CD3^+$ T-lymphocytes were inhibited in antibiotic-treated chickens. Similarly, in studies with mice, the number of $CD3^+$ T-cells substantially decreased after antibiotic treatment [70], and the germ-free mice displayed a reduction in $CD8^+$ T-cell numbers in the intraepithelial lymphocyte (IEL) compartment of the small intestine [71, 72]. These results at least corroborate the significant role of the partial bacterial signals in the maintenance of certain T-lymphocytes.

To verify the role of microbiota in different succession stages in shaping the immune system, the commensal microbiota from ileum mucosa and lumen was administered to newborn chicks. Inoculating the microbiota from 14-day-old donor birds mostly contributed to the development of innate immunity in the early growth phase, while that from 35-day-old donor birds mainly promoted the development of T-cell subsets until the late-growth phase, revealing that transplanting microbiota in various succession stages has distinct immunoregulatory actions and duration. This process might occur because in the early stage of growth, which is accompanied by the rapid developmental maturation of innate immunity, microorganisms predominantly drive the development of innate immunity, whereas adaptive immunity gradually develops and matures in the late growth period, and microorganisms induce adaptive immune competence at that time [73, 74].

Generally, the protection of chickens against *Salmonella* infection increases with age of the chicken [30, 31], which may be related to the maturing bacterial impact on the immune system [75]. As alluded to earlier, newly hatched inoculated chickens with gut microbiota sourced from 14- or 35-day-old donors showed diverse immunomodulatory actions, and accordingly exhibited various immune responses to pathogens, but they were equally protected against *Salmonella* infection, in line with the previous research [21, 29–31]. These findings demonstrate that cross-talk between microbiota composition and immune cells is highly associated with the establishment of immune competence and anti-infection capabilities.

Although the microbiota transplantation from healthy donors has been effective for helping chicks fight infection, the safety of microbiota transplantation still needs further investigation because it is possible to be infected with other pathogens or have adverse reactions after microbiota transplantation [76]. Thus, we look forward to identifying more potent therapeutic microorganisms that are compatible with the host and can modulate the host immune system in a well-controlled, physiological manner. Symbionts have the ability to become permanent members of the microbiota and therefore represent the most promising candidates. Although a few studies have established the ability of distinct microbes to drive intestinal immune cells to specific fates, we chose five strains with minimal background research that were derived from the chicken intestine in immune functions and inoculated them into antibiotic-treated birds. Extensive immunophenotyping and transcriptomic analysis were performed to mine the immunomodulatory gut symbionts. Indeed, screening of five strains yielded a number of activities, both anticipated and unanticipated. For example, we observed Rl not only dampens pro-inflammatory signals but also promotes the generation of T-reg cells. Similar results were reported in gnotobiotic mice in which members of *Clostridiales* enhance T-reg cell abundance and mediate anti-inflammatory properties in the intestine [16, 42]. We also noted that Bc and Fs could lead to an increase in the proportion of $CD8^+$ T-cells, which could contribute to an increase in the immunomodulatory capacities of young chickens against pathogenic bacterial infection. *Blautia* is considered a potential new probiotic genus and plays certain roles in metabolic diseases, inflammatory diseases, and biotransformation [77]. Studies have reported that the elderly chickens showed a lower *Blautia* abundance compared with the adults, and its decrease may be related to an age-related decline in immune function known as immunosenescence [78]. *Fournierella* also is an important member of the commensal flora of animals, and while there has been little research on its functions, our data demonstrate it may be involved in the maintenance of intestinal homeostasis.

We found that the immunomodulatory effects of the symbionts show substantial diversity and redundancy. We noted that all of those five bacterial strains have multiple immunomodulatory effects. Furthermore, many immunological changes were caused

by more than one symbiont. It has been previously reported that the broad diversity and redundancy of immunological alterations permit many different microbes to provide the balance needed to promote overall host health and buffer change in stability in ecosystem processes caused by reduced microbial diversity [79, 80]. Consistently, a recent study systematically analyzed host immunologic adaptation to monocolonization with each of 53 individual bacterial species, elucidating that most microbes exerted several specialized, complementary, and redundant transcriptional and immunomodulatory effects [79].

This study prompted an intriguing investigation into the way the microbiota influences immune cell development and functions in addition to the identification of microbiota members with immunomodulatory roles. Our results underscore that the microbiota at each succession stage has a distinct influence on the maturation of the immune system, and the early establishment of a diverse core microbiome could impede *Salmonella* colonization and invasion in the gut. This process establishes the foundation for targeted immunological interventions aiming to combat infectious diseases and promote animal health and production. However, much remains to be understood regarding the acquisition of bacterial microbiota and their interactions with the immune systems of developing host. Having identified individual effector strains, future research will be needed to unlock the microbial components responsible and the molecular mechanisms of host/microbial interactions for fully utilizing these bioresources. It would be interesting to study the combinatorial effects of immunomodulatory microbes both in a gnotobiotic setting and also under conventional conditions.

MATERIALS AND METHODS

Animals

One-day-old Arbor Acres male chicks and breeding eggs were obtained from Beijing Dafa Chia Tai Co. LTD and housed in a breeding base in Zhuozhou, Hebei Province, China. All birds were fed ad libitum and had free access to water throughout the experiments. Room temperature and humidity were automatically controlled according to Arbor Acres Broiler Feeding Management. The feed was formulated according to the instructions of the Chinese chicken feeding standard (NY/T-33–2004). Feed composition and nutrient levels were presented in Table S1.

Antibiotic administration

A total of 400 chicks were randomly divided into two groups. Each group contained 10 replicates with 20 chicks per replicate. Chicks in control group (Control) were fed basal diet, and the antibiotic group (Antibiotic) were fed basal diet added with 100 mg/kg aureomycin for 35 days. Samples were taken at 1, 7, 14, 21, 28, and 35 days of age. The spleen, thymus, peripheral blood, and bursa of Fabricius were harvested for flow cytometry analysis. The distal ileal mucosa and contents were collected for subsequent DNA extraction.

Preparation for ileal microbiota suspension

Ileum microbiota suspensions, MT14 and MT35, were obtained from healthy and non-antibiotic fed Arbor Acres male broiler chickens at 14 and 35 days of age (150 chickens pooled per treatment), respectively. Microbiota suspensions were obtained and stored by a modified method according to Hu et al. [28]. In brief, broiler chickens were sacrificed after which the ileum was collected and tied at both ends. The tied ileum was transferred into an anaerobic operation apparatus immediately and the ileum mucosa and contents were scraped gently into a sterile tube. After adding sterile saline and mixing, the ileal content suspension was obtained and then filtered with 10, 18, 35, and 60 mesh sieves followed by filtration three more times with 60 mesh sieves. The filtered suspension was centrifuged at 6000 × *g* for 15 min, and then the supernatant was removed. The sediment was re-suspended and mixed with fresh sterile saline containing 10% glycerol and subsequently, placed in a 15 ml centrifuge tube and stored at −80 °C for later use. When microbiota transplantation is required, frozen microbiota suspensions should be thawed at 37 °C (water bath). PBS can be added to adjust the concentration of the thawed microbiota suspensions to 10^9 CFU/ml.

Microbiota transplantation

A total of 168 1-day-age male chicks were randomly allocated into Control, MT14, and MT35 groups. Each group contained seven replicates with eight chicks per replicate. Chicks in three groups were orally inoculated with (1) PBS (Control), (2) a pre-prepared ileal microbiota suspension from 14-day-old broiler chickens (MT14), and (3) a pre-prepared ileal microbiota suspension from 35-day-old broiler chickens (MT35) 200 µl/bird/day at the first and fourth day after birth. The animal samples were collected at 7, 21, and 35 days of age. The distal ileal mucosa and contents were collected for subsequent DNA extraction. Sections of distal ileum were collected for flow cytometry analysis and RNA extraction. The peripheral blood was harvested for flow cytometry analysis.

Salmonella Typhimurium challenge

A total of 76 1-day-age male AA chicks were randomly assigned into four groups with 19 chicks in each group. The treatments were divided into several groups: (1) NC group: PBS + sterile saline, (2) PC group: PBS + *Salmonella* Typhimurium (ST) challenge, (3) PMT14 group: MT14 + ST challenge, and (4) PMT35 group: MT35 + ST challenge. Treatments using PBS, MT14, and MT35 were the same as described above. Chicks undergoing the *Salmonella* Typhimurium challenge were orally gavaged with 1 ml of *S*. Typhimurium (1×10^8 CFU/ml) at 5 and 6 days of age, and chicks in the NC group were gavaged with 1 ml of sterile saline instead. The ST challenged chicks were reared in isolated compartments to avoid cross-infection. On day 7 (1-day post-infection) and day 14 (7-days post-infection), seven birds, randomly selected from each group, were sacrificed and sampled. Samples of terminal ileum, ileal mucosa and contents were collected and stored at −80 °C before analysis.

Bacterial cultivation, isolation, and identification

Fresh chicken intestine samples were serially diluted in PBS (10^{-2} to 10^{-8}) and plated on Gifu anaerobic medium (GAM) agar. The plate was incubated at 37 °C for 3 weeks and checked daily for new colonies. Individual colonies were transferred to nitrogen-filled liquid medium and incubated at 37 °C until the liquid became hazy. All operations were performed in an anaerobic workstation filled with a gas mixture of 95% CO_2, 4.75% N_2 and 0.25% H_2. The identities of all collected isolates were confirmed by amplifying the 16S rRNA gene using primers 27F (5′- AGA GTT TGA TCA TGG CTC A- 3′) and 1492F (5′- TAC GGT TAC CTT GTT ACG ACT T -3′). The genomes of the isolated strains *Blautia coccoides* (CML164), *Bacteroides xylanisolvens* (CML384), *Fournierella* sp002159185 (CML151), *Romboutsia lituseburensis* (CML 137), and *Megamonas funiformis* (CML154) were sequenced. The genome map of *B. coccoides* (CML164), *B. xylanisolvens* (CML384), *F.* sp002159185 (CML151), and *R. lituseburensis* (CML137) performed with proksee (https://proksee.ca/). The ecological distribution analysis was performed with protologger (http://www.protologger.de/) based on the previous study [81]. The habitats we examined include activated sludge, bovine gut, chicken gut, coral, freshwater, human gut, human lung, human oral, human skin, human vagina, insect gut, marine, marine sediment, mouse gut, pig gut, plant, rhizosphere, soil and wastewater.

Inoculation of bacterial strain

The breeding egg is disinfected by dipping it into povidone-iodine for 5 min after which it was then transferred to an incubator placed in a HEPA-filtered isolation room and sprayed with povidone-iodine once a day. After hatching, male chicks with similar body weights were immediately transferred to six sterile isolators for feeding. Each isolator contained 15 chicks. During the first 6 days of feeding, sterilized drinking water was combined antibiotics (200 ppm ampicillin, 200 ppm metronidazole, 200 ppm neomycin, and 100 ppm vancomycin) for gut bacteria elimination. The addition of antibiotics was stopped at day 7. On day 8, chicks in the six isolators were fed sterile saline and bacterial strain *B. xylanisolvens*, *F.* sp002159185, *R. lituseburensis*, *B. coccoides*, and *M. funiformis* (1 ml/bird/day) with the bacterial quantity of 10^{7-8} CFU/ml. All feeds were treated with cobalt irradiation sterilization. Samples were taken after 14 days of bacterial inoculation. The terminal ileum and spleen were collected for flow cytometry analysis and RNA extraction.

DNA extraction and 16S rRNA gene amplicon amplification and sequencing

In the trials, the ileal digesta and mucosa of broiler chickens were collected for microbiome analysis. DNA extraction was conducted with QIAamp Fast DNA Stool Mini Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. Then, the barcoded primer Pro341F: 5′-CCTACGGGNBGCAS-CAG-3′ and Pro805R: 5′-GACTACNVGGGTATCTAATCC-3′ was used to amplify the V3-V4 region of bacterial 16S rRNA using a polymerase chain reaction (PCR). PCR products were purified with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, USA). Amplicon libraries were generated with Qubit 2.0 (Life Technologies, CA, USA) and sequenced on a HiSeq PE250 (Illumina, CA, USA) to obtain paired-end reads. According to the overlaps of the paired-end reads, long reads were merged with FLASH (version 1.2.11). After filtration and chimera removal, clean reads were obtained for further analysis.

Bioinformatics analysis

Clean reads with exactly the same sequence were sorted according to their abundance, and singletons were filtered out. The SILVA database (v138) was utilized to taxonomically classify ASVs. Alpha diversity analysis, including Shannon and Simpson, principal coordinate analysis (PCoA) and bacteria relative abundance were performed with Quantitative Insights Into Microbial Ecology (QIIME). Regularized canonical correlation analyses (RCCA) were performed with the Mixomics package 6.10.8 [82] to unravel specific correlations between immune index and microorganisms in all samples. The maturity curve of the microflora was determined according to the method described in previous study [69]. For the training of the random forest model, a smoothing spline function was fitted between microbiota age and chronologic age of the host for the control. The model was then applied to other groups. When the curve reached its plateau, the microbiota age was defined as 100% maturity.

Real-time PCR

Approximately 1 cm segment of ileum was collected and frozen at −80 °C for RNA extraction. Total RNA was extracted from ileum tissue with Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. A NanoDrop-2000 spectrophotometer (Thermo Scientific, MA, USA) was used to detect the purity and concentration of RNA. Qualified RNA was subsequently used to synthesize cDNA with Primer Script RT Reagent kit (Takara, Shiga, Japan). Using cDNA as a template, quantitative real-time PCR (qPCR) was performed in QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, CA, USA) using SYBR Premix Ex Taq kit (Takara) in accordance with the manufacturer's guidelines. Primers of target genes used in the current study are shown in Table S2. The thermocycling protocol for the qPCR followed several steps: (1) 95 °C for 5 min, (2) 40 cycles of denaturation at 95 °C for 5 s, and (3) annealing and extension at 60 °C for 34 s. Melt curve analysis was performed to identify a PCR product's specificity and purity. All samples were determined in triplicate. $2^{-\Delta\Delta Ct}$ method was used to analyze the data, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to standardize the mRNA level.

Quantification of *Salmonella* in ileum digesta

Quantification of *Salmonella* content in ileal digesta was determined according to the methods described in Wise and Siragusa's study [83]. Total bacterial DNA was extracted from 0.2 g intestinal digesta and *S*.Typhimurium cultures with Stool Mini Kit (Gene-Better DNA, Beijing, China). DNA was stored at −20 °C for further use. Standard curves were constructed using DNA extracted from *S*. Typhimurium cultures. *Salmonella*-specific primers invA-F (5′-TTTATCAATAGCCTCAAACCC-3′) and invA-R (5′-TGCGTCACCCTCTAACTCATC-3′) were used to amplify DNA concentration. PCR products were recovered using a DNA purification kit (Beijing Biomed Gene Technology, Beijing, China), recombined with PCR®2.1 plasmid vector (TA Cloning Kit, Transgene, Beijing, China), and then transfected into *Escherichia coli* DH-5α receptor cells (Transgene) to construct standard plasmids. The plasmid DNA concentration was then measured using a nucleation assay (Nano-DROP 1000). The copy number of the target gene was determined according to the following formula: DNA (copy) = (6.02×10^{23}) (copy/mol) × DNA quantity (g)) / (DNA length (bp) × 650 (g/mol/bp)). Using ileal digesta DNA as a template, fluorescence quantitative PCR was performed according to the instructions of the SYBR® Premix Ex TaqTM kit (Takara). At the same time, standard plasmids were gradient diluted and added to each 384-well PCR plate to create the standard curve. The number of *Salmonella* in ileal digesta was presented as $\log_{10}$ gene copies per gram of digestion.

Determination of ileum mucosal sIgA

The concentration of sIgA in mucosa was determined using an ELISA kit provided by Bethyl Laboratories (Montgomery, TX, USA). sIgA detection was conducted according to the manufacture description. Briefly, mucosal tissue is homogenized in ice-cold PBS four times of its volume. After homogenization, the suspension was centrifuged at 5000 RPM and 4 °C for 20 min. Subsequently, the supernatant was used for sIgA and total protein determination. The concentration of sIgA was presented as μg/mg*protein.

RNA-seq analysis

Ileal tissue RNA was extracted with TRIzol reagent (Invitrogen). The concentration and integrity of extracted RNA was detected by Nanodrop 2000 (Thermo Scientific) and Bioanalyzer 2100 (Agilent Technologies, CA, USA) respectively. Then, the RNA was purified and interrupted, and subsequently used as templates to synthesize the cDNA through PCR. After purifying, the cDNA was added with the final reagent and put into the PCR instrument for reaction. After the reaction, the connector was connected immediately. Primer connector sequence: Adpter3 = "AGATCGGAA GAG-CACACGTCTGAACTCCAGTCAC," Adpter5 = "AGATCGGAAGAGCGTCGTG-TAGG GAAAGAGTGT". Then, the processed cDNA was subjected to PCR amplification and purification. The purified product was inspected with QSEP-400 and then sequenced in Illumina NovaSeq 6000 Platform. Trimmomatic was used to trim the adaptor sequences and poor-quality reads [84]. The DESeq2 program in R was used to standardize the levels of gene expression as reads per kilobase per million mapped reads (RPKM) and calculate the differentially expressed genes (DEGs) [85]. $|\log_2 FC| > 1$, and $p < 0.05$ was set as the threshold for DEGs. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed using the R package. The immune-related genes were integrated from ImmPort and KEGG databases.

Preparation of lymphocytes and flow cytometry

Spleen, thymus, and Bursa of Fabricius were mechanically disrupted, and single-cell suspensions were made. Peripheral blood mononuclear cells were extracted using density gradient centrifugation following the manufacturer's guidelines (TBD, Tianjin, China). Single-cell suspensions of ileum were prepared as previously described [79]. Antibodies used are listed in Table S3. Staining was performed for 30 min on ice in PBS containing 1% fetal bovine serum. Cells were acquired with a Coulter XL (Beckman Coulter, CA, USA), and analysis was performed with FlowJo and FCS Express 6 Flow software.

Statistical analysis

SPSS 20.0 was used for statistical analysis. Two-tailed unpaired Student's *t* test was used to evaluate the difference between the control group and the experimental group (antibiotic treatment, microbiota transplantation, and bacterial strain inoculation). Single-factor analysis and Duncan multiple comparative analysis were used in the *Salmonella* challenge trial. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered statistically significantly different.

DATA AVAILABILITY

The raw 16S rRNA gene sequencing data of luminal microbiota from the antibiotic-untreated broiler chickens were obtained from NCBI BioProject PRJNA817429 (unpublished data from our own laboratory), and additional raw 16S rRNA gene sequencing data have been deposited in NCBI BioProject PRJNA904673. The genome data have been deposited in NCBI BioProject PRJNA903494 and PRJNA902159. The transcriptome data have been deposited in NCBI BioProject PRJNA904665. Raw datasets used for multicolor flow cytometry and qRT-PCR are available on FigShare (https://doi.org/10.6084/m9.figshare.21825042).

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AUTHOR CONTRIBUTIONS

DL designed the project. YL carried out the experimental work and drafted the manuscript. MZ carried out bioinformatic analyses. YF, XY, ZL, PL and FW collected samples. All the other authors revised and edited the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

All the experiments were reviewed and approved by China Agricultural University Animal Care and Use Committee (statement no. AW30112202-1-1).

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