



OPEN

The effect and potential mechanism of inulin combined with fecal microbiota transplantation on early intestinal immune function in chicks

Yang Song¹, Yibo Cui¹, Yumeng Wang¹, Taiping Wang¹, Yue Zhong¹, Jingsheng Liu^{2,3} & Xin Zheng¹

Our previous research found that fecal microbiota transplantation (FMT) and inulin synergistically affected the intestinal barrier and immune system function in chicks. However, does it promote the early immunity of the poultry gut-associated lymphoid tissue (GALT)? How does it regulate the immunity? We evaluated immune-related indicators in the serum, cecal tonsil, and intestine to determine whether FMT synergistic inulin had a stronger impact on gut health and which gene expression regulation was affected. The results showed that FMT synergistic inulin increased TGF- β secretion and intestinal goblet cell number and *MUC2* expression on day 14. Expression of *BAFFR*, *PAX5*, *CXCL12*, and *IL-2* on day 7 and expression of *CXCR4* and *IL-2* on day 14 in the cecal tonsils significantly increased. The transcriptome indicated that *CD28* and *CTLA4* were important regulatory factors in intestinal immunity. Correlation analysis showed that differential genes were related to the immunity and development of the gut and cecal tonsil. FMT synergistic inulin promoted the development of GALT, which improved the early-stage immunity of the intestine by regulating *CD28* and *CTLA4*. This provided new measures for replacing antibiotic use and reducing the use of therapeutic drugs while laying a technical foundation for achieving anti-antibiotic production of poultry products.

Keywords Fecal microbiota transplantation, Inulin, Immunity, Transcriptome, Chick

In poultry, the efficient vertical transmission of gut symbiotic bacteria to offspring is also crucial for the rapid development of the offspring microbiota and host development, which can protect the host from intestinal pathogens¹. Many reports indicated vertical transmission through the egg exists in poultry, but transmission from hens to chicks is limited². Exposure of newly hatched chicks to adult gut contents is protective against *Salmonella* infection³. However, in modern intensive culture, the egg, as isolated individuals, is sent to the incubator for artificial incubation through fumigation and disinfection, increasing the possibility of deviation in the delivery of intestinal bacteria from hens to chicks. The risk of chick disease is greatly enhanced, thus seeking a way to enhance immunity in the early stages of chicks is crucial for their postnatal health.

In recent years, fecal microbiota transplantation (FMT) has been studied in poultry. On the one hand, utilizing microbiota to ferment undigestible carbohydrates and reduce gastric emptying time to increase the nutritional value and feed intake^{4,5}. On the other hand, FMT is used as a tool to regulate host health through competitive exclusion and antagonism characteristics of gut microbiota to induce early life programming of receptors. Ma, et al.⁶ showed that FMT changed the gut microbiota, increasing the abundance of *Lactobacillus*, which improved the growth performance of chickens by balancing Th17/Treg cells. Fecal microbiota from healthy adult hens can be transplanted into chicks to promote the development of their intestinal microbiota structure and enhance their resistance to *Salmonella* infection⁷. Considering that the intestine is always exposed to a variety of external

¹College of Animal Science and Technology, Jilin Agricultural University, No.2888 Xincheng Road, Nanguan District, Changchun 130118, Jilin, China. ²College of Food Science and Engineering, Jilin Agricultural University, Changchun 130118, Jilin, China. ³National Engineering Research Center for Wheat and Corn Deep Processing, Changchun 130118, Jilin, China. ✉email: zhengxin@jlau.edu.cn

substances from the outside and could potentially be in danger from harmful pathogens at any point. Thus, the mucosal immune system is essential for preventing pathogen invasion of the gut and preserving intestinal homeostasis⁸. According to research by Lee, et al.⁹, the gut microbiota regulated the quantity and function of CD4⁺CD8⁺CD25⁺ and CD4⁺CD8⁺CD25⁺ T cells in the cecal tonsil. Intestinal microbiota can induce regulatory T cell responses, increase intestinal IgA plasma cells, participate in B cell class transition, and develop mucosal antibody repertoire^{10–13}. FMT might be an effective strategy to affect immunological responses later in life because of the interaction between the microbiota and the host immune system. However, some reports have shown that FMT may damage the intestinal barrier to a certain extent and induce intestinal inflammation, which is potentially dangerous to the health of the recipient¹⁴. Therefore, it is necessary to find a method to help FMT fully exert its beneficial effects and avoid the occurrence of adverse effects.

Inulin is a widely recognized prebiotic and the only one that has received the EU health declaration to improve intestinal function¹⁵. Inulin has non-digestibility and a very low caloric value, but it can be utilized by microbiota, its most significant nutritional effect is to stimulate the growth of *Bifidobacteria* in caecal contents and improve the host's microbial balance¹⁶. Additionally, inulin improved IgA levels and mucin mRNA expression in the gut of broilers¹⁷. Adding inulin to the basic diet can significantly reduce the counts of *E. coli* and *Salmonella*¹⁸. The immune system, growth and nutrient utilization, and gut microbiota of broilers are all positively impacted by the feeding of *Lactobacillus* and inulin¹⁹. There is insufficient research on the effects of applying FMT and inulin simultaneously in the early stages of poultry life. Due to the colonization of gut microbiota starting from birth or hatching, it is crucial to establish a beneficial microbial community as soon as possible for the health of chicks²⁰. This study determined the donor of FMT, and the positive effects of inulin combined with FMT on the microbiota and immune organ index of chicks through preliminary results²¹. Meanwhile, we also found that the combination of FMT and inulin has a synergistic effect on improving the immune system function and intestinal immunity of chicks. On this basis, we focused on conducting transcriptome analysis of the ileum and observing the development of gut-associated lymphoid tissue (GALT). Here, we hypothesized that the combination of FMT and inulin improved the immunity of the gut and its related lymphoid tissues in chicks, with the aim of determining whether FMT synergistic inulin had a stronger impact on gut health and which gene expression regulation was affected. Our results provided new measures for replacing antibiotic use and reducing the use of therapeutic drugs while laying a technical foundation for achieving anti-antibiotic production of poultry products.

Results

Effects of inulin and FMT combination on cytokines in chick serum.

We analyzed the secretion levels of IL-6, IL-18, and TGF- β in chick serum on days 7 and 14 (Fig. 1). The serum results showed that compared with the CON group, the content of IL-6 on day 7 and TGF- β on day 14 in the INU group was significantly increased by 19.14% ($P=0.001 < 0.01$) and 9.78% ($P=0.022 < 0.05$), respectively. In addition, the level of IL-18 on day 14 showed the opposite (18.76%) ($P < 0.001$). The levels of TGF- β significantly

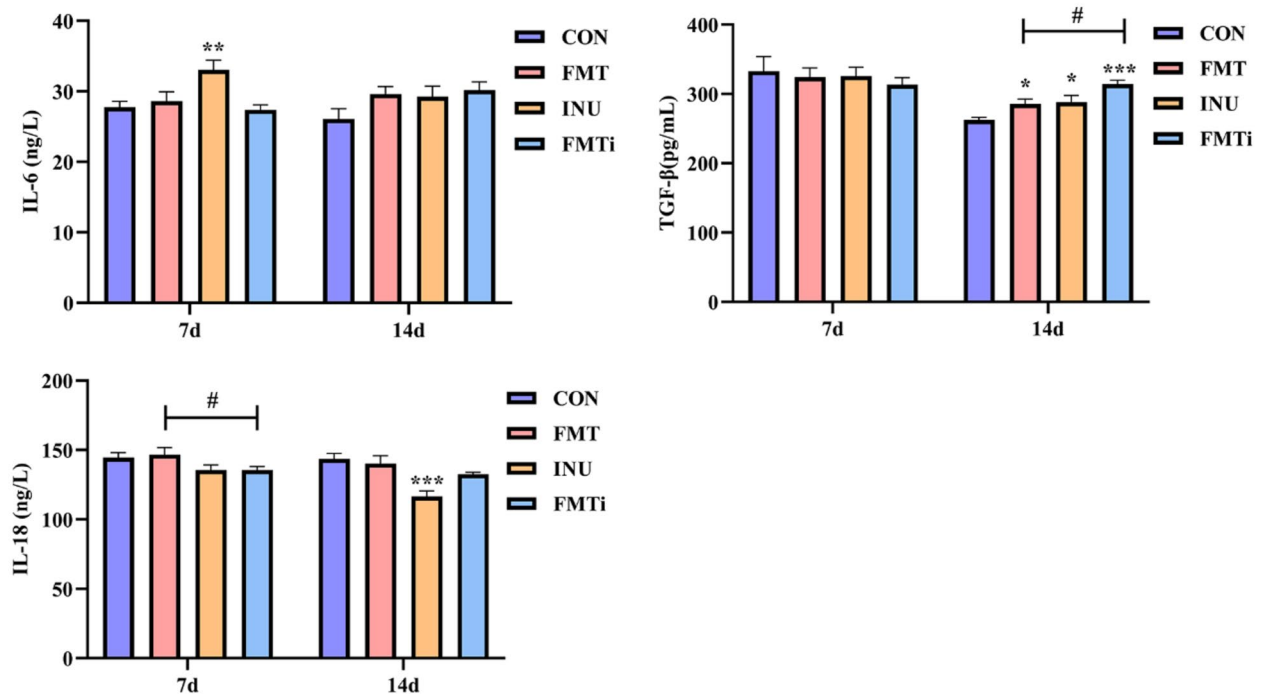


Figure 1. The secretion levels of IL-6, IL-18, and TGF- β were determined by ELISA in the serum of chicks among different treatment groups on days 7 and 14. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the CON group. # $P < 0.05$, versus the FMT group. $n = 10$.

increased by 8.81% and 19.66% in the FMT group ($P=0.042<0.05$) and FMTi group ($P<0.001$) on day 14. Compared with the FMT group, the content of IL-18 in the FMTi group significantly decreased by 7.58% on day 7 ($P=0.047<0.05$), while TGF- β levels significantly increased by 9.97% on day 14 ($P=0.034<0.05$). There was no statistical difference in the other indicators.

Inulin combined with FMT promoted the ileal barrier function in chicks

The goblet cells in the intestine can secrete mucus. According to the results in Fig. 2a, the number of goblet cells showed no significant difference among the treatment groups on day 7. Compared to the CON group, the number of goblet cells was significantly increased in the FMTi group ($P<0.001$), which was significantly higher in the FMTi group than in the FMT group on day 14 ($P=0.009<0.001$). Figure 2b examined the relative gene expression of *MUC2* in the ileum and found that its expression level was significantly higher in the FMT group on day 7 ($P=0.004<0.01$) and the FMTi group on day 14 ($P<0.001$) than in the CON group. In addition, compared with the FMT group, the relative expression level of *MUC2* in the FMTi group was significantly increased ($P=0.025<0.05$).

Morphological observation of cecal tonsils in chicks

The results in Fig. 3 indicated that the area of the GC in the cecal tonsils increased with time. On day 7, the area of GC in the FMTi group was significantly higher than that in the CON group ($P=0.024<0.05$) or the FMT group ($P=0.006<0.01$). On day 14, compared with the CON group, the area of GC in the INU group ($P=0.009<0.01$) and FMTi group ($P=0.003<0.01$) showed the same trend.

Inulin combined with FMT improved the immune function of the cecal tonsil in chicks

The immune response-related indicators in the cecal tonsils were detected by q-PCR. According to Fig. 4a, on day 7, the relative expression of *IL-2* was significantly increased in the FMT group compared to the CON group ($P=0.003<0.01$). The relative expression levels of *PAX5* ($P=0.005<0.01$), *CXCL12* ($P=0.027<0.05$), and *IL-2* ($P=0.002<0.01$) showed the same trend in the INU group. This result was similar to the gene expression levels of *BAFFR* ($P<0.001$), *PAX5* ($P=0.012<0.05$), *CXCL12* ($P<0.001$), *IL-2* ($P<0.001$), and *IL-4* ($P=0.002<0.01$).

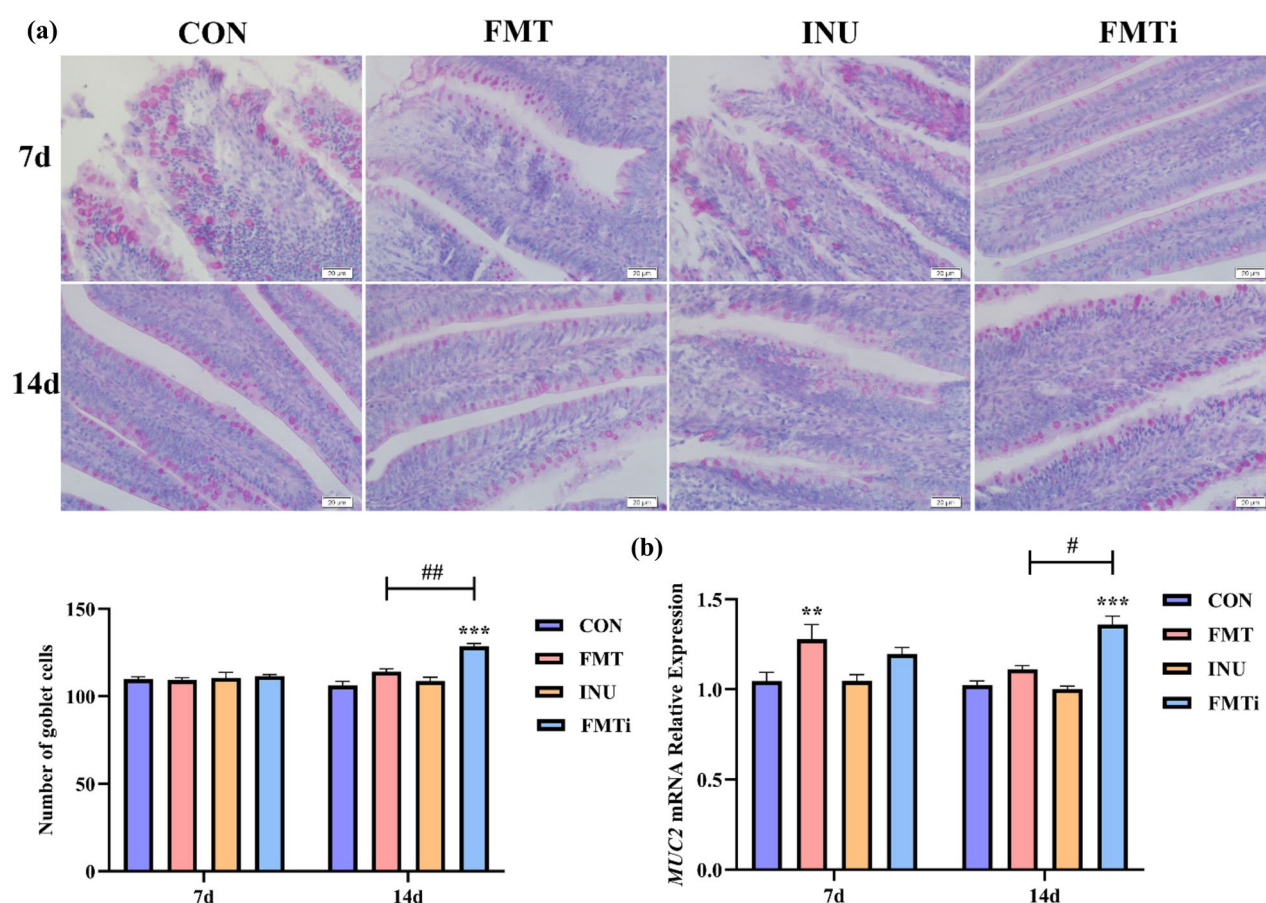


Figure 2. The combined effect of inulin and FMT on intestinal barrier function. (a) The number of goblet cells in the ileum among treatment groups on days 7 and 14. Bar = 20 μ m. (b) The relative gene expression of *MUC2* in the ileum on days 7 and 14. ** $P<0.05$, *** $P<0.001$, versus the CON group. # $P<0.05$, ## $P<0.01$, versus the FMT group. n = 10.

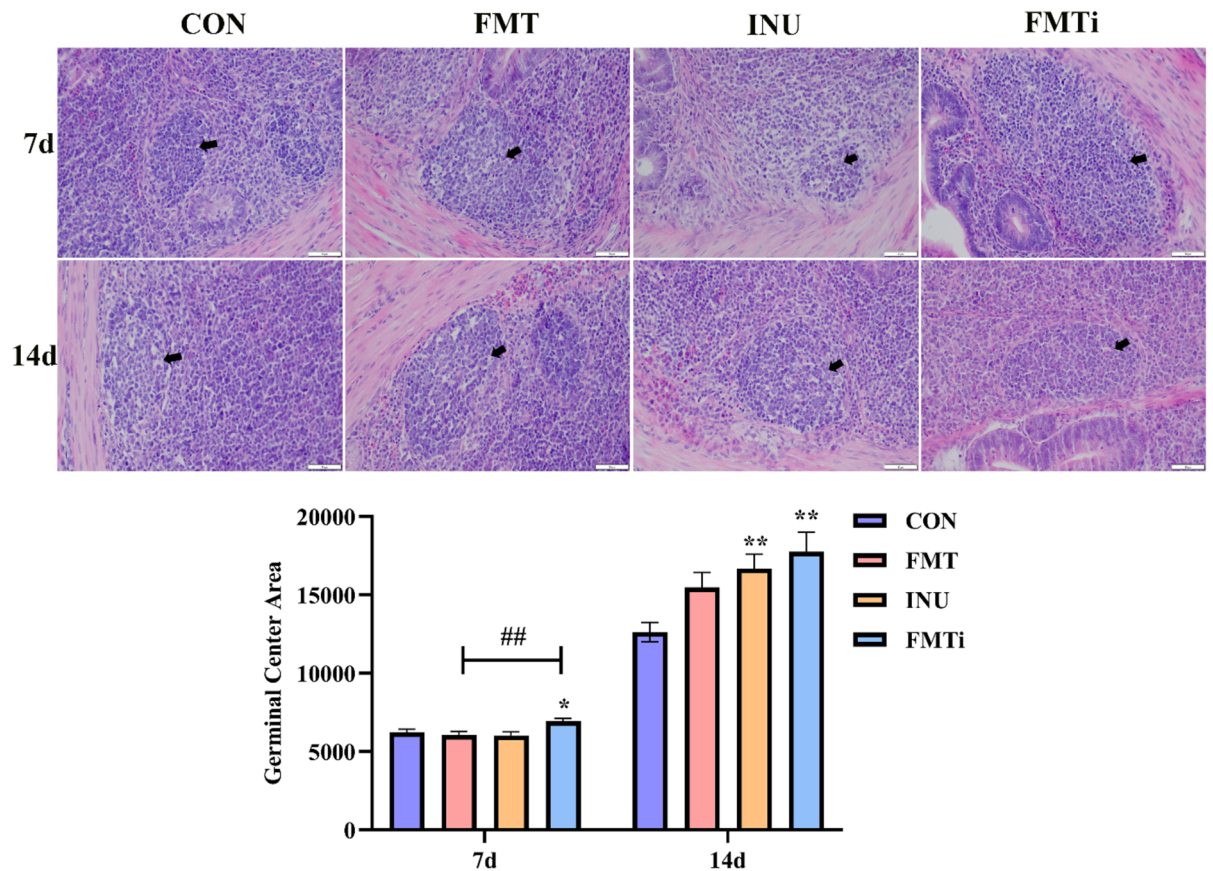


Figure 3. The area of the germinal center (GC, black arrow) in the cecal tonsil of chicks on days 7 and 14. Bar = 50 μ m. * P < 0.05, ** P < 0.01, versus the CON group. ## P < 0.01, versus the FMT group. n = 10.

in the FMTi group. It was worth noting that the gene expression levels of *BAFFR* (P = 0.001 < 0.01), *PAX5* (P = 0.024 < 0.05), *CXCL12* (P < 0.001), and *IL-2* (P < 0.001) in the FMTi group were significantly higher than those in the FMT group. On day 14, the relative expression levels of *CXCR4* (P = 0.007 < 0.01), *IL-2* (P < 0.001), *IL-4* (P = 0.001 < 0.01), and *Bu-1* (P = 0.046 < 0.05) in the FMTi group were significantly increased compared to the CON group. In addition to *IL-2* (P = 0.014 < 0.05), *IL-4* (P = 0.003 < 0.01) showed a significant increase in the INU group, while *IL-4* (P = 0.007 < 0.01) also showed the same trend in the FMT group. In addition, the expression of *CXCR4* (P = 0.001 < 0.01) and *IL-2* (P < 0.001) in the FMTi group was significantly higher than in the FMT group. From the immunohistochemical results in Fig. 4b, we found that the expression level of IgA⁺ had no significant differences among the treatment groups on day 7 but was significantly increased in the FMT group (P = 0.003 < 0.01) and FMTi group (P = 0.008 < 0.01) compared to the CON group on day 14.

Combination with inulin and FMT on transcriptome changes in the gut was related to intestinal immune responses in chicks

Based on the above experimental results, we found that the combined effect of inulin and FMT was superior to other treatments, and the overall effect on day 14 was better than that on day 7. So, we performed RNA-seq on the ileum of chicks from the CON group and FMTi group on day 14. We filtered differentially expressed genes in the ileum on day 14 and found 54 upregulated DEGs and 82 downregulated DEGs in the FMTi group (P < 0.05) (Fig. 5a). GO enrichment analysis was performed on the expressed genes, and 432 terms were significantly enriched in 2633 GO terms (P < 0.05), of which 16.90% terms were in molecular function (MF), 7.18% terms in cellular component (CC), and 75.93% terms in biological process (BP) (Fig. 5b). In the biological process category, TOP30 terms were selected, and it was found that differentially expressed genes with enrichment factors (DEG number/total gene number in terms) were mainly involved in TOP5 terms, which were protein ADP ribosylation (4/26), response to corticotropin-releasing hormone (2/2), cellular response to corticotropin-releasing hormone stimulus (2/2), activation of immune response (9/241), and organic hydroxy compound biosynthetic process (6/124). The remaining terms significantly enriched in Top30 are shown in Fig. 5c.

Correlation analysis between DEGs and immune indicators

Subsequently, we found a total of 7 differentially expressed genes related to immune response from Fig. 5a, including 5 upregulated genes (*CTLA4*, *TRIL*, *FOXJ1*, *HSP90AA1*, *C7*) and 2 downregulated genes (*CD28*, *NR1D1*).

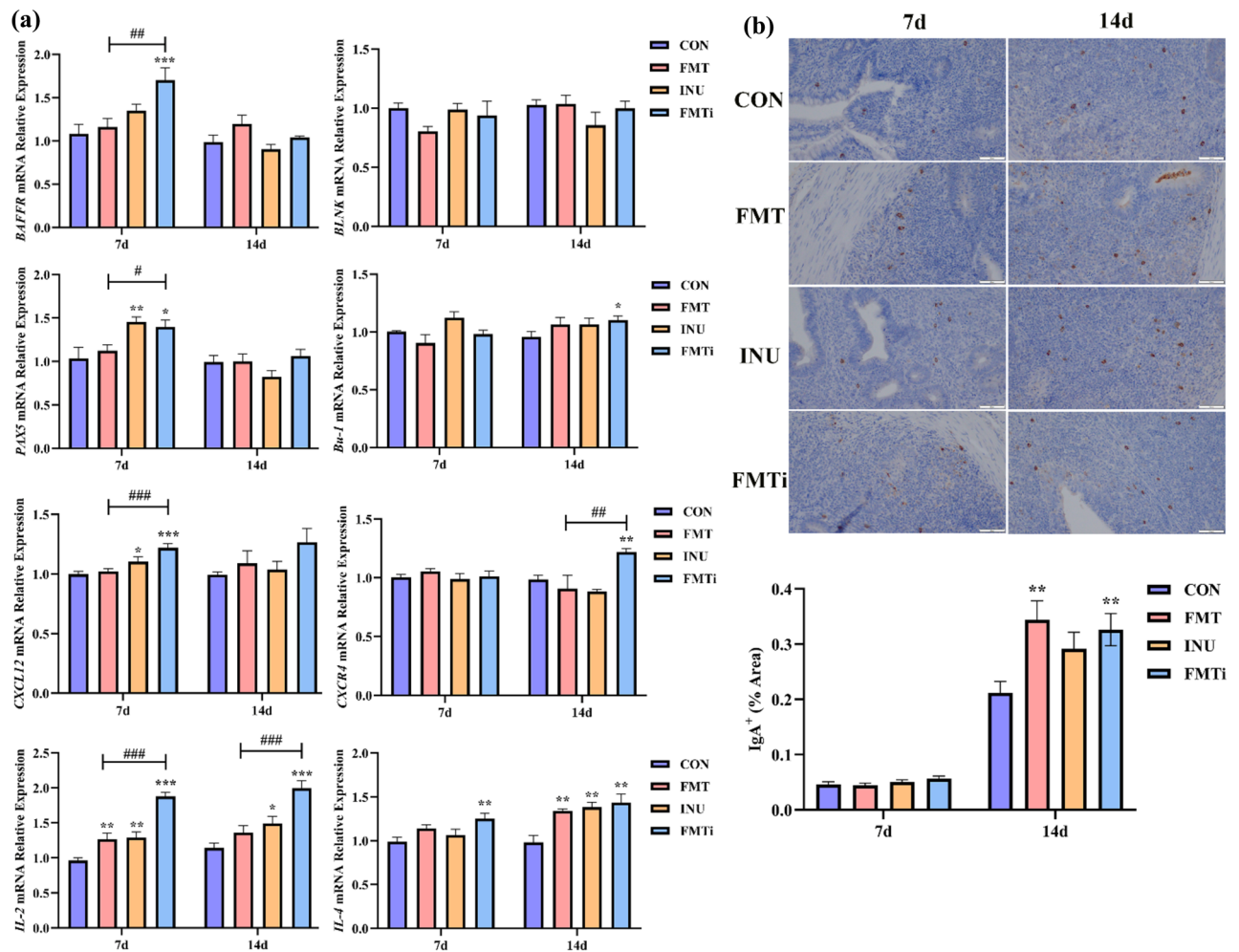


Figure 4. The immune level of the cecal tonsil in chicks. (a) Relative gene expression of *BAFFR*, *PAX5*, *BLNK*, *Bu-1*, *CXCL12*, *CXCR4*, *IL-2*, and *IL-4* on days 7 and 14. (b) Expression of IgA-positive cells on days 7 and 14. Bar = 50 μ m. * P < 0.05, ** P < 0.01, *** P < 0.001, versus the CON group. # P < 0.05, ## P < 0.01, ### P < 0.001, versus the FMT group. n = 10.

A correlation analysis was performed between the FPKM values of these DEGs and serum immunity indicators, or the immune response of the intestine and GALT (Fig. 6a,b). The results in Fig. 6a showed that *IL-18* had a significant negative correlation with *CTLA4* (P = 0.009 < 0.01), *FOXJ1* (P = 0.024 < 0.05), and *HSP90AA1* (P = 0.009 < 0.01), which had an opposite correlation with *NR1D1* (P = 0.002 < 0.01). *TGF- β* had a significant negative correlation with *CTLA4* (P = 0.004 < 0.01), *FOXJ1* (P = 0.016 < 0.05), and *HSP90AA1* (P = 0.028 < 0.05).

In the ileum, Fig. 6b showed that the gene expression of *MUC2* was significantly positive correlated with *CTLA4* (P = 0.014 < 0.05), *FOXJ1* (P = 0.016 < 0.05), and *C7* (P = 0.006 < 0.01) and had a significant negative correlation with *CD28* (P = 0.018 < 0.05) and *NR1D1* (P = 0.031 < 0.05). The number of goblet cells exhibited a positive association with *CTLA4* (P = 0.001 < 0.01), *FOXJ1* (P = 0.042 < 0.05) and *HSP90AA1* (P = 0.013 < 0.05) and a negative correlation with *CD28* (P = 0.028 < 0.05) and *NR1D1* (P = 0.004 < 0.01) significantly. The positive expression of IgA in the ileum was significantly correlated with DEGs (P < 0.05). For the development and immunity of cecal tonsil, we discovered that there were significant positive correlations between the area of the germinal center and *CTLA4* (P = 0.039 < 0.05), *FOXJ1* (P = 0.008 < 0.01), and *HSP90AA1* (P = 0.021 < 0.05). There was a significant negative correlation between the IgA⁺ cell and *CD28* (P = 0.017 < 0.05) and *NR1D1* (P = 0.037 < 0.05), at the same time, showing an opposite trend with *CTLA4* (P = 0.035 < 0.05).

The intestinal immune network for IgA production in the ileum and validation of RNA-seq

KEGG enrichment analysis found that these differential genes were mainly related to the intestinal immune network for IgA production and the cell adhesion molecules signaling pathway (Fig. 7a). We tested genes (*CD28*, *CTLA4*, and *CD4*) associated with the pathway of the intestinal immune network for IgA production and the cell adhesion molecules signaling pathway. As shown in Fig. 7b, compared with the CON group, the FPKM values of *CTLA4* (P = 0.005 < 0.01) and *CD4* (P = 0.004 < 0.01) were significantly increased in the FMTi group, and *CD28* (P = 0.002 < 0.01) showed opposite results. Results in RNA-seq and q-PCR showed the same pattern of expression for the three genes. The consistent expression of the DEGs indicated the reliability of RNA-seq data.

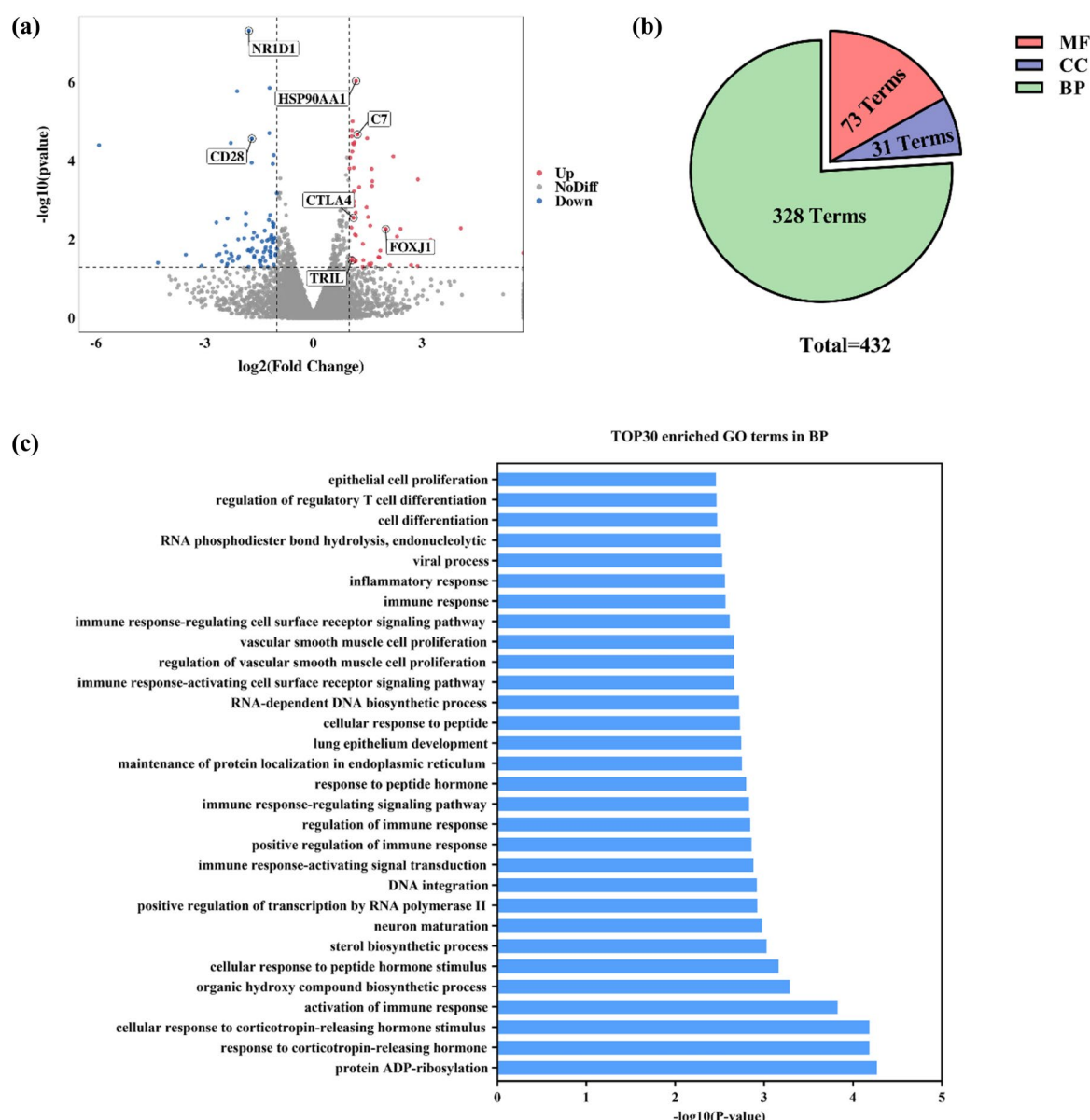


Figure 5. The gut transcriptome of chicks between the CON group and the FMTi group was related to gut immune responses. (a) DEGs in the ileum transcriptome on day 14. (b) The significantly enriched proportion of GO terms in the MF, CC, and BP categories of the ileum transcriptome on day 14. (c) TOP30 enriched GO terms with rich factors (DEG number/total gene number in terms) in the BP category for DEGs in the ileum transcriptome on day 14. $n = 5$.

Genes related to these pathways (*CD28*, *CTLA4*, *IL-10*, *TGF- β* , *CXCR4*, and *CXCL12*) were measured on days 7 and 14 in Fig. 7c. The combination of inulin and FMT significantly increased the relative mRNA expression of *IL-10* ($P = 0.031 < 0.05$) and *CTLA4* ($P = 0.048 < 0.05$) on day 14. The relative gene expression level of *CD28* significantly increased in the FMTi group on day 7 ($P = 0.015 < 0.05$) and showed an opposite trend on day 14 ($P = 0.033 < 0.05$). Additionally, the positive expression level of IgA in Fig. 7d showed that there were no differences between the groups on day 7, and its expression level from the ileum in the FMTi group was significantly higher than that in the CON group on day 14 ($P < 0.001$).

Discussion

The excessive production of pro-inflammatory cytokines (IL-6, IL-18) is an indication of inflammation, while the increase in anti-inflammatory cytokines (TGF- β , IL-10) is the degree to which inflammation is eliminated through an effective host immune response during chicken intestinal inflammation^{22–24}. The serum results showed that compared with the CON group, the content of IL-6 significantly increased in the INU group on day 7. On day 14, the levels of TGF- β in the FMT group, INU group, and FMTi group were significantly higher than those in the CON group, while the secretion of IL-18 showed the opposite result in the INU group. Compared with the FMT group, the level of IL-18 in the FMTi group was significantly decreased on day 7, while the level

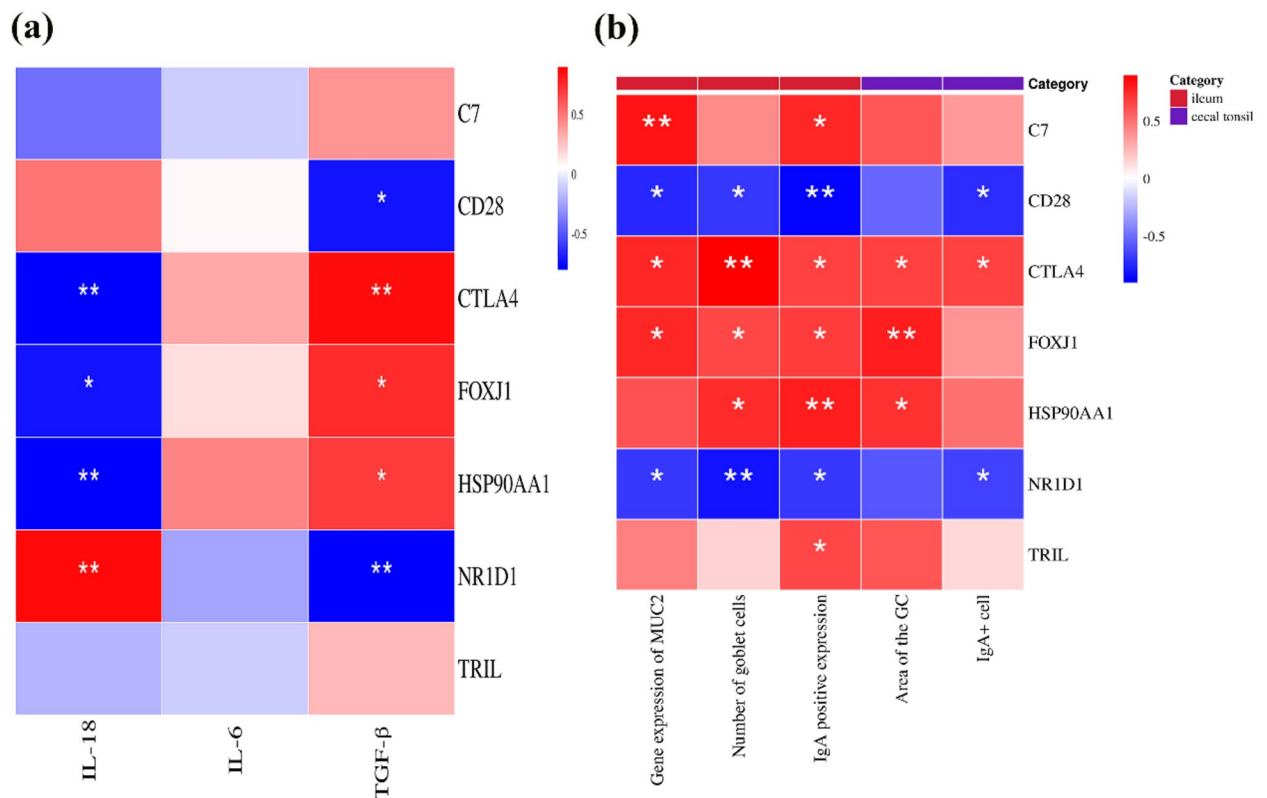


Figure 6. The correlation analysis between DEGs and serum immune indicators (a) or immunity of the ileum and cecal tonsil (b) on day 14. * $P < 0.05$, ** $P < 0.01$. $n = 5$.

of TGF- β was significantly increased on day 14. This indicated that the chicks in each treatment group did not cause inflammation. However, can inulin combined with FMT promote early maturation of bird immunity to reduce the risk of disease? How does it regulate the immune ability of birds?

We considered the gut mucosal system to be the entrance point because the gut microbiota and the host's gut mucosa maintain a symbiotic interaction. The intestinal epithelium mucosal barrier, GALT, and secretory IgA (sIgA) plasma cells make up the intestinal mucosa immune system²⁵. The primary lymphoid tissue within GALT in chickens is the cecal tonsil, which contains a significant number of T lymphocytes. T lymphocytes have the ability to release cytokines, maintain intestinal immune homeostasis, and encourage epithelial cells to produce antimicrobial peptides. On the other hand, they can also stimulate B lymphocytes to develop into plasma cells and secrete IgA²⁶. Additionally, the germinal center of the cecal tonsil also receives IgA induction. It has been demonstrated that sIgA, which is produced by IgA cells, has a role in the immunological barriers of the intestine²⁷. In our results, chicks treated with inulin or inulin combined with FMT significantly increased the germinal center area of the cecal tonsil on day 14 or days 7 and 14, and the expressions of IgA from the FMT group and FMTi group were also significantly enhanced on day 14. Although IgA cells are not the only immune cells, they can reflect B lymphocyte function²⁸. As a result, the expression of IgA cells was used in this study as a proxy for the immunological capacity of the cecal tonsil. We preliminarily concluded that inulin combined with FMT promoted the function of B-secreting cells in the cecal tonsil to enhance immune function.

The core components of humoral immunity, B cells and their antibodies, function as a part of the adaptive immune system to defend against a nearly infinite variety of pathogens. Bu-1 is a B cell marker that accompanies the development of B cells²⁹. Schneider, et al.³⁰ suggested that BAFFR was expressed predominantly or exclusively in B cells and provides survival signals to immature and mature B cells. Sharma, et al.³¹ found that *in ovo* delivery of *lactobacilli* induced BAFF and BAFF-R expressions and stimulated and extended the proliferative capacity of B cells. Mikkola, et al.³² demonstrated that PAX5 acted as the factor maintaining B cell identity and was essential for B cell development. Based on the above research, we found that inulin alone or combined with FMT significantly increased the relative mRNA expression of *BAFFR*, *PAX5*, and *Bu-1* on day 7 or 14. Cheng, et al.²⁹ believed that the expression of *CXCR4*, *BLNK*, *PAX5*, and *BAFFR* is associated with B cell differentiation. This confirmed that inulin alone or combined with FMT can stimulate the function of B cells. The development and function of lymphoid tissue depend on directed cell migration, and chemokines provide guideposts for cell movement and localization within lymphoid tissues³³. Within the chemokine family, CXCL12 provides key localization cues for B lymphocytes during B cell development and/or B cell immune responses through its receptor CXCR4^{34,35}. With the continuous expansion of the field of B cell research, IL-4 has also been proven to be an important product secreted by B cells to regulate immune responses³⁶. Multiple reports have found that the main cellular source of IL-4 is the GC B cell^{37,38}. Inhibiting the synthesis of proinflammatory modulators, IL-2 is a crucial cytokine that performs crucial functions in promoting the proliferation of B and T lymphocytes³⁹. In our results,

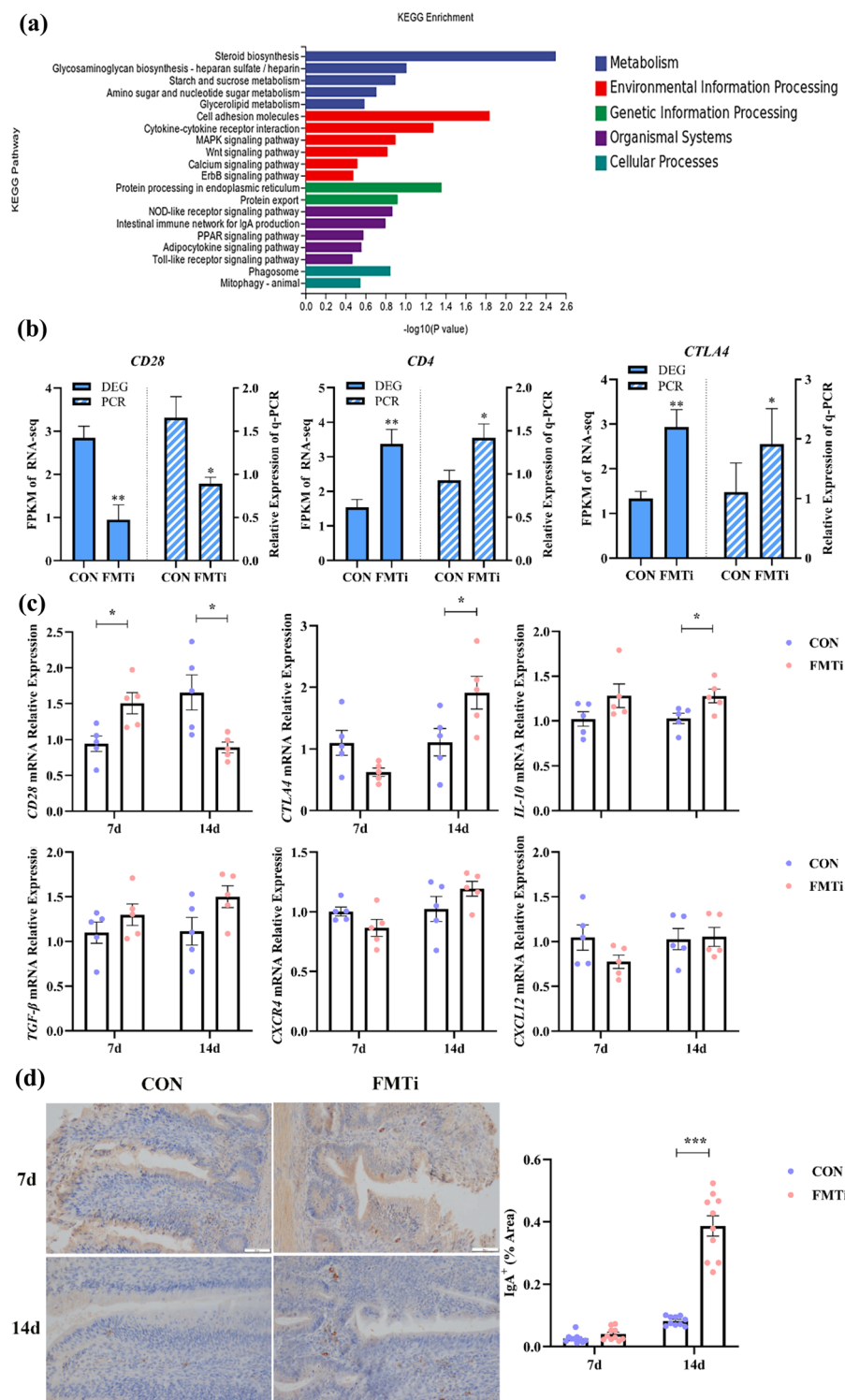


Figure 7. KEGG enrichment analysis and validation of the intestinal immune network for IgA production between the CON group and the FMTi group. **(a)** KEGG enrichment analysis of the ileum transcriptome on day 14^{50–52}. $n = 5$. **(b)** FPKM values and relative gene expressions of *CD28*, *CTLA4*, and *CD4* were compared on day 14. $n = 5$. **(c)** The gene expression of *CD28* and *CTLA4* in the ileum and the expression levels of genes (*IL-10*, *TGF-β*, *CXCL12*, and *CXCR4*) related to the intestinal immune network for IgA production on days 7 and 14. $n = 5$. **(d)** The expression of IgA-positive cells in the ileum on days 7 and 14. $n = 10$. Bar = 50 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the CON group.

genes that reflect immunity (*IL-2*, *IL-4*, *CXCL12*, etc.) in each group showed a significant increase to varying degrees starting on day 7, it was worth noting that inulin combined with FMT gave the best results. This further confirmed our initial conclusion. Traced to the ileum, the level of IgA also increased on day 14, with an increase in the number of goblet cells, and the mRNA expression of *MUC2* indicated the barrier function was enhanced. From this, we concluded that inulin combined with FMT improved intestinal immune function, but it was still unclear which intrinsic factors regulate immune response, which was crucial for revealing more details on the specific characteristics it causes.

Next, we further analyzed how inulin combined with FMT regulated intestinal immune function through transcriptome analysis. The results show that TOP 30 GO terms in BP were related to immunity. In fact, most B-cell responses require the “help” of T-cells to generate the “competence” hierarchy, in which T-cells are the “final arbiters” of the response⁴⁰. We found that the differentially expressed genes involved in immunity were mainly enriched in the intestinal immune network for IgA production and the cell adhesion molecules signaling pathway. Therefore, we tested the main genes involved in the pathway and found that the expression of *CD28* on day 7, *CTLA4*, and *IL-10* on day 14 in the FMTi group significantly increased, while *CD28* significantly decreased on day 14. DEGs in the intestine were correlated with the regulation of cytokines and the immunity of GALT. A family of immunoglobulin-related receptors, including *CD28* and *CTLA4*, are in charge of various aspects of T cell immune regulation⁴¹. Naïve CD4 and CD8 T cells express the costimulatory molecule *CD28*, which is necessary for triggering T cell responses, promoting T cell differentiation, supporting the generation of cytokines, and regulating metabolism^{42,43}. *CTLA4* serves as a negative regulator of T cell responses and is the primary regulator of Treg, in opposition to *CD28*^{44,45}. Enhancing Treg activity and maintaining gut homeostasis depend on *IL-10* signaling⁴⁶. The level of *CD28* first increased and then decreased over time, while the expression of *CTLA4* significantly increased on day 14 in the result, indicating that inulin combined with FMT stimulated T cell responses on day 7 and enhanced the inhibitory function of Treg on day 14, by expressing inhibitory factors (*CTLA4*, *IL-10*) to prevent T cell overactivation and maintain intestinal immune homeostasis.

In summary, the synergistic effect of FMT and inulin promoted the early development of intestinal lymphatic tissue, enhanced intestinal immune function, and screened out *CD28* and *CTLA4* as important candidate genes for regulating intestinal immunity. Normal intestinal immune activity was promoted by the synergistic effect of FMT and inulin, providing new measures for replacing antibiotic use and reducing the use of therapeutic drugs while laying a technical foundation for achieving anti-antibiotic production of poultry products. Next, we will expand the sample size, focus on maternal FMT, and screen special bacteria to serve the healthy production of large-scale chicks, and conduct an in-depth exploration of candidate genes.

Materials and methods

Animal management

Chicks were provided by the Changchun Institute of Agricultural Science and Technology. The one-day-old laying-type chicks (Hy-line Brown, male, initial weight 38.37 ± 0.17 g) were managed as recipients ($n = 80$). The chicks were randomly divided into four groups, with 20 chicks in each group. There were 4 pens with 5 chicks each that were identical for each group. For 1–6 days, the control group (CON) was fed a basic diet (Changchun Hefeng Feed Co., Ltd., Jilin, China) and an equal amount of water, the FMT group was fed a basic diet and the mixture of fecal microbiota suspension and water, the inulin group (INU) was treated with 1% inulin (10 g inulin was added in 1 kg of the basic diet) and an equal amount of water, and the FMT combined with inulin group (FMTi) was fed 1% inulin and the mixture of fecal microbiota suspension and water. It was noticed that stopping drinking and starvation was required for 2 h before treatment, then providing their diet and water after the equal amount of water or bacterial suspension was depleted. For 7–14 days, each group was fed a basic diet and drank water freely. The basic diet and management of chicks are shown in Supplementary Tables S1 and S2. The chicks were ensured to have free access to feed during the experimental period (1–14 days). On days 7 and 14, 40 chicks ($n = 10$ per group at each time point) were humanely euthanized respectively and the ileum and cecal tonsil were collected. A part of the tissue sample was fixed in 4% paraformaldehyde for morphological observation, while the rest was preserved at -80°C for subsequent examination. All the methods were performed following the relevant guidelines and regulations.

Preparation and supplement of fecal microbiota suspension

Twenty healthy hens (Hy-line Brown, 6-month-old) without a history of gastrointestinal diseases and antibiotic treatments, were used as FMT donors in this experiment. Their fresh feces were collected without the white part in sterile centrifuge tubes and were immediately treated under anaerobic conditions. Feces were mixed with sterile saline (1:2) and homogenized, filtered with sterile gauze and a 0.25 mm strainer. The supernatant was obtained by centrifuge (800g, 3 min) and mixed with 10% sterile glycerol on ice for 60 min to allow the glycerol to seep into the bacterial cells and ensure microbial survival during storage (-80°C). To prevent freezing cycles for the single inoculation, the fecal suspension was separated into aliquots. Keeping a low temperature throughout the process. On the day of inoculation, 10 mL fecal bacterial suspension was mixed with water in 1:8, and chicks freely drank a mixture (90 mL) of water and bacterial suspension daily fresh as soon as possible for a short time. This process lasted for 6 days.

ELISA assay

The blood ($n = 10$ per group at each time point) was collected on days 7 and 14, and centrifuged (3500g, 15 min) to obtain serum. Three technical replicates were conducted to calculate the contents of Interleukin-6 (IL-6), Transforming growth factor- β (TGF- β), and Interleukin-18 (IL-18) in serum according to the directions of the ELISA kit (Shanghai Enzyme Immune Biotechnology Co., Ltd., China).

Observation of goblet cells in the intestine

The intestinal tissues (days 7 and 14) were cut into 5 µm histological sections to measure the number of mucins per unit area with Periodic Acid-Schiff staining (n = 10 per group at each time point). The optical microscope (Olympus, Tokyo, Japan) was used to observe each slide's five visual fields.

Morphology of cecal tonsil in chicks

The cecal tonsil of chicks was dehydrated, embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin. The area of the germinal center (GC) in the cecal tonsil was measured by the Olympus light microscope and the Olympus MicroSuite™ Imaging software (Olympus, Tokyo, Japan). Five visual fields on each slide (n = 10 per group at each time point) were observed.

q-PCR analysis

RNA (n = 10 per group at each time point) was extracted from the ileum and cecal tonsil of chicks on days 7 and 14 according to the instructions of the RNAiso Plus kit (TaKaRa Bio Inc., Shiga, Japan). The extracted RNA was quantified by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and reverse transcribed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa Bio Inc.) to cDNA. Finally, TB Green® Premix Ex Taq™ II (TaKaRa Bio Inc.) connected Real-Time PCR detection system (Bio Rad, Hercules, CA, USA) in CFX to detect the relative expression. In the experiment, *GAPDH* was set as the housekeeping gene, and the relative mRNA expression of target genes (*Mucin-2* (*MUC2*), *C-X-C chemokine receptor 4* (*CXCR4*), *C-X-C chemokine ligand 12* (*CXCL12*), *Interleukin-10* (*IL-10*), *TGF-β*, *CD28*, *CD4*, and *Cytotoxic T-lymphocyte antigen 4* (*CTLA4*) in the ileum and the relative mRNA expression of target genes (*CXCR4*, *CXCL12*, *B-cell linker protein* (*BLNK*), *B-cell activating factor receptor* (*BAFFR*), *B-cell lineage specific activator* (*PAX5*), *Bu-1*, *Interleukin-2* (*IL-2*), *Interleukin-4* (*IL-4*)) in the cecal tonsil were calculated by $2^{-\Delta\Delta C_t}$. Three technical replicates were carried out. A reaction system of 20 µL was carried out in a thermal cycling program. The thermal cycling program consisted of an initial denaturation step at 95 °C for 30 s followed by 39 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, and extension at 65 °C for 5 s with a final extension step of 5 s at 95 °C. The primer sequences are in Supplementary Table S3.

Immunohistochemical observation of secretory IgA

Slides of ileum and cecal tonsil (days 7 and 14, 5 µm) were transferred to xylene and different concentration gradients of alcohol (3 × 3 min) and then washed in PBS (PH = 7.4). The slides were subjected to the antigen retrieval procedure by boiling sodium citrate antigen buffer for 20 min and washed in PBS after cooling to room temperature (3 × 3 min) before incubating with the peroxylase blocking solution (Fuzhou Meixin Biotechnology Development Co., Ltd., Fujian, China) at 37 °C for 10 min. The next 20 min of incubation at 37 °C in the goat serum after washing in PBS. The slides were incubated overnight at 4 °C with the mouse anti-chicken IgA primary antibodies (1:100, SouthernBiotech, Cat. No. 8330-01, USA). The slides received PBS instead of the primary antibody as negative controls. After being exposed to the biotinylated secondary antibody goat anti-mouse IgG (Fuzhou Meixin Biotechnology Development Co., Ltd., Fujian, China) for 20 min at 37 °C, the slices were incubated with streptavidin (Fuzhou Meixin Biotechnology Development Co., Ltd., Fujian, China) for 10 min at 37 °C. Under dark conditions, the slices were immersed in diaminobenzidine hydrochloride (DAB, Fuzhou Meixin Biotechnology Development Co., Ltd., Fujian, China) to visualize the immunoreaction. Microscopically, the reaction was stopped by immersion in distilled water after the brown staining was visualized. Slices were counterstained with hematoxylin and hydrochloric acid alcohol color separation, washed with tap water, dehydrated in ethanol, and cleared in xylene.

The slides per tissue sample were measured and observed using the Olympus light microscope (n = 10 per group at each time point). The IgA-positive areas in five different microscope fields with each tissue were measured with Image J and averaged.

Intestinal RNA extraction and RNA-seq

On day 14, RNA-seq was used for the analysis of gene expression in the ileum (n = 5 for each group) from the CON group and the FMTi group. Total RNA (n = 5) from the ileum was separated using the TRIzol reagent (Invitrogen). A Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; NC2000), agarose gel electrophoresis, and a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA; Cat # G2939BA) were used to measure the quantity, integrity, and quality of the RNA. The OD260/280, OD260/230, 28S/18S, and RNA Integrity Number (RIN) were used to evaluate the quality of extracted RNA. The values of 10 samples were in the range of $2.11 < \text{OD260/280} < 2.16$, $2.341 < \text{OD260/230} < 2.369$, $1.8 < 28\text{S}/18\text{S} < 2.5$, $8.9 < \text{RIN} < 10$, all of which meet the high-throughput sequencing requirement (Supplementary Table S4).

A TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA; Cat# RS-122-2001) was used for building the sequencing libraries. Then, 3 µg of RNA was utilized as the basic material for making RNA samples. Purification, fragmentation, reverse transcription, and amplification of the RNA were all performed (Supplementary Fig. S1). Using the Illumina PCR Primer Cocktail, DNA fragments with adaptor molecules ligated on both ends were selectively enriched. On the Agilent Bioanalyzer 2100 System, a high-sensitivity Agilent DNA assay (Agilent; Cat# 5067-1511) was used to perform quality control and then sequenced on an Illumina NovaSeq platform.

Bioinformatics analyses of RNA-seq data

The samples were sequenced to produce image files, which were transformed by the software embedded into the sequencing equipment to generate raw data (FASTQ). The sequencing data contained some adaptors and low-quality reads, which caused significant interference in subsequent information analysis. Therefore, Cutadapt (version 1.15) was used to further filter the sequencing data⁴⁷. The *Gallus gallus* reference genome (GRCg6a) and gene annotation files were acquired from Ensembl for data mapping analysis (http://asia.ensembl.org/Gallus_gallus/Info/Index). HISAT2 (version 2.0.5) was used to map the filtered reads to the reference genome. The HTSeq (version 0.9.1) statistics were used to compare the read count values on each gene as the original expression levels of the genes. In order to ensure comparability of gene expression levels between different genes and samples, fragments per kilobases per mapped read (FPKM) were used to standardize the expression levels⁴⁸. The difference expression of genes was analyzed by DESeq (version 1.30.0) with screened conditions as follows: expression difference multiple $|\log_2\text{FoldChange}| > 1$, significant P -value < 0.05 . At the same time, we used the R language Pheatmap (version 1.0.8) software package to perform bi-directional clustering analysis of all different genes in the samples. We mapped all the genes to Terms in the Gene Ontology (GO) database (<http://geneontology.org>) and calculated the numbers of differentially enriched genes (DEGs) in each Term. Using topGO to perform GO enrichment analysis on the differential genes, calculate the P -value by hypergeometric distribution method (the standard of significant enrichment is P -value < 0.05), and find the GO term with significantly enriched differential genes to determine the main biological functions performed by differential genes. ClusterProfiler (version 3.4.4) software was used to carry out the enrichment analysis of the Kyoto Encyclopaedia of Genes and Genomes (KEGG, <https://www.kegg.jp>) pathway of differential genes, focusing on the significant enrichment pathway with P -value < 0.05 .

Statistical analysis

We conducted a Spearman correlation analysis between the FPKM value of the screened DEGs and serum immunity indicators, or the immunity of the intestine and GALT. All results were shown as mean \pm standard error (SEM). For the analysis among the four groups, a Kruskal–Wallis test was performed for the data with SPSS 26.0. For comparison between the two groups, the Student's T -test was performed when the variance was equal. Otherwise, the Welch test was used. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant versus the CON group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were considered statistically significant versus the FMT group.

Ethics approval

This experiment was approved by the Animal Ethics Committee of Jilin Agricultural University (No. 201705001) and complied with the latest version of the ARRIVE guidelines⁴⁹.

Data availability

The raw sequences were deposited in the SRA database under accession number SRP463296: PRJNA1021316 (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1021316?reviewer=2nslmmrtfbi2vgu2u4bqjt6h1>).

Received: 1 March 2024; Accepted: 17 July 2024

Published online: 23 July 2024

References

- Sommer, F. & Bäckhed, F. The gut microbiota—masters of host development and physiology. *Nat. Rev. Microbiol.* **11**, 227–238. <https://doi.org/10.1038/nrmicro2974> (2013).
- Shterzer, N. *et al.* Vertical transmission of gut bacteria in commercial chickens is limited. *Anim. Microbiome* **5**, 50. <https://doi.org/10.1186/s42523-023-00272-6> (2023).
- Varmuzova, K. *et al.* Composition of gut microbiota influences resistance of newly hatched chickens to *Salmonella enteritidis* infection. *Front. Microbiol.* **7**, 957. <https://doi.org/10.3389/fmicb.2016.00957> (2016).
- Hu, F. *et al.* Effects of antimicrobial peptides on growth performance and small intestinal function in broilers under chronic heat stress. *Poult. Sci.* **96**, 798–806. <https://doi.org/10.3382/ps/pew379> (2017).
- Elokil, A. A. *et al.* Early life microbiota transplantation from highly feed-efficient broiler improved weight gain by reshaping the gut microbiota in laying chicken. *Front. Microbiol.* **13**, 1022783. <https://doi.org/10.3389/fmicb.2022.1022783> (2022).
- Ma, Z. *et al.* Fecal microbiota transplantation improves chicken growth performance by balancing jejunal Th17/Treg cells. *Microbiome* **11**, 137. <https://doi.org/10.1186/s40168-023-01569-z> (2023).
- Kempf, F. *et al.* Gut microbiota composition before infection determines the *Salmonella* super- and low-shedder phenotypes in chicken. *Microb. Biotechnol.* **13**, 1611–1630. <https://doi.org/10.1111/1751-7915.13621> (2020).
- Zhang, Y., Wang, Z., Dong, Y., Cao, J. & Chen, Y. Effects of different monochromatic light combinations on Cecal microbiota composition and Cecal Tonsil T lymphocyte proliferation. *Front. Immunol.* **13**, 849780. <https://doi.org/10.3389/fimmu.2022.849780> (2022).
- Lee, I. K. *et al.* Regulation of CD4(+)CD8(–)CD25(+) and CD4(+)CD8(+)CD25(+) T cells by gut microbiota in chicken. *Sci. Rep.* **8**, 8627. <https://doi.org/10.1038/s41598-018-26763-0> (2018).
- Geuking, M. B. *et al.* Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* **34**, 794–806. <https://doi.org/10.1016/j.immuni.2011.03.021> (2011).
- Moreau, M. C., Ducluzeau, R., Guy-Grand, D. & Muller, M. C. Increase in the population of duodenal immunoglobulin A plasma-cytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. *Infect. Immun.* **21**, 532–539. <https://doi.org/10.1128/iai.21.2.532-539.1978> (1978).
- He, B. *et al.* Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* **26**, 812–826. <https://doi.org/10.1016/j.immuni.2007.04.014> (2007).
- Häpfelmeier, S. *et al.* Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science* **328**, 1705–1709. <https://doi.org/10.1126/science.1188454> (2010).

14. Brunse, A. *et al.* Effect of fecal microbiota transplantation route of administration on gut colonization and host response in preterm pigs. *ISME J* **13**, 720–733. <https://doi.org/10.1038/s41396-018-0301-z> (2019).
15. Gibson, G. R. *et al.* Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* **14**, 491–502. <https://doi.org/10.1038/nrgastro.2017.75> (2017).
16. Nabizadeh, A. The effect of inulin on broiler chicken intestinal microflora, gut morphology, and performance. *J. Anim. Feed Sci.* **21**, 725–734. <https://doi.org/10.22358/jafs/66144/2012> (2012).
17. Xia, Y. *et al.* Effects of dietary inulin supplementation on the composition and dynamics of cecal microbiota and growth-related parameters in broiler chickens. *Poult. Sci.* **98**, 6942–6953. <https://doi.org/10.3382/ps/pez483> (2019).
18. Gurram, S. *et al.* Supplementation of chicory root powder as an alternative to antibiotic growth promoter on gut pH, gut microflora and gut histomorphometry of male broilers. *PLoS One* **16**, e0260923. <https://doi.org/10.1371/journal.pone.0260923> (2021).
19. Wu, X. Z., Wen, Z. G. & Hua, J. L. Effects of dietary inclusion of Lactobacillus and inulin on growth performance, gut microbiota, nutrient utilization, and immune parameters in broilers. *Poult. Sci.* **98**, 4656–4663. <https://doi.org/10.3382/ps/pez166> (2019).
20. Amit-Romach, E., Sklan, D. & Uni, Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poult. Sci.* **83**, 1093–1098. <https://doi.org/10.1093/ps/83.7.1093> (2004).
21. Song, Y. *et al.* Donor selection for fecal bacterial transplantation and its combined effects with inulin on early growth and ileal development in chicks. *J. Appl. Microbiol.* <https://doi.org/10.1093/jambio/txad099> (2023).
22. Lynagh, G. R., Bailey, M. & Kaiser, P. Interleukin-6 is produced during both murine and avian Eimeria infections. *Vet. Immunol. Immunopathol.* **76**, 89–102. [https://doi.org/10.1016/s0165-2427\(00\)00203-8](https://doi.org/10.1016/s0165-2427(00)00203-8) (2000).
23. Kogut, M. H. & Arsenault, R. J. A role for the non-canonical Wnt- β -catenin and TGF- β signaling pathways in the induction of tolerance during the establishment of a *Salmonella enterica* Serovar Enteritidis persistent Cecal infection in chickens. *Front Vet Sci* **2**, 33. <https://doi.org/10.3389/fvets.2015.00033> (2015).
24. Peng, X. *et al.* Lactobacillus rhamnosus alleviates intestinal inflammation and promotes microbiota-mediated protection against Salmonella fatal infections. *Front. Immunol.* **13**, 973224. <https://doi.org/10.3389/fimmu.2022.973224> (2022).
25. Kogut, M. H., Lee, A. & Santin, E. Microbiome and pathogen interaction with the immune system. *Poult. Sci.* **99**, 1906–1913. <https://doi.org/10.1016/j.psj.2019.12.011> (2020).
26. Peralta, M. F. *et al.* Gut-associated lymphoid tissue: A key tissue inside the mucosal immune system of hens immunized with *Escherichia coli* F(4). *Front. Immunol.* **8**, 568. <https://doi.org/10.3389/fimmu.2017.00568> (2017).
27. Lammers, A. *et al.* Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. *Dev. Comp. Immunol.* **34**, 1254–1262. <https://doi.org/10.1016/j.dci.2010.07.001> (2010).
28. Li, J., Wang, Z., Cao, J., Dong, Y. L. & Chen, Y. X. Role of monochromatic light on development of cecal tonsil in young broilers. *Anat. Rec. (Hoboken)* **297**, 1331–1337. <https://doi.org/10.1002/ar.22909> (2014).
29. Cheng, J. *et al.* B lymphocyte development in the bursa of Fabricius of young broilers is influenced by the gut microbiota. *Microbiol. Spectr.* **11**, e0479922. <https://doi.org/10.1128/spectrum.04799-22> (2023).
30. Schneider, K. *et al.* Chicken BAFF—A highly conserved cytokine that mediates B cell survival. *Int. Immunol.* **16**, 139–148. <https://doi.org/10.1093/intimm/dxh015> (2004).
31. Sharma, S. *et al.* In ovo feeding of probiotic lactobacilli differentially alters expression of genes involved in the development and immunological maturation of bursa of Fabricius in pre-hatched chicks. *Poult. Sci.* **103**, 103237. <https://doi.org/10.1016/j.psj.2023.103237> (2023).
32. Mikkola, L., Heavey, B., Horcher, M. & Busslinger, M. Reversion of B cell commitment upon loss of Pax5 expression. *Science* **297**, 110–113. <https://doi.org/10.1126/science.1067518> (2002).
33. Shi, G. X., Harrison, K., Wilson, G. L., Moratz, C. & Kehr, J. H. RGS13 regulates germinal center B lymphocytes responsiveness to CXCL12 and CXCL13. *J. Immunol.* **169**, 2507–2515. <https://doi.org/10.4049/jimmunol.169.5.2507> (2002).
34. Nagasawa, T. *et al.* Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXCL12 chemokine PBSF/SDF-1. *Nature* **382**, 635–638. <https://doi.org/10.1038/382635a0> (1996).
35. Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I. & Littman, D. R. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595–599. <https://doi.org/10.1038/31269> (1998).
36. Scala, G., Kuang, Y. D., Hall, R. E., Muchmore, A. V. & Oppenheim, J. J. Accessory cell function of human B cells. I. Production of both interleukin 1-like activity and an interleukin 1 inhibitory factor by an EBV-transformed human B cell line. *J. Exp. Med.* **159**, 1637–1652. <https://doi.org/10.1084/jem.159.6.1637> (1984).
37. Harris, D. P. *et al.* Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat. Immunol.* **1**, 475–482. <https://doi.org/10.1038/82717> (2000).
38. Johansson-Lindbom, B. & Borrebaeck, C. A. Germinal center B cells constitute a predominant physiological source of IL-4: Implication for Th2 development in vivo. *J. Immunol.* **168**, 3165–3172. <https://doi.org/10.4049/jimmunol.168.7.3165> (2002).
39. Lee, K. W. *et al.* Effects of direct-fed microbials on growth performance, gut morphology, and immune characteristics in broiler chickens. *Poult. Sci.* **89**, 203–216. <https://doi.org/10.3382/ps.2009-00418> (2010).
40. Edner, N. M., Carlesso, G., Rush, J. S. & Walker, L. S. K. Targeting co-stimulatory molecules in autoimmune disease. *Nat. Rev. Drug Discov.* **19**, 860–883. <https://doi.org/10.1038/s41573-020-0081-9> (2020).
41. Rowshanravan, B., Halliday, N. & Sansom, D. M. CTLA-4: A moving target in immunotherapy. *Blood* **131**, 58–67. <https://doi.org/10.1182/blood-2017-06-741033> (2018).
42. Broadley, I., Pera, A., Morrow, G., Davies, K. A. & Kern, F. Expansions of cytotoxic CD4(+)CD28(-) T cells drive excess cardiovascular mortality in rheumatoid arthritis and other chronic inflammatory conditions and are triggered by CMV infection. *Front. Immunol.* **8**, 195. <https://doi.org/10.3389/fimmu.2017.00195> (2017).
43. Alegre, M. L., Frauwirth, K. A. & Thompson, C. B. T-cell regulation by CD28 and CTLA-4. *Nat. Rev. Immunol.* **1**, 220–228. <https://doi.org/10.1038/35105024> (2001).
44. Kennedy, A. *et al.* Differences in CD80 and CD86 transendocytosis reveal CD86 as a key target for CTLA-4 immune regulation. *Nat. Immunol.* **23**, 1365–1378. <https://doi.org/10.1038/s41590-022-01289-w> (2022).
45. Liu, Y. & Zheng, P. Preserving the CTLA-4 checkpoint for safer and more effective cancer immunotherapy. *Trends Pharmacol. Sci.* **41**, 4–12. <https://doi.org/10.1016/j.tips.2019.11.003> (2020).
46. Shouval, D. S. *et al.* Interleukin 10 receptor signaling: Master regulator of intestinal mucosal homeostasis in mice and humans. *Adv. Immunol.* **122**, 177–210. <https://doi.org/10.1016/b978-0-12-800267-4.00005-5> (2014).
47. Kechin, A., Boyarskikh, U., Kel, A. & Filipenko, M. cutPrimers: A new tool for accurate cutting of primers from reads of targeted next generation sequencing. *J. Comput. Biol.* **24**, 1138–1143. <https://doi.org/10.1089/cmb.2017.0096> (2017).
48. Anders, S., Pyl, P. T. & Huber, W. HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169. <https://doi.org/10.1093/bioinformatics/btu638> (2015).
49. Percie du Sert, N. *et al.* The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Br. J. Pharmacol.* **177**, 3617–3624. <https://doi.org/10.1111/bph.15193> (2020).
50. Kanehisa, M. & Goto, S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30. <https://doi.org/10.1093/nar/28.1.27> (2000).

51. Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M. & Ishiguro-Watanabe, M. KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res.* **51**, D587–d592. <https://doi.org/10.1093/nar/gkac963> (2023).
52. Kanehisa, M. Toward understanding the origin and evolution of cellular organisms. *Protein Sci.* **28**, 1947–1951. <https://doi.org/10.1002/pro.3715> (2019).

Acknowledgements

This work was supported by the key technology research project of the Changchun Key R&D Program under Grant [21ZGN16]; the National Key R&D Program of China under Grant [2021YFD2101003-2]; the Science and Technology Development Plan Program of Jilin Province under Grant [20220203096SF]. The authors thank the Personal Biotechnology Co., Ltd. (Shanghai, China) for assistance with the sequencing and bioinformatics analyses with the online analysis platform Personalbio GenesCloud (<https://www.genescloud.cn>).

Author contributions

Y.S., J.L., and X.Z. conceived and designed the experiments. Y.S., Y.C., Y.W., T.W., and Y.Z. performed the experiments. Y.S. analyzed the data. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-67881-2>.

Correspondence and requests for materials should be addressed to X.Z.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2024