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Immunomodulatory effect of nutrients on intestinal disorders and immunity

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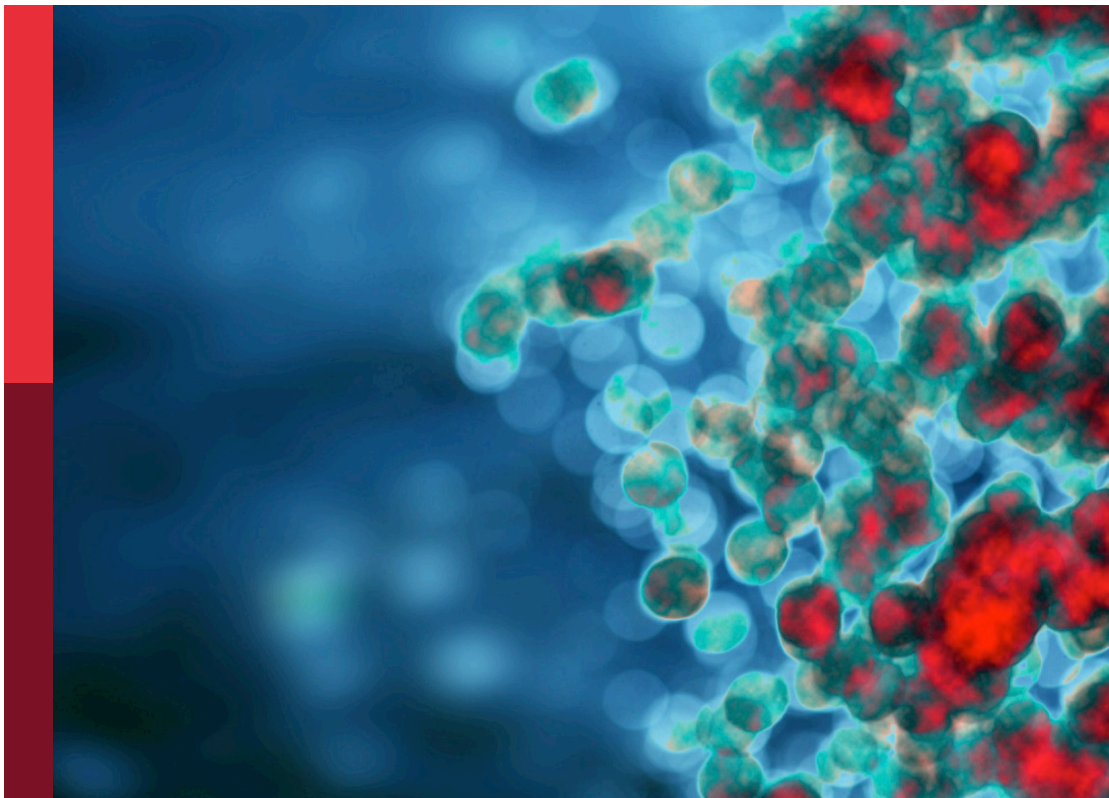

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Immunomodulatory effect of nutrients on intestinal disorders and immunity

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Editorial: Immunomodulatory effect of nutrients on intestinal disorders and immunity

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KEYWORDS

nutrient, intestinal disorders, immunomodulation, immune aging, metabolism

Editorial on the Research Topic
Immunomodulatory effect of nutrients on intestinal disorders and immunity

Nutrition and immunity have emerged as active areas of research within the field of basic nutrition science, representing an interdisciplinary discipline that bridges nutrition and immunology. While traditional immunology focuses on the immune system's defenses against exogenous substances, research on nutrition and immunity explores the relationship between dietary nutrients, dietary factors, nutritional status, and immune system function. Related research extends further, to examine the immune-mediated effects and interventions of nutrients on homeostasis and disease (1). Dietary nutritional components include macronutrients such as carbohydrates, fats, and proteins, micronutrients such as zinc (Zn) and iron (Fe), and dietary additives such as flavonoids and polyphenolic compounds. It's promising to conduct multidimensional studies to investigate the immunomodulatory functions and disease intervention effects of a variety of dietary nutritional components (2). It is imperative to elucidate the interactions between dietary components and the immune system, along with their intermediary roles in both health and disease. The identification of molecular mechanisms of nutritional immunoregulatory effects from a genetic perspective provides the foundation for multidisciplinary research, which aims to improve immune system function through nutritional interventions, ultimately promoting health and well-being.

The research on the exploration of the interactions between dietary components and gut microbiota is emphasized in the context of gut immunity, energy metabolism, and systemic immunity, which could systematically investigate the secretion and metabolic patterns of functional components like dietary functional factors, and nutrients, along with their health effects (3). For example, it is anticipated that research on infant immunity will be proposed to comprehensively examine the dynamic changes of nutrient human milk oligosaccharides (HMOs) and probiotics in breast milk during different stages of pregnancy, thereby elucidating their mechanisms in regulating gut immunity. Moreover, the bioactive mechanisms of dietary polyphenols on gut microbiota, intestinal homeostasis, and systemic inflammation are the intense field for dietary intervention.

The study is proposed to investigate the individual differences in metabolism and health effects of plant compounds. Focusing on energy metabolism and neurodegeneration, it is promising to evaluate the non-nutritional bioactivities and their efficacy in chronic disease

interventions. The interaction of gut metabolism and immune function requires the systematic investigation of the interaction mechanisms and health effects of dietary functional factors, such as prebiotics and probiotics (4, 5). It is helpful to elucidate the immunoregulatory functions of dietary components and their role in maintaining systemic health homeostasis (6). By exploring the regulatory effects and mechanisms of dietary polyphenols and probiotics on innate and adaptive immunity, research is establishing health intervention effects based on the interactions between gut metabolism and the host system. Research is also clarifying the metabolic differences and application characteristics of specific metabolites. Studies are aimed at examining the immune regulatory functions of dietary components in the context of chronic diseases and provide a systematic analysis of their immune-enhancing effects, offering a scientific basis and data support for dietary-based immune regulation and immunity-based precision interventions.

In light of the increasing concern about aging and aging related dysbiosis, it is crucial to focus on nutrient mediated intervention for healthy aging and the reversal of age-related diseases (7, 8). Moreover, immune aging drives the functional decline of multiple systems and organs, which is closely related to systemic inflammation, recurrent infections, and age-related diseases in elderly individuals. Thus, delaying it is essential to contribute to healthy aging (9). Studies have found that a diverse diet can help reverse immune aging. For instance, supplementation with 2'-fucosyllactose (2'-FL) can significantly ameliorate age-related osteoporosis, metabolic syndrome, and systemic oxidative stress (10). It is speculated that 2'-fucosyllactose targets the mechanism related to immune aging. In any case, it is both necessary and important to expand the fundamental research on immunology and to investigate the results of nutritional immunology to develop novel intervention strategies for health and disease by leveraging their immunomodulatory effect.

Author contributions

YW: Investigation, Resources, Writing – original draft. AL: Investigation, Writing – review & editing. YH: Conceptualization, Investigation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immunomodulation of nutritional formula containing epigallocatechin-3-gallate, ginseng extract, and polydextrose on inflammation and macrophage polarization

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Single nutrient likes polyphenol or dietary fiber have been exhaustively investigated to validate their positive intervention in health or disease. Meanwhile, the common interaction of inner systems with the nutrient complex has not been well elucidated, which raises the scientific issue of the modulatory effect of the nutrient complex on immunity. The representative prebiotics of epigallocatechin-3-gallate (EGCG), ginseng extract, and polydextrose (PDX) were selected on behalf of the classification of polyphenol, flavone or polysaccharides, and dietary fiber to generally cover the daily food intake in this study to explore their intervention in inflammation and macrophage polarization. The intervention of selected nutrients on inflammation and macrophage polarization has been evaluated against macrophages to unveil their comprehensive effects. The synergistic effect of selected nutrients was demonstrated by inhibiting M1 macrophage polarization and the promotion of M2 macrophage polarization. Then, the nutrient formula was set up to verify the intervention effect, and the results revealed the significant inhibition of cell inflammation and the effect on cell proliferation through promoting the cell cycle in the G2 phase. The nutrient complex could inhibit M1 macrophage polarization to inhibit M1-mediated inflammation and promote M2 macrophages for anti-inflammatory effect and enhance cell phagocytosis. Moreover, the varied intervention effects of the nutrient complex with different formulas could be summarized. In general, the formula containing EGCG, ginseng extract, and PDX was demonstrated to possess an enhanced immunomodulatory effect on cell inflammation and macrophage polarization, which could potentially inspire the investigation of complex nutrients in health and diseases.

KEYWORDS

EGCG, ginseng extract, polydextrose, nutrient complex, macrophage

1 Introduction

It has been widely accepted that varied nutrients are required for daily exercise to benefit the health and function of inner systems, including the immune system. Numerous studies have explored the intervention effect of individual nutrients on health or disease to report their positive effects (1–3). However, the common interaction of the inner systems with the nutrient

complex has not been well investigated, nor have its comprehensive effects on health or diseases, hence emphasizing the need for investigation. Thus, the scientific issue concerning the effect of the nutrient complex on macrophage phenotypes was raised to explore the enhanced immune function in this study. The distinct dietary patterns from the west to the east comprise varied nutrients, which increases the difficulty of research proposals to include different types of nutrients as exhaustively as possible. Meanwhile, the widely applied food types all over the world can be classified as representative health-beneficial nutrients or prebiotics such as polyphenols, flavones, and dietary fibers, which address the functionality of the representative food nutrients of tea polyphenols, ginseng, and polydextrose (PDX) that are consumed worldwide. Thus, the representative prebiotics of epigallocatechin-3-gallate (EGCG), ginseng extract, and PDX were selected on behalf of the classification of polyphenol, flavone or polysaccharides, and dietary fiber to generally cover the daily food intake worldwide. More importantly, the intervention effects of the selected nutrients have been stated with the identical effects of anti-oxidative stress, anti-inflammation, and immunity enhancement, etc.; in particular, a large number of studies have shown that these compounds have excellent functions in immune regulation, such as reducing the release of inflammatory factors from macrophages and potentially enlightening the enhanced intervention of their complex (4–6).

The novelty of tea polyphenols relies on the frequent daily intake around the world to elicit intervention on homeostasis and human health. As the most abundant polyphenol in green tea, EGCG has been thoroughly evaluated to identify its effect on inner homeostasis or the progression of various diseases. Previous reports revealed the alleviating effect of EGCG on inflammation-related chronic disease through regulating the process of lipid metabolism (7, 8). Other research unveiled the relieving effect of EGCG on colonic inflammation in a colitis mouse model (9). Relevant studies provided evidence concerning the autoxidation of EGCG and the mediated production of reactive oxygen species (10). The intervention effect of EGCG on macrophages has been reported in a study about acute lung injury, and the results indicated the inhibition of inflammatory macrophage phenotypes (11). Furthermore, the alteration of macrophage polarization from M1 to M2 phenotype was verified in non-alcoholic fatty liver disease (NAFLD) mice (12). Thus, the immune modulatory effect of EGCG has been raised in previous studies.

For selection of flavone resources, ginseng and its extract have been highlighted and focused on to analyze their dominant contents and beneficial effects. The wide application of ginseng as a functional food ingredient or traditional herbal medicine has addressed its beneficial effects on health through regulating inflammation, immunity, and oxidation, etc. To date, it has been developed as frequently ingested prebiotics in food to maintain the health of the inner systems. The published data have demonstrated the main actives of ginseng as ginseng flavone, ginsenosides, and ginseng polysaccharides. Their bio-activities have been widely investigated to report the inhibitory effect on varied diseases such as tumor and inflammation (13–15). The inhibition of ginsenoside on tumor angiogenesis and growth was concluded in an experiment on nude mice (16). Huang et al. reported the immune enhancement of ginseng polysaccharides through sensitizing the tumor immune therapy against the immune checkpoint (17). Yang et al. (18) have studied the intervention effect of ginseng extract on inflammation and oxidative stress in RAW264.7 cells and in a dextran sulfate sodium (DSS)-induced colitis mouse model, revealing that ginseng extract can exert both anti-inflammatory and anti-oxidative functions by targeting the MAPK/NF-κB and p62-Nrf2-Keap1 pathways, as well as autophagy, *in vitro* and *vivo*.

The alternative nutrient recommended for daily intake is dietary fiber, which has been verified to have a positive effect on gastrointestinal homeostasis. As a kind of soluble dietary fiber containing randomly bonded glucose polymer, PDX is thought to be a food prebiotic with impact on health and disease after digestion and fermentation by intestinal microbiota (19). Several cohort studies indicated the alleviating effect of PDX, from fecal bulk to soft stools, as the result of raising defecation frequency (20, 21). Luoto et al. (22) demonstrated the significant inhibitory effect of PDX on viral infection in the respiratory tract of infants. A previous study also revealed that PDX can modulate the gut microbiota and attenuate serum triglyceride and cholesterol levels in mice fed with a western diet for 14 days (23). Hu et al. (24) reported that PDX alleviated serum lipopolysaccharide (LPS) levels and macrophage infiltration in epididymal adipose tissue and resulted in macrophage polarization toward the M2 phenotype, as the result of preventing and treating obesity in high-fat-diet-fed mice, specifically in alleviating glucolipid metabolism disorders and adipose tissue inflammation.

Host immunity consists of two types of defensive strategies: innate and adaptive immunity. As the most studied and focused cells, macrophages are classified as monocytes generally originated from progenitors in bone marrows and identified as innate immune cells with high homogeneity and plasticity (25, 26). Macrophages are classified as different formats in organs and tissue and as recruited or resident cells to be involved the general immune defense or tissue reoccurrence. Macrophage polarization is considered a vital process to determine the functionality of macrophages under different conditions, with the classically activated M1 phenotypes and alternatively activated M2 macrophages. M1 and M2 macrophages play different roles in different processes through regulating different signaling pathways to finally show distinct or even opposite functions. Under a normal state, M1 macrophages are mainly responsible for the phagocytotic capacity to eliminate invaded pathogens or bacteria (27–29). Meanwhile, the inflammatory response is generally understood to potentially aggravate inflammatory diseases. M2 macrophages are believed to be evolved in parasite elimination, tissue remodeling, and allergic diseases (30). Thus, the modulation of macrophage polarization can produce varied biological effects in different microenvironments.

Herein, the nutrient formula, including the prebiotics of EGCG, ginseng extract, and PDX, was prescribed to simulate the daily intake of nutrients to reveal the intervention effect on macrophage polarization. The results demonstrated the positive intervention of the formula on inflammation by inhibiting the release of pro-inflammatory cytokines. More importantly, the immunomodulatory effect on macrophage polarization was validated.

2 Materials and methods

2.1 Materials

Ginseng extract (Ginsenosides ≥80%, designated as R) and EGCG (designated as E) with purity higher that 94% were purchased from Shanghai Novanat Co., Ltd. (Shanghai, China). PDX (designated as D)

with purity higher that 90% was purchased from Tate & Lyle Trading Co., Ltd. (Shanghai, China). The complex of selected nutrients was proposed based on the experimental design and designated as the combination of the abbreviation of the corresponding nutrient (ERD). Lipopolysaccharide (LPS) was purchased from Santa Cruz Biotechnology (California, United States). Cell Counting Kit-8 (CCK-8) reagent was purchased from Meilunbio (Dalian, China). Anti-mouse CD16/32, APC-conjugated anti-mouse F4/80, FITC-conjugated anti-mouse CD86, and PE-conjugated anti-mouse CD206 antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, United States) for flow cytometry analysis. Recombinant mouse IFN-γ and IL-4 were provided by Thermo Fisher Scientific. Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-1β analysis were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Neutral red was from sourced from Aladdin (Shanghai, China). An apoptosis kit was purchased from Thermo Fisher Scientific. All reagents used in this study were of analytical grade unless otherwise indicated.

2.2 Cell culture and treatment

The murine macrophage cell line RAW264.7 was from a laboratory collection and was cultured in a DMEM medium (Gibco, Grand Island, NY, United States) with a supplementation of 10% fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin-streptomycin (P/S, Gibco). Cells were seeded in 24-well plates at 3×10^5 cells/mL for the following treatment by nutrients with or without induction to M1 or M2 phenotypes by supplementing 100 ng/mL LPS and 20 ng/mL IFN-γ for 24 h or IL-4 to a final concentration of 20 ng/mL, respectively (31). Upon culturing or treatment with nutrients, all cells were incubated in a humidified incubator at 37°C with 5% CO_2 supplementation. Adherent cells were gently blown down with 5 mL of serum-free medium, collected by centrifugation at 300 × g for 5 min, and subjected to the following investigation.

2.3 Cell viability

Cell viability upon the incubation with EGCG, ginseng extract, or PDX was determined by utilizing CCK-8 solution to generally provide the value concerning the intervention concentration of the complexed nutrients (32). Generally, the RAW264.7 cells were cultured in a 96-well plate at (1×10^5 cells/mL) for 24 h and then incubated with different concentrations (0, 1, 5, 10, 20, 40, 60, 80, 100 μg/mL) of EGCG, ginseng extract, or PDX for 24 h. A blank control group was set up as the wells without cells. After washing with sterile PBS, fresh DMEM medium containing 10 μL of CCK-8 solution was added and incubated for another 2 h. The absorbance was measured at 450 nm (the data were read using SparkControl Magellan 3.1) by using a microplate reader (TECAN Infinite M200 Pro NanoQuant, Switzerland) to evaluate the survival rates of the RAW264.7 cells.

2.4 Cell apoptosis and cell cycle analysis

The intervention effect of the nutrient complex on cell apoptosis or cell cycles was determined by flow cytometry. In general, RAW264.7 cells were cultured in a 24-well plate at (3×10^5 cells/mL) for 24 h and then incubated with different concentrations of nutrient complex [ERD-I-H: (E: 2.2 μg/mL, R: 3.9 μg/mL, D: 100 μg/mL); ERD-I-M: (E: 1.1 μg/mL, R: 1.9 μg/mL, D: 50 μg/mL); ERD-I-L: (E: 0.22 μg/mL, R: 0.39 μg/mL, D: 10 μg/mL); ERD-II-H: (E: 7.5 μg/mL, R: 75 μg/mL, D: 100 μg/mL); ERD-II-M: (E: 3.8 μg/mL, R: 37.6 μg/mL, D: 50 μg/mL); and ERD-II-L: (E: 0.75 μg/mL, R: 7.5 μg/mL, D: 10 μg/mL)] for 24 h. A blank control group was set up as the well without nutrient complex supplementation. Cells were then scrapped and collected for the staining with annexin V and propidium iodide (PI) for cell apoptosis analysis. Cell cycle analysis was accomplished by following the well-established protocol (33). Cells were firstly fixed with 75% ethanol and then stained with PI with the presence of RNaseA (20 μg/mL) in 60 min. Cells were then analyzed with BD FACSDiva Software.

2.5 Cytokine analysis

The concentrations of TNF-α and IL-1β in the cell culture supernatant were determined with commercial ELISA kits by following the guidelines provided by manufacturer. The standard curve was determined for each test. The absorbance value at 450 nm (the data were read using SparkControl Magellan 3.1) was measured with a microplate reader (TECAN Infinite M200 Pro NanoQuant, Switzerland).

2.6 Flow cytometry analysis

RAW264.7 cells were collected after centrifugation at 300 × g for 5 min. Then, the cells were resuspended in 100 μL of cold PBS and pre-incubated with anti-mouse CD16/32 antibody on ice for 10 min to block and prevent non-specific binding with Fc receptors. The cells were washed with PBS by centrifuging at 300 × g for 5 min at 4°C and incubated with APC-conjugated anti-mouse F4/80 and FITC-conjugated anti-mouse CD86 at 4°C for 30 min for staining of M1 macrophages with vortex every 15 min. Excess antibody was removed by washing the cells with cold PBS. The cells were permeabilized in fixation/permeabilization solution (BD Bioscience) for 20 min at 4°C and rinsed in BD perm/wash buffer (BD Bioscience) by centrifuging at 400 × g for 5 min at 4°C. Then, the cells were incubated with PE-conjugated anti-mouse CD206 at 4°C for 30 min to effectively label cells of M2 phenotypes. Excess antibody was removed by washing the cells with cold PBS, and the cells were collected and suspended in 500 μL PBS. Flow cytometric analysis was performed with a BD Symphony A1 (BD, New Jersey, United States) to acquire 10,000 cells in each group for analysis. The data were analyzed with FlowJo software (34).

2.7 Macrophage phagocytosis

The phagocytic ability of RAW264.7 macrophages after treating with nutrient complex was determined by the neutral red assay (35, 36). Briefly, the cells seeded in 96-well plates (1×10^5 cells/mL) were stimulated into M1 phenotype and treated with different doses of the nutrient complex (ERD-I-H: (E: 2.2 μg/mL, R: 3.9 μg/mL, D: 100 μg/mL); ERD-I-M: [E: 1.1 μg/mL, R: 1.9 μg/mL, D: 50 μg/mL); ERD-I-L: (E: 0.22 μg/mL, R: 0.39 μg/mL, D: 10 μg/mL); ERD-II-H: (E: 7.5 μg/

mL, R: 75 μg/mL, D: 100 μg/mL); ERD-II-M: (E: 3.8 μg/mL, R: 37.6 μg/mL, D: 50 μg/mL); and ERD-II-L: (E: 0.75 μg/mL, R: 7.5 μg/mL, D: 10 μg/mL)] for 24 h simultaneously. The control group was set up as the cells without treating with the nutrient complex. The baseline was determined by setting up the well without cultured cells. After 2 h-incubation with 100 μL of 0.1% neutral red solution, the remaining fraction was removed after washing with ice-cold PBS, and 200 μL of cell lysis buffer (glacial acetic acid: anhydrous acetic acid = 1:1) was added to wells to allow the complete lysis of cells during the following 2 h at room temperature. The absorbance value of the cell lysate was measured at 540 nm (the data were read using SparkControl Magellan 3.1) with a microplate reader (TECAN Infinite M200 Pro NanoQuant, Switzerland).

2.8 Statistical analysis

All data were shown as mean ± standard deviation (SD). A two-tailed, unpaired t-test was used to compare the significance of the two groups. One-way ANOVA followed by a Dunnett test (alpha value = 0.05) was used to determine the statistical significance in multiple comparisons. p-value ≤0.05 was considered statistically significant and indicated with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). Statistical analysis was conducted by using GraphPad Prism 7.0 software (GraphPad software, La Jolla, CA, United States).

3 Results

3.1 Effect of single nutrients on cell viability and inflammation

Single nutrients of EGCG, ginseng extract, and PDX have been widely validated as supplements to enhance internal homeostasis or maintain health. In order to verify the intervention effect of the complex of these nutrients, cell viability was assessed as the first step to provide research evidence for the concentration set-up. After treatment of RAW264.7 cells with the corresponding nutrient, cell viability was determined by CCK-8 analysis. As shown in Figure 1A, the result indicated the cytotoxicity of EGCG with a concentration higher than 20 μg/mL. Meanwhile, ginseng extract and PDX were

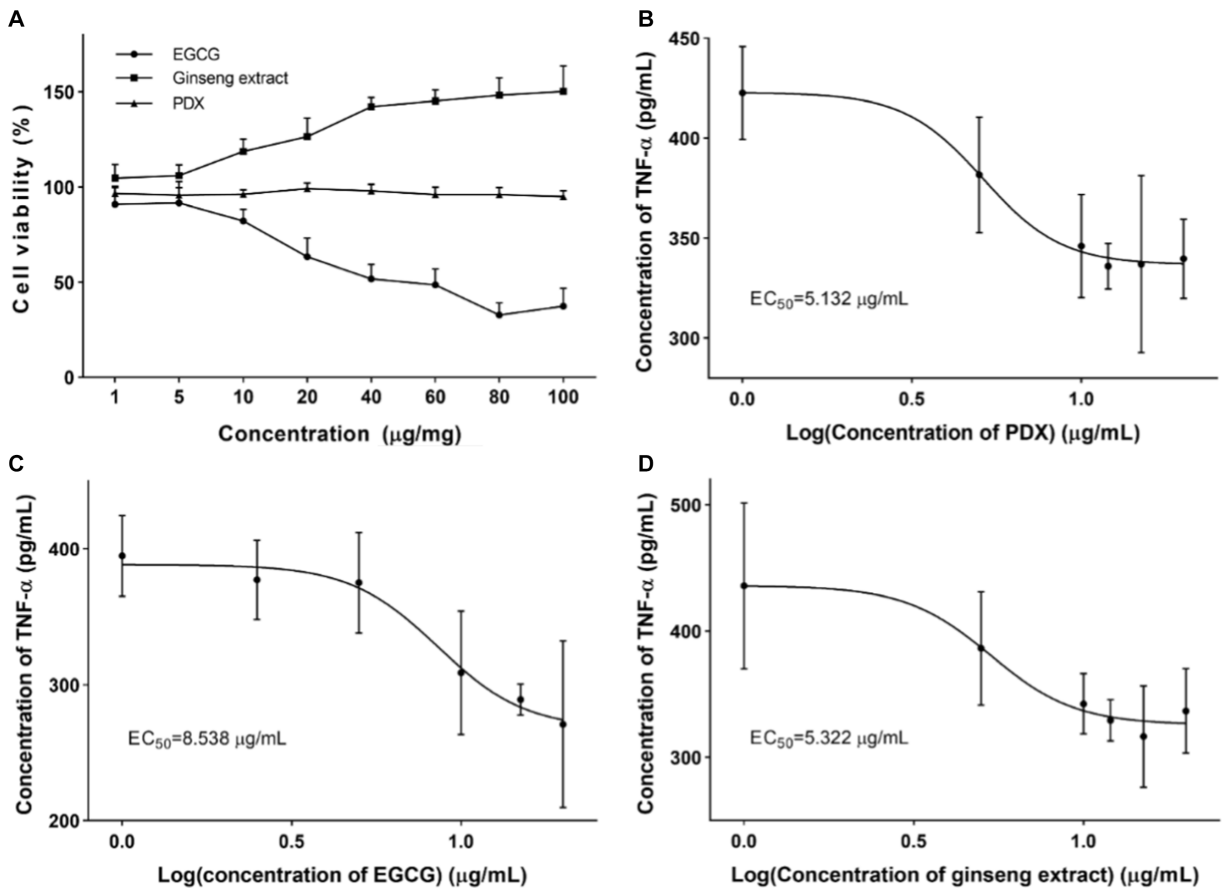

FIGURE 1
Cell viability and intervention on cell inflammation after treating with corresponding nutrient. **(A)** Effect of EGCG, ginseng extract, and PDX on cell viability was determined after treating RAW264.7 cells with corresponding nutrient to provide data for initial ratio determination. **(B)** Effect of EGCG on the level of TNF-α in cultural supernatant was determined after treating RAW264.7 cells with varied concentration of EGCG. **(C)** Effect of ginseng extract on the level of TNF-α in cultural supernatant was determined after treating RAW264.7 cells with varied concentration of ginseng extract. **(D)** Effect of PDX on the level of TNF-α in cultural supernatant was determined after treating RAW264.7 cells with varied concentration of PDX.

demonstrated to not have a significant effect on cell viability, and ginseng extract was observed to have an effect of promoting cell proliferation in a dose-dependent manner. PDX was verified to not have a significant effect on cell viability even at the concentration of 100 μg/mL. The intervention of different nutrients on cell inflammation was assessed by determining the level of TNF-α after treating LPS-stimulated RAW264.7 with the corresponding nutrient, and the concentration corresponding to half of the effect (EC_{50}) was analyzed to provide an initial formula of the complex. The results revealed the dramatic decrease of TNF-α after treating with different nutrients, which indicated the intervention of different nutrients on cell inflammation. EC_{50} was calculated for each nutrient and was used as the initial formulation (Figures 1B–D). The initial nutrient complex was set based on the EC_{50} of individual component as EGCG: ginseng extract: PDX = 1.67: 1.04: 1, which will be used for the initial evaluation of the enhanced effect of the nutrient complex.

3.2 Synergistic effect of nutrients

In order to verify the synergistic effect of nutrients or the complex on immunity, the analysis against macrophage polarization was proposed to verify the effect. The intervention on cell polarization was finalized after stimulating RAW264.7 cells with either IFN-γ and LPS or IL-4 to promote M1 or M2 macrophages, respectively. RAW264.7 cells were then cultured and treated with a certain concentration of nutrients or complex (EGCG: 20 μg/mL, ginseng extract: 12.5 μg/mL, PDX: 12 μg/mL) by following the experimental design. Cells were then applied for macrophage polarization analysis after labeling the corresponding cluster of differentiation (CD) with corresponding antibodies to indicate the percentage of M1 ($F4/80^+$ $CD86^+$) or M2 macrophages ($F4/80^+$ $CD206^+$). As shown in Figure 2A, the percentage of macrophages with M1 phenotypes was dramatically decreased after treating with nutrient complex, and the enhanced inhibitory effect of the

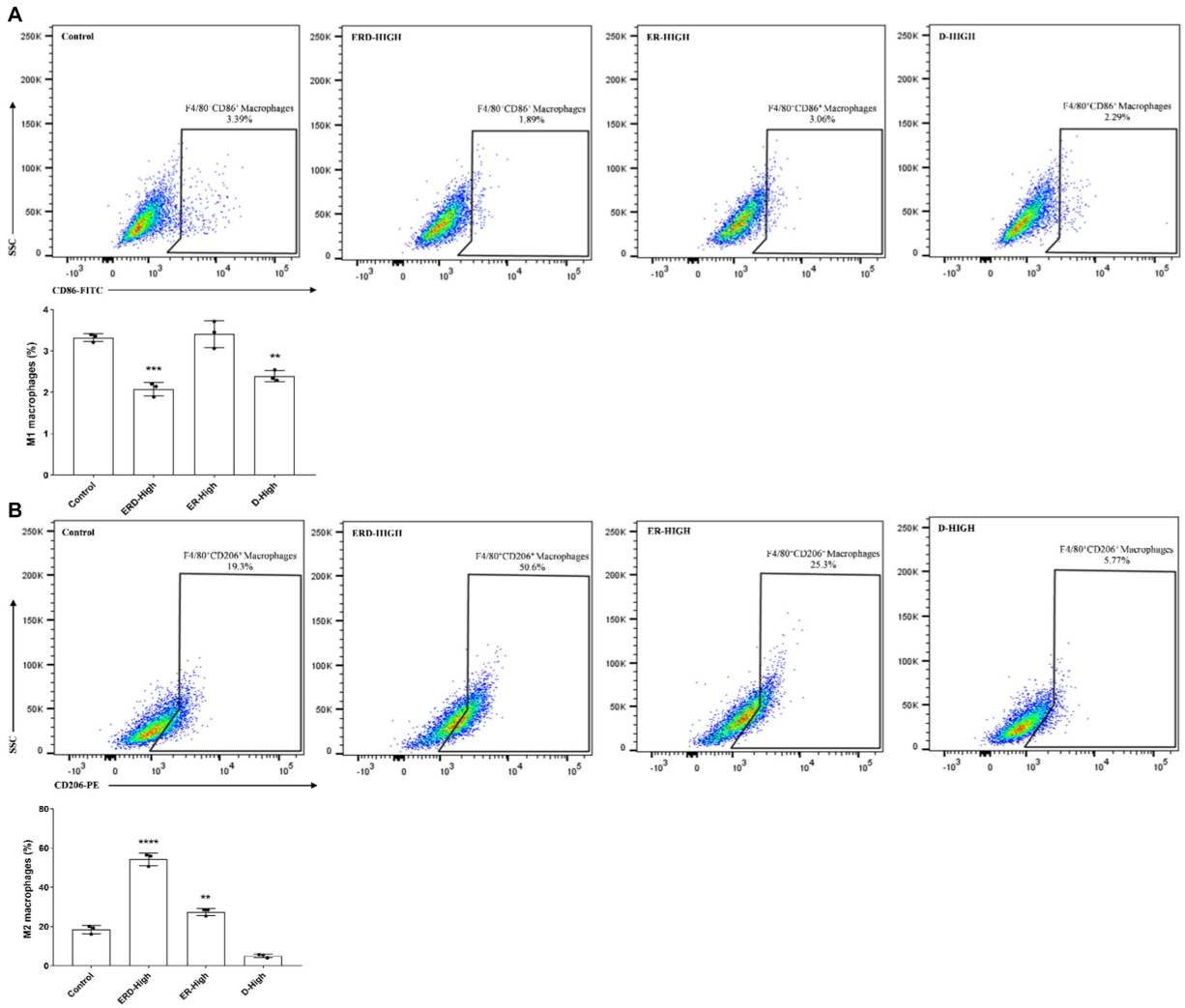

FIGURE 2
Synergistic effect of nutrients. **(A)** Effect of nutrient complex on M1 macrophage polarization was determined by flow cytometry analysis of RAW264.7 cells after treating with certain concentration of nutrients. The synergistic effect was verified by comparing the groups of the nutrient complex with single or paired nutrient groups to address the effect of PDX. **(B)** Effect of nutrient complex on M2 macrophage polarization was determined by flow cytometry analysis of RAW264.7 cells after treating with certain concentration of nutrients. The synergistic effect was verified by comparing the groups of the nutrient complex with single or paired nutrient groups to address the effect of PDX.

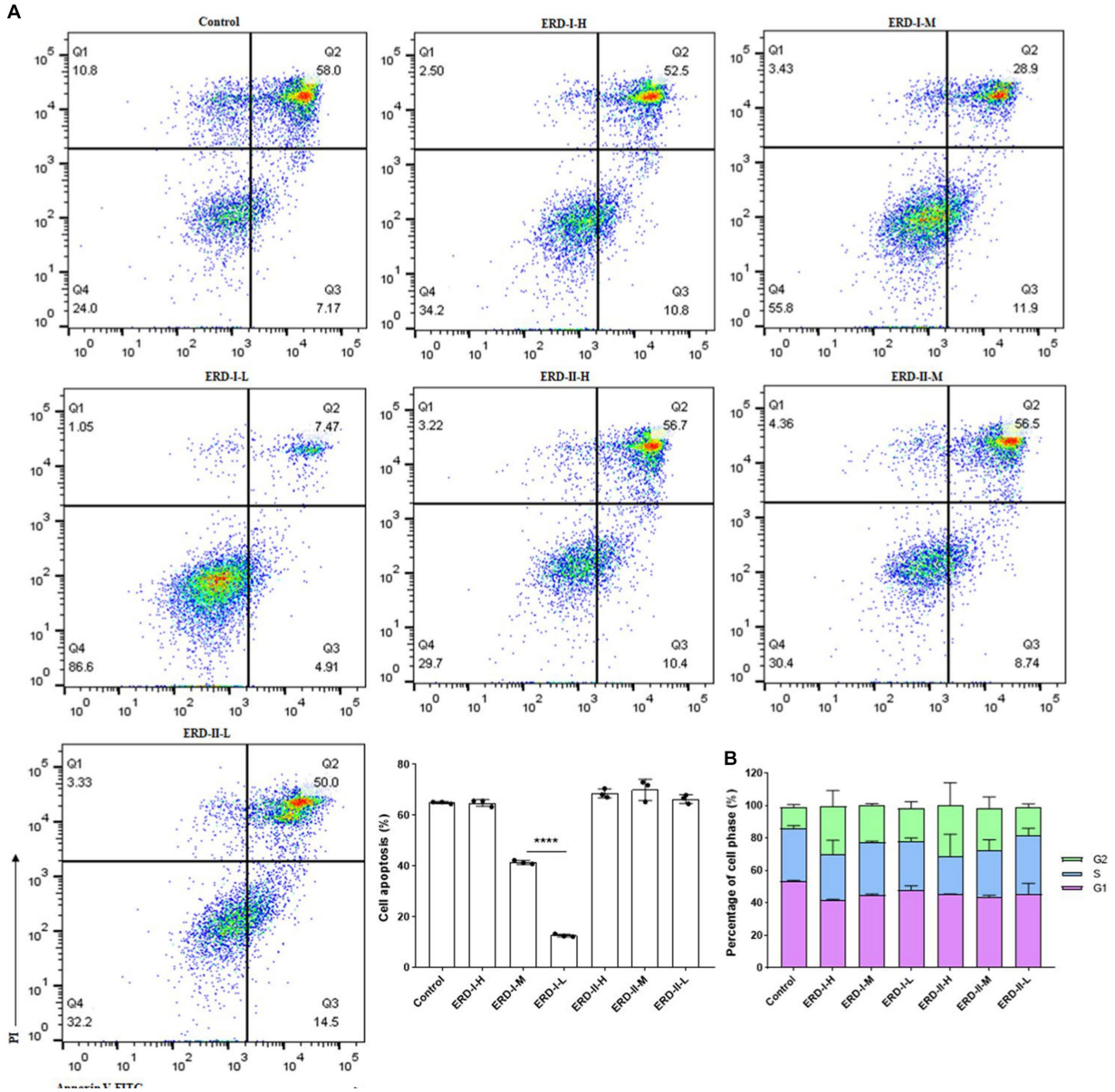

FIGURE 3
Effect of the nutrient complex on cell apoptosis and cycle. **(A)** Effect of nutrient formulas on cell apoptosis was determined by treating RAW264.7 cells with certain concentrations of formulas I and II. The significant inhibition of nutrient formulas on cell apoptosis could be summarized for formula I in a dose-dependent manner, whereas no significant effect of formula II was observed. **(B)** Effect of nutrient formulas on cell cycles was determined by treating RAW264.7 cells with certain concentrations of formulas I and II. The significant promotion of nutrient formulas on cell cycles to the G2 phase could be summarized for formulas I and II in a dose-dependent manner in general.

complexed nutrients was demonstrated by comparing with the groups of ER or D, which emphasized the contribution of PDX. The percentage of M2 macrophage was significantly raised (Figure 2B), which indicated the promotion of nutrient complex on M2 macrophages and laterally revealed the inhibition of the nutrient complex on inflammation. Meanwhile, the enhanced effect on M2 macrophage polarization was observed by comparing the results from the nutrient complex with that of single or double nutrients as well. In general, the synergistic effect of nutrients was summarized by analyzing the intervention effect of the nutrient complex on macrophage polarization.

3.3 Effect of the nutrient complex on cell apoptosis and cycle

Based on the results concerning the synergistic effect of nutrients, two alternative formulas of the nutrient complex with the ratio of EGCG: ginseng extract: PDX = 1: 1.79: 46.16 (formula I coined ERD I) and 1: 10: 13.3 (formula II coined ERD II) were set up to highlight the effect of PDX in ERD I and the effect of both ginseng extract and PDX in ERD II. Then, the intervention effect of these nutrient complexes on cell growth or proliferation was analyzed. The concentration was set up to avoid the cytotoxicity of EGCG and

classified as the concentration of PDX: high for 100 μg/mL, medium for 50 μg/mL, and low for 10 μg/mL. The concentration of EGCG and ginseng extract was calculated from the value of PDX based on the defined corresponding formula. Cell apoptosis was analyzed by following the steps described above, and the effect on cell cycles was determined by performing flow cytometry. The result indicated that formula I inhibited the cell apoptosis dramatically, whereas no significant effect of formula II was observed (Figure 3A), which indicated the significant contribution of PDX on cell promotion, and the increased concentration of ginseng extract was considered to contribute negatively. Cell cycle analysis revealed the promotion of both formulas on cells in the G2 phase, which unveiled the promotion of both formulas on cell proliferation (Figure 3B) to maintain the cells in the phase of reproduction, thus potentially contributing to self-renewal capacity.

3.4 Effect of the nutrient complex on inflammation-related cytokine

The effect of formulas I and II on cell inflammation was reflected by determining the level of inflammation-related cytokines, namely, TNF-α and IL-1β, to indicate the inflammation status of cells. RAW264.7 cells were firstly stimulated with LPS and IFN-γ to establish an inflammatory model and then treated with different concentrations of the nutrient complex. The level of corresponding cytokines was determined by ELISA with commercial kits, and the results revealed a significant inhibition on the level of TNF-α (Figure 4A) and IL-1β (Figure 4B) upon treatment with a corresponding nutrient formula. The intervention effect of the nutrient complex on the corresponding cytokines was determined in a dose-dependent manner in general. The results revealed no significant variation of the two formulas on the level of cytokines, which demonstrated the intervention of the formulas on cell inflammation.

3.5 Effect of the nutrient complex on macrophage polarization

The effect of the nutrient complex on macrophage polarization was analyzed with flow cytometry by following the steps described above. In general, cells were stimulated to the M1 phenotype macrophage after incubation with IFN-γ and LPS, with M2 macrophage stimulated with IL-4. Then, cells were treated with different concentrations of formulas I and II. The percentage of M1 and M2 macrophages were quantified, and the results revealed the significant inhibition of both formulas on M1 polarization, which indicated the intervention of the nutrient complex on inflammation-related macrophages (Figure 5A). Meanwhile, both formulas could promote the polarization of macrophages to the M2 phenotype to exhibit an anti-inflammation effect (Figure 5B).

3.6 Effect of the nutrient complex on macrophage phagocytosis

Phagocytotic capacity represents the ability of macrophages in innate immune defense. In order to verify the intervention effect of the nutrient complex on macrophage-mediated immune defense, cell phagocytosis was analyzed by using neutral red assay. After the treatment of cells with different formulas of the nutrient complex, cells were applied for neutral red assay. The phagocytotic capacity was determined after recording the absorbance value. As shown in Figure 6, the results indicated the intervention of both formulas on phagocytosis to reveal the increased phagocytosis after treatment with both formulas, though no significance was observed. Meanwhile, the enhanced effect of formula I was observed by comparing with the control or formula II groups, which suggested the better intervention of formula I to address the functionality of PDX rather than the increased concentration of ginseng extract.

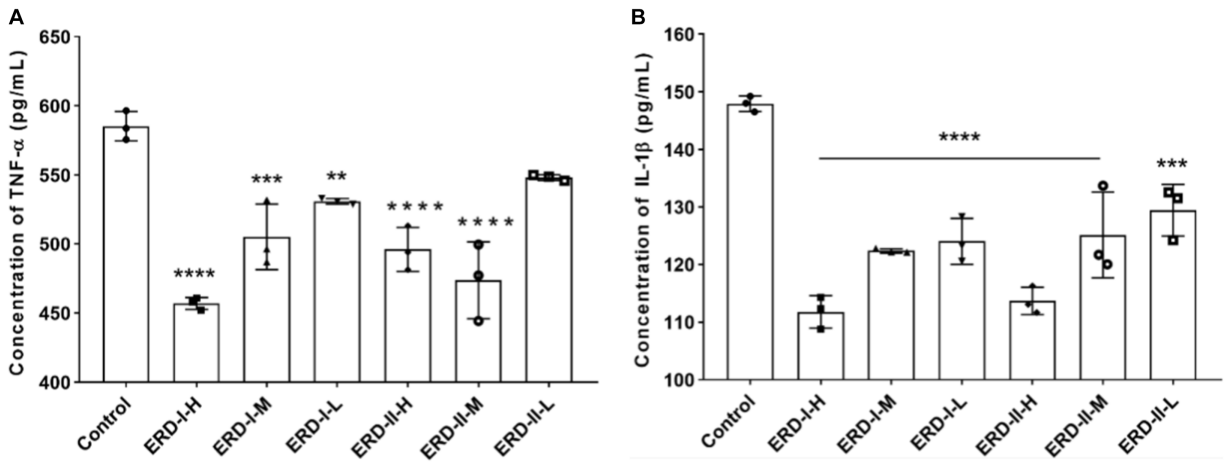

FIGURE 4
Effect of nutrient formulas on secretion of inflammation-related cytokines. **(A)** Effect of nutrient formulas on the level of TNF-α in cultural supernatant was determined after treating RAW264.7 cells with certain concentrations of formulas I and II. **(B)** Effect of nutrient formulas on the level of IL-1β in cultural supernatant was determined after treating RAW264.7 cells with certain concentrations of formulas I and II.

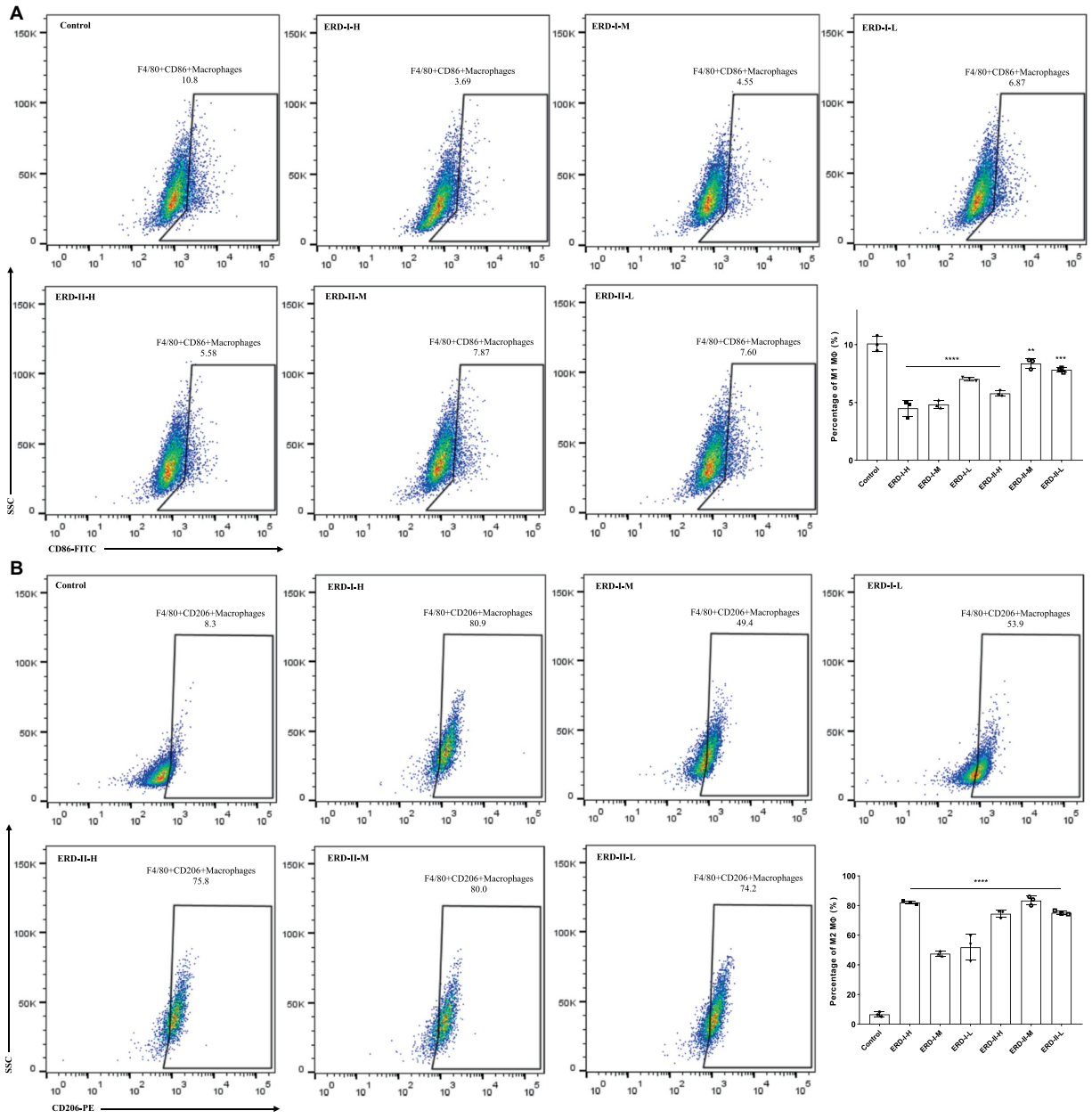

FIGURE 5
Effect of nutrient formula on macrophage polarization. **(A)** Effect of nutrient formulas on the macrophage polarization to M1 phenotype was determined by flow cytometry analysis of RAW264.7 cells after treating with certain concentrations of nutrient formulas I and II. **(B)** Effect of nutrient formula on the macrophage polarization to M2 phenotype was determined by flow cytometry analysis of RAW264.7 cells after treating with certain concentrations of nutrient formulas I and II.

4 Discussion

The daily intake of varied types of food nutrients is recommended to fulfill the requirement of health maintenance (37). This general investigation has explored the intervention of single nutrients on health and disease from distinct aspects to validate their bioactivities or provide recommended ingestion (38, 39). However, the complex formula including varied types of nutrients has not been reported thoroughly to indicate its comprehensive effect or synergistic contribution on certain healthy states. Thus, we have proposed to evaluate the complex effect of the nutrients EGCG, ginseng extract, and PDX as the representatives of polyphenol, flavone or polysaccharides, and dietary fiber to generally cover the daily food intake. The comprehensive effect has been evaluated by determining the bioactivities of the complex formula on cell viability, inflammation, or macrophage polarization. The results are expected to provide data to inspire following investigations on complex formulas or indicate the supplementation of corresponding nutrients in foods.

The distinct bioactivities of selected nutrients were observed by determining cell viability after treating with corresponding nutrients individually to indicate the maintained or promoted effect of PDX and ginseng extract, respectively. Meanwhile, the significant effect on cell

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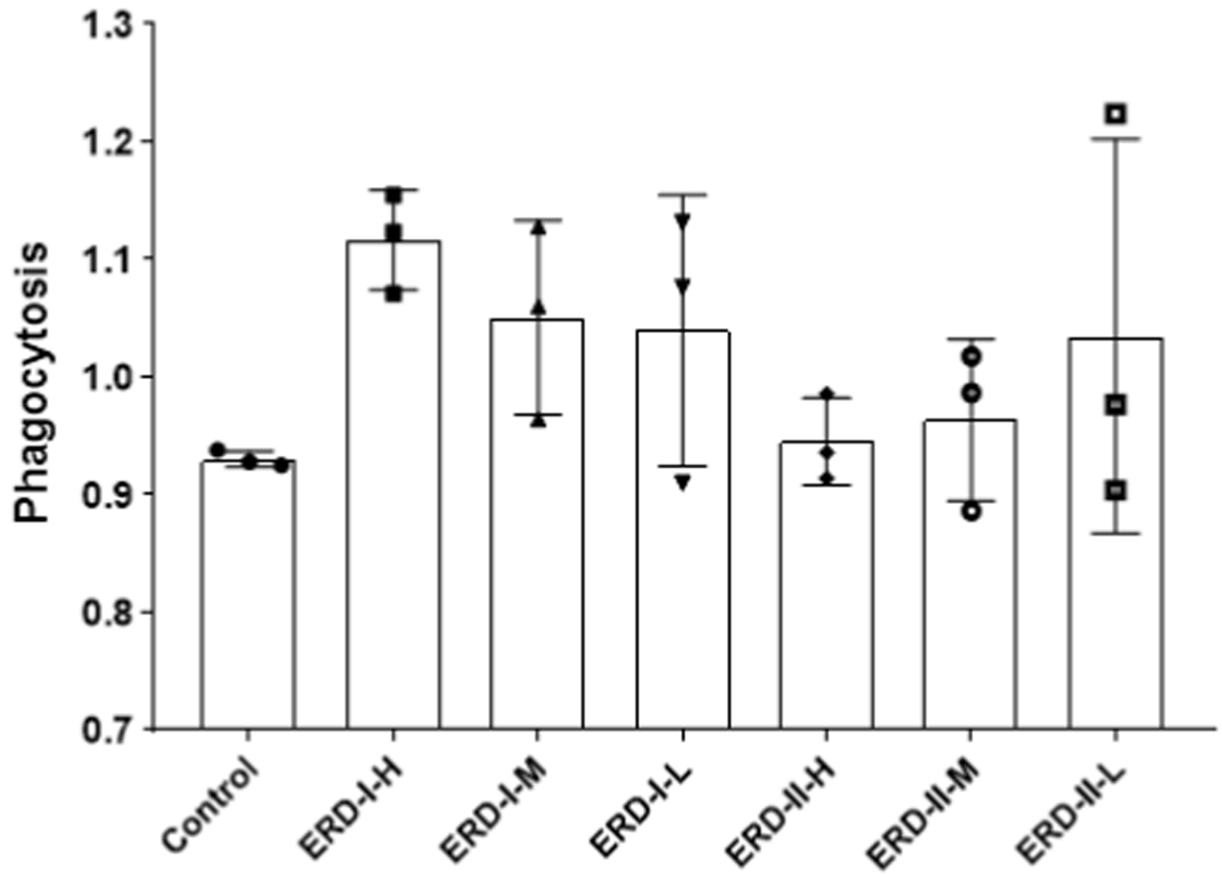

FIGURE 6
Effect of nutrient formulas on macrophage phagocytosis. Cells were treated with certain concentrations of nutrient formulas of I and II and then analyzed by neutral red assay.

viability was visualized upon exposure to a high concentration of EGCG. Thus, the recommended dosage for each nutrient was determined based on the effect of EGCG to form the initial formula of the nutrients for baseline investigation on cell inflammation or functionalization. The results indicated the synergistic effect of the fixed ratio on certain cell activities of macrophage polarization to M1 or M2 phenotypes, which reflected the enhanced efficiency of the formula on the promoted effect on M2 polarization to indicate the anti-inflammatory effect. Thus, it is evident that the complexed nutrient could potentially enhance the intervention effect.

Moreover, the nutrient complex with different formulas could possibly exhibit varied intervention effects. Thus, the alternative ratio of selected nutrients has been designed to emphasize the contents of either PDX (ERD I) or ginseng extract (ERD II) to exhaustively explore the intervention of the defined formula. The results revealed the distinctive effect of the different formulas on cell apoptosis or cycles and the effect on macrophage polarization. It was revealed that ERD I showed a dramatically inhibitory effect on inflammation, cell apoptosis and M1 polarization, which demonstrated an enhanced effect upon ingestion of raised level of PDX by comparing with the formula with a decreased ratio of PDX and increased concentration of ginseng extract. The effect of different formulas on the expression level of the inflammation-related cytokines TNF-α and IL-1β has verified the reinforced impact of ERD I. Thus, it was demonstrated that the complex formula with enhanced ingestion of PDX is expected to maximize the beneficial effect of the complex formula to potentially indicate the supplementation in food manufactures. The data are expected to inspire future research and application to focus on not only the selected nutrients in complex formulas but also the ratio of each nutrient.

5 Conclusion

Due to the necessity to explore the comprehensive effects of nutritional formulas on either health or disease, the representative prebiotics of EGCG, ginseng extract, and PDX were selected on behalf of the classification of polyphenol, flavone or polysaccharides, and dietary fiber in this study to generally cover the daily food intake to reveal the intervention effects on cell inflammation and macrophage polarization *in vitro*. It was demonstrated that the nutrient complex produced a synergistic effect on certain cell activities of inhibition on M1 macrophage polarization and promotion on M2 macrophage polarization. The intervention effect was then verified by treating cells with two fixed formulas, which revealed the inhibition on M1 macrophage polarization and M1-mediated inflammation and promotion on M2 macrophages for anti-inflammatory effect, as well as a generally enhanced effect on cell phagocytosis. In general, the

formula containing EGCG, ginseng extract, and PDX was demonstrated to possess an enhanced immunomodulatory effect on cell inflammation and macrophage polarization. The results are expected to provide research evidence for future supplementation in food with prescribed functionality or inspire further research on the effect of nutritional formulas on health and diseases.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

YW: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. YH: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. ZN: Resources, Writing – review & editing. XZ: Methodology, Writing – review & editing. DF: Investigation, Writing – review & editing. XJ: Methodology, Writing – review & editing. HL: Methodology, Writing – review & editing. SW: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. YZ: Resources, Writing – review & editing.

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Conflict of interest

ZN and YZ was employed by Shanghai M-Action Health Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Arginine alleviates *Clostridium perfringens* α toxin-induced intestinal injury *in vivo* and *in vitro* via the SLC38A9/mTORC1 pathway

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Introduction: *Clostridium perfringens* α toxin is a main virulence factor responsible for gut damage in animals. Arginine is a functional amino acid exhibiting significant immunoregulatory activities. However, the effects and immunoregulatory mechanisms of arginine supplementation on α toxin-induced intestinal injury remain unclear.

Methods: *In vivo*, 256 male Arbor Acres chickens were randomly assigned to a 2×2 factorial arrangement, involving diet treatments (with or without 0.3% arginine supplementation) and immunological stress (with or without α toxin challenge). *In vitro*, IEC-6 cells were treated with or without arginine in the presence or absence of α toxin. Moreover, IEC-6 cells were transfected with siRNA targeting mTOR and SLC38A9 to explore the underlying mechanisms.

Results and discussion: The results showed that *in vivo*, arginine supplementation significantly alleviated the α toxin-induced growth performance impairment, decreases in serum immunoglobulin (Ig)A and IgG levels, and intestinal morphology damage. Arginine supplementation also significantly reduced the α toxin-induced increase in jejunal proinflammatory cytokines interleukin *(IL)-1β*, *IL-6* and *IL-17* mRNA expression. *Clostridium perfringens* α toxin significantly decreased jejunal mechanistic target of rapamycin *(mTOR)* and solute carrier family 38 member 9 (*SLC38A9)* mRNA expression, while arginine supplementation significantly increased *mTOR* and *SLC38A9* mRNA expression. *In vitro*, arginine pretreatment mitigated the α toxin-induced decrease in cell viability and the increase in cytotoxicity and apoptosis. Arginine pretreatment also alleviated the α toxin-induced upregulation of mRNA expression of inflammation-related cytokines *IL-6*, C-X-C motif chemokine ligand (*CXCL*)*10*, *CXCL11* and transforming growth factor-β (*TGF-β*), as well as apoptosis-related genes B-cell lymphoma-2 associated X protein (*Bax*), B-cell lymphoma-2 (*Bcl-2*), B-cell lymphoma-extra large (*Bcl-XL*) and cysteinyl aspartate specific proteinase 3 (*Caspase-3*) and the ratio of *Bax* to *Bcl-2*. Arginine pretreatment significantly increased the α toxin-induced decrease in *mTOR*, *SLC38A9*, eukaryotic translation initiation factor 4E (eIF4E)-binding

protein 1 (*4EBP1*) and ribosomal protein S6 kinase (*S6K*) mRNA expression. Knockdown SLC38A9 and mTOR largely abrogated the positive effects of arginine pretreatment on α toxin-induced intracellular changes. Furthermore, SLC38A9 silencing abolished the increased *mTOR* mRNA expression caused by arginine pretreatment. In conclusion, arginine administration attenuated α toxin-induced intestinal injury *in vivo* and *in vitro*, which could be associated with the downregulation of inflammation via regulating SLC38A9/mTORC1 pathway.

KEYWORDS

arginine, *Clostridium perfringens* α toxin, intestinal inflammation, cell apoptosis, SLC38A9/mTORC1 pathway, broiler chickens, IEC-6 cells

Introduction

Clostridium perfringens, a Gram-positive, anaerobic, opportunistic pathogen, is ubiquitous in diverse environments, including in soil, sewage, and the gastrointestinal tracts of humans and animals (1). *C. perfringens* has been implicated in a wide range of serious diseases in muscle, the gastrointestinal tract, and other organs and tissues. These disorders include gas gangrene, necrotizing enteritis, enterotoxemia, foodborne or non-foodborne poisoning of diarrhea, and necrotic enteritis (2). Necrotic enteritis is a gastrointestinal disease in poultry, exhibiting in two forms: acute and chronic. The acute form is characterized by anorexia, severe morbidity, and substantial mortality. The chronic form is marked by slowed growth performance, lowered feed efficiency, and lethargy (3). *C. perfringens* α toxin is recognized as a key virulence factor in the pathogenesis of avian necrotic enteritis (4–6). This toxin exhibits phospholipase C and sphingomyelinase activities, resulting in extreme cytotoxicity and myotoxicity. Furthermore, it can induce hemolysis, necrosis, damage to cell membranes, the generation of superoxide radicals and inflammatory cytokines (7–10).

L-arginine is a functional amino acid in both humans and animals, serving as an important precursor for the synthesis of nitric oxide, polyamines and creatine during immune responses. Numerous studies have highlighted the significant benefits of L-arginine in preserving intestinal health across various disease models (11–13). Zheng et al. (13) reported that arginine supplementation exerted beneficial effects on the intestine by regulating arginine metabolism and reducing inflammatory cytokine expression in piglets under oxidative stress. Lan et al. (11) discovered that L-arginine effectively alleviated intestinal inflammation induced by lipopolysaccharide by inhibiting the TLR4/NF-κB and MAPK pathways while enhancing β-Defensin expression both *in vivo* and *in vitro*. Moreover, our previous study demonstrated that dietary arginine supplementation alleviated serum arginine depletion induced by *C. perfringens* challenge, restored normal arginine transport and catabolism in the intestine, mitigated the inflammatory response, and suppressed the JAK-STAT signaling pathway in broiler chickens, thereby safeguarding their intestinal health (14).

Mechanistic target of rapamycin complex 1 (mTORC1) is a conserved multi-protein complexes sensitive to changes in intracellular levels of amino acids, glucose and lipids, as well as extracellular stimuli such as cytokines, growth factors, and Toll-like receptors (15). It plays a critical role in regulating essential physiological functions, including protein synthesis, cellular metabolism, immune responses, inflammatory reactions, and apoptosis (16, 17). Arginine is known to promote diverse physiological effects largely through the activation of mTORC1 (18–20). Recent discoveries have identified solute carrier family 38 member 9 (SLC38A9) as an arginine sensor located upstream of mTORC1, thereby positively regulating the mTORC1 signaling pathway (21–23). Through the activation of mTOR, arginine triggers its downstream targets, including eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) and ribosomal protein S6 kinase (S6K), which play key roles in protein synthesis (24, 25) and the modulation of intestinal inflammation (26). However, whether the SLC38A9/mTORC1 signaling pathway plays an important role in the protective effects of arginine against α toxin challenge remains unclear.

Therefore, this study was conducted to determine the effects of arginine administration on intestinal injury induced by the α toxin challenge *in vivo* and *in vitro*. Further, the possible immunoregulatory mechanism based on the SLC38A9/mTORC1 pathway was explored. This research contributes to the understanding of nutritional interventions for enhancing host resilience to pathogenic challenges.

Materials and methods

Experimental design and animals

The experiments were approved by the Animal Care and Use Committee of Qingdao Agricultural University (No. DKY20220905). The experimental design followed a 2×2 factorial arrangement. A total of 256 one-day-old male Arbor Acre broilers

with similar initial body weight were selected and categorized into four groups. The groups were as follows: CON group, birds fed a basal diet without α toxin challenge; ARG group, birds fed a basal diet supplemented with 0.3% arginine without α toxin challenge; ATX group, birds fed a basal diet and subjected to α toxin challenge; ARG+ATX: birds fed a basal diet supplemented with 0.3% arginine and subjected to α toxin challenge. Each group was divided into 8 replicates, each containing 8 chickens. At 15, 17, 19 and 21 days of age, the birds were intraperitoneally injected with α toxin (Sigma, P7633; in groups ATX and ARG+ATX) at a dosage of 0.1 U/kg of body weight or an equal volume of phosphate-buffered saline (PBS; in groups CON and ARG). The experimental diet was made in mash form and formulated in accordance with the China feeding standard of chicken (NY/T 33-2004). The diet composition and nutritional levels are provided in Supplementary Table 1. L-arginine with a purity of 98.5% was purchased from CJ CheilJedang Corporation in South Korea, while L-alanine with a purity of 98% was obtained from Anhui Huaheng Biotechnology Co., Ltd. The room temperature was maintained at 33°C for the first week and gradually decreased to 26°C in the third week. The relative humidity was controlled at 50%~70%. The experiment was conducted using a three-tier cage system with 23 h of light and 1 h of darkness each day. The broiler chickens had ad libitum access to feed and water. Standard management and immunization procedures were employed throughout the experimental period.

Growth performance

On d 14 and d 21, feed consumption and body weight for each replicate were assessed. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated for the periods from d 1 to 14 and from d 15 to 21.

Sample collection

On the 21 d of age in broiler chickens, 3 h after toxin injection, one chicken was randomly selected from each replicate. Aseptic blood samples were drawn from the bird's wing veins, and serum was obtained by centrifugation for 10 min at 3000 r/min at 4°C. The serum was kept for further analysis at -20°C. Subsequently, the chicken was euthanized by exsanguination from the neck vein, and the intestines were isolated. A small section from the midsection of the jejunum was collected and fixed in a 4% paraformaldehyde solution for the preparation of hematein-eosin-stained slides. Furthermore, tissue from the midsection of the jejunum was collected, rapidly frozen in liquid nitrogen, and kept at -80°C for gene expression level determination.

Measurement of serum immunoglobulins (Ig) levels

The levels of IgA (M1244L96), IgG (M1245L96), and IgM (M1246L96) in the serum were measured using chicken ELISA kits (Shanghai Meilian Biological Engineering Co. Ltd., Shanghai, China), following the manufacturer's instructions.

Intestinal morphology analysis

Jejunal segments were immersed in a 4% polyformaldehyde phosphate buffer for 48 h to ensure proper fixation. The segments were dehydrated, embedded in paraffin, and sectioned to 4 μm thickness before being stained with hematoxylin and eosin. The microscopic images were captured using a Leica model DMi8 microscope (Leica, Wetzlar, Germany) and analyzed with image analysis software (version 4.2, Leica Application Suite, Leica, Wetzlar, Germany). Villus height was measured from the apex of the villus to the junction of the villus and the crypt. Crypt depth was determined as the vertical distance from the base of the crypt up to the junction of the villus and crypt, and the villus height-to-crypt depth ratio (VCR) was then calculated. Ten complete villi and their associated crypts were determined in each section at a magnification of 50×.

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California, USA). The concentration and purity of RNA samples were determined using an NanoPhotometer NP80 (Implen, München, Germany). Subsequently, 1 μg of total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio Inc., Dalian, China). The systhesized cDNA was stored at -20°C for further use. Quantitative PCR was performed to analyze the mRNA expression of target genes. Primers were synthesized by Shanghai Sangon Biotech Co., Ltd. Quantitative PCR was performed using the TB Green® Premix Ex TaqTM (Takara Bio Inc., Dalian, China) following the manufacturer's instructions. The amplification was carried out on a CFX96 Real-Time PCR Detection Systems (Bio-rad, Hercules, CA). The PCR protocol included an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method with *GAPDH* as the reference gene. The primer sequences for the target and reference genes *in vivo* and *in vitro* are presented in Supplementary Tables 2, 3, respectively.

Cell culture and treatment

The rat small intestinal epithelial cell line IEC-6 was acquired from Peking Union Medical College (Beijing, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT) containing 5% (vol/vol) fetal bovine serum (Gibco, Carlsbad, CA), 100 U/mL penicillin, 100 mg/mL streptomycin (Solarbio, Beijing, China) and 0.01 mg/mL bovine insulin (Solarbio, Beijing, China). The cells were incubated at 37°C with 5% CO_2. Upon reaching 80% confluence, the cells were divided into 4

groups: Con group (cells cultured in arginine-free DMEM without α toxin), Arg group (cells cultured with 4 mM arginine without α toxin), Tox group (cells cultured in arginine-free DMEM followed by 50 U/L α toxin incubation, and Arg+Tox group (cells cultured with 4 mM arginine for 24 h, followed by incubation with both 50 U/L α toxin and 4 mM arginine for an additional 4 h).

In the RNA interference experiment, the primer sequences for small interfering RNA (siRNA) targeting mTOR were as follows: 5'-GGCAUAUGGUCGAGAUUUATT-3' and 5'-UAAAUCUCG ACCAUAUGCCTT-3'. The primer sequences for siRNA targeting SLC38A9 were as follows: 5'-GGCUCUGCCUAUAAACUUATT-3' and 5'-UAAGUUUAUAGGCAGAGCCTT-3'. The siRNAs were transfected into IEC-6 cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, the culture medium was replaced with arginine-free DMEM, and the cells were treated with either 0 or 4 mM arginine for 24 h, followed by α toxin or PBS treatment for another 4 h.

Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) test kit (Dojindo, Japan). In 96-well culture plates, approximately 1×10^4 cells were seeded per well and incubated in complete medium for 36 h. Subsequently, the cells were washed twice with PBS. IEC-6 cells were pre-treated with either 0 or 4 mM arginine for 24 h, followed by treatment with PBS or 50 U/L of α toxin. After 3 h of α toxin treatment, the CCK-8 reagent was added to each well and incubated for 1 h at 37°C. The absorbance was determined using a microplate reader (TECAN, Infinite M Nano, Männedorf, Switzerland) at 450 nm. The absorbance values were normalized to those of the Con group.

Lactic dehydrogenase (LDH) activity assay

LDH activity in the cell culture supernatants was determined to evaluate cytotoxicity using a commercially available LDH assay kit (Beyotime Biotechnology, Beijing, China) following the manufacturer's protocol.

Flow cytometry analysis

The Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Dojindo, Japan) was used to determine the rate of cell apoptosis following the manufacturer's instructions. Cells were cultured in 12-well plates at a density of 1×10^5 cells per well for 72 h. Subsequently, the cells were treated with either 0 or 4 mM arginine, followed by PBS or α toxin exposure. After treatment, the cells were harvested, washed twice with PBS, and resuspended in Annexin V binding solution to make a cell suspension with a final concentration of 1×10^6 cells/mL. FITC-labeled Annexin V and PI were added to the cell suspension, followed by an incubation in the dark at room temperature for 15 minutes. An additional Annexin V binding solution was then added, and the samples was analyzed within 1 h using a flow cytometer (BD Bioscience, BD FACS Calibur, USA).

TUNEL assay

Approximately 1×10^4 cells were seeded per well in 96-well culture plates and incubated for 36 h. After treatment with arginine and α toxin, cell apoptosis was evaluated using the One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The cells were observed under a fluorescence microscope (DMi8; Leica Microsystems, Wetzlar, Germany), and four random fields in each well were selected for cell counting using Image Pro Plus software.

Statistical analysis

In the *in vivo* experiment, the general linear model procedure of SPSS version 25.0 (SPSS Inc., Chicago, IL) was used to evaluate the main effects of arginine supplementation, α toxin challenge, and their interaction. If a significant interaction effect was observed, one-way ANOVA and Duncan's multiple comparison were used to examine the differences across the groups. For the *in vitro* experiment, data between two groups were analyzed by Student's *t* test. Statistical significance was considered at $P< 0.05$. Graphs were generated using GraphPad Prism 8 Software (GraphPad Software Inc., La Jolla, CA, USA), with error bars representing the standard error of the mean (SEM).

Results

Arginine alleviated α toxin-induced growth performance impairment in broiler chickens

As shown in Table 1, no changes were observed in the ADG, ADFI and FCR of broiler chickens from d 1 to 14 in response to arginine supplementation, α toxin challenge and their interaction ($P > 0.05$). There was no significance observed in the body weight of d 14 ($P > 0.05$). At 21 d of age, the α toxin challenge significantly decreased the body weight ($P = 0.001$), while arginine supplementation significantly increased the body weight ($P < 0.05$). The α toxin challenge significantly decreased the ADG and ADFI of broiler chickens from d 15 to 21 ($P < 0.05$), while the addition of arginine significantly increased the ADFI ($P < 0.05$). The FCR from d 15 to 21 was not affected by arginine supplementation, α toxin challenge and their interaction ($P > 0.05$).

Arginine attenuated α toxin-induced reduction in serum immunoglobulins levels in broiler chickens

As presented in Table 2, the α toxin challenge significantly decreased serum IgA and IgG levels ($P < 0.05$), which were

TABLE 1 Effects of arginine supplementation on growth performance of broiler chickens.

Items	1-14d				15-21d			
	ADG, g	ADFI, g	FCR	BW, g	ADG, g	ADFI, g	FCR	BW, g
CON	20.58	30.16	1.47	328.04	44.82	65.76	1.47	603.33
ARG	20.56	30.57	1.49	342.29	45.94	69.30	1.51	619.38
ATX	20.39	30.09	1.51	323.28	42.08	61.76	1.44	565.42
ARG+ATX	20.39	29.57	1.45	335.18	42.42	64.06	1.52	587.19
SEM	0.187	0.484	0.021	3.838	0.455	0.794	0.017	5.600
Main effect								
Arg								
–	20.48	30.12	1.49	325.50	43.45	63.89	1.46	584.38
+	20.48	30.07	1.47	338.46	44.18	66.68	1.51	600.98
α toxin								
–	20.57	30.38	1.48	334.62	45.38	67.53	1.49	611.35
+	20.39	29.83	1.48	328.83	42.25	62.99	1.48	577.86
P value								
Arg	0.984	0.954	0.709	0.100	0.332	0.034	0.100	0.041
α toxin	0.651	0.599	0.964	0.445	<0.001	0.002	0.764	0.001
Interaction	0.989	0.650	0.373	0.879	0.602	0.640	0.653	0.746

CON, birds received a basal diet. ARG, birds received a basal diet supplemented with 0.3% arginine. ATX, birds received a basal diet and subjected to a C. perfringens challenge. ARG+ATX, birds received a basal diet supplemented with 0.3% arginine and subjected to a C. perfringens challenge. ADG, average daily gain. ADFI, average daily feed intake. FCR, feed conversion ratio. BW, body weight. Eight replicates per group.

increased by arginine supplementation ($P < 0.05$). The serum IgM level was not affected by arginine supplementation, α toxin challenge and their interaction ($P > 0.05$).

Arginine mitigated α toxin-induced intestinal morphology injury in broiler chickens

Figure 1 illustrates that the intestinal structure was nearly normal in the CON and ARG groups, while severely disrupted in the ATX group, as indicated by villi atrophy, villus tip loss, capillary hemorrhages and lymphocytes infiltration. The ARG+ATX group partially alleviated the intestinal morphology damage. Table 3 demonstrates that the effects of arginine supplementation and α toxin challenge had a significant interaction on the villus height, crypt depth and VCR in the jejunum of broiler chickens ($P < 0.05$). The villus height in the ARG group was the highest among the four groups, with no significant differences observed among the other three groups ($P > 0.05$). Compared with the CON and ARG groups, the ATX group significantly increased the crypt depth ($P < 0.05$). However, compared with the ATX group, the ARG+ATX group significantly reduced the crypt depth ($P < 0.05$). The ARG group had the highest VCR among the four groups, while the ATX group had the lowest. Compared to the ATX group, the ARG+ATX group significantly increased the VCR ($P < 0.05$).

Arginine alleviated α toxin-induced intestinal inflammatory response and upregulated the mRNA expression of mTOR and SLC38A9 in broiler chickens

As presented in Table 4, the mRNA expression levels of *interleukin (IL)-1β*, *IL-6*, *IL-8*, and *IL-17* was significantly increased by the α toxin challenge ($P < 0.05$). Arginine supplementation significantly reduced the mRNA expression levels of *IL-1β*, *IL-6*, and *IL-17* ($P < 0.05$). The mRNA expression of *mTOR* and *SLC38A9* was significantly decreased by the α toxin challenge ($P < 0.05$), while arginine supplementation significantly increased *mTOR* mRNA expression ($P < 0.05$).

Arginine alleviated the decrease in cell viability and the increase in cytotoxicity induced by α toxin in IEC-6 cells

Figure 2A shows that treatment with α toxin challenge alone caused a significant decrease in cell viability compared with the Con group ($P < 0.001$). However, the Arg+Tox group significantly increased the cell viability compared to the Tox group ($P < 0.001$). As shown in Figure 2B, compared to the Con group, the Tox group significantly elevated the release of LDH from IEC-6 cells

TABLE 2 Effects of arginine supplementation on serum immunoglobulin (Ig) levels of broiler chickens.

Items	IgA, μg/mL	IgG, μg/mL	IgM, μg/mL
CON	27.59	40.26	3.12
ARG	39.72	41.45	3.12
ATX	24.77	37.12	3.04
ARG+ATX	32.69	40.48	3.08
SEM	1.617	0.495	0.031
Main effect			
Arg			
–	26.18	38.69	3.08
+	35.88	40.9	3.10
α toxin			
–	33.66	40.85	3.12
+	29.09	39.04	3.06
P value			
Arg	<0.001	0.011	0.768
α toxin	0.043	0.021	0.351
Interaction	0.364	0.199	0.691

CON, birds received a basal diet. ARG, birds received a basal diet supplemented with 0.3% arginine. ATX, birds received a basal diet and subjected to a C. perfringens challenge. ARG+ATX, birds received a basal diet supplemented with 0.3% arginine and subjected to a C. perfringens challenge. Eight replicates per group.

($P < 0.001$), indicating an increase in cytotoxicity. However, the Arg+Tox group significantly reduced this cytotoxicity ($P < 0.05$).

Arginine inhibited α toxin-induced cell apoptosis in IEC-6 cells

As illustrated in Figure 3, compared to the Con group, treatment with α toxin alone significantly increased the apoptosis rate of cells ($P < 0.001$). However, the Arg+Tox group led to a reduction in the apoptosis rate of cells compared with the Tox group ($P < 0.05$).

As depicted in Figure 4, TUNEL assays indicated that arginine-treated cells without α toxin challenge had lower cell apoptosis rate than the Con group ($P < 0.001$). However, the Tox group significantly increased the cell apoptosis rate compared to the Con group ($P < 0.001$). Pretreatment with arginine in the challenged cells resulted in a reduction of cell apoptosis ($P < 0.001$).

Arginine suppressed the increased mRNA expression of inflammation and apoptosis-related genes in IEC-6 cells induced by α toxin

As presented in Figures 5A–E, when compared to the Con and Arg groups, the Tox group significantly increased the mRNA expression levels of pro-inflammatory cytokines *IL-6* and *tumor necrosis factor alpha (TNF-α)*, as well as chemokines *C-X-C motif chemokine ligand 10* (*CXCL10)* and *C-X-C motif chemokine ligand 11* (*CXCL-11*), along with anti-inflammatory cytokines *transforming growth factor-β* (*TGF-β)* ($P < 0.001$). However, pretreatment with arginine in cells cultured with α toxin significantly reduced the mRNA expression levels of *IL-6*, *CXCL-10*, *CXCL-11* and *TGF-β*, when compared to cells treated with α toxin challenge alone ($P < 0.05$).

Figures 5F–J shows that compared to the Con group, the α toxin challenge alone significantly increased the mRNA expression levels of pro-apoptotic factors *B-cell lymphoma-2 associated X protein (Bax)* and *cysteinyl aspartate specific proteinase 3 (Caspase-3)*, as well as anti-apoptotic factors *B-cell lymphoma-extra large (Bcl-XL)* ($P < 0.01$). Pretreatment with arginine prior to α toxin challenge significantly reduced the mRNA expression levels of *Bax*, *B-cell lymphoma-2 (Bcl-2)*, *Bcl-XL* and *Caspase-3* ($P < 0.01$), compared to cells subjected to α toxin challenge alone. In addition, the α toxin challenge significantly elevated the *Bax/Bcl-2* ratio ($P < 0.01$), and these effects were effectively reversed by pretreatment with arginine prior to the toxin challenge ($P < 0.05$).

Arginine upregulated the mRNA expression of genes in SLC38A9/mTORC1 pathway in α toxin-treated IEC-6 cells

Compared with cells in the Con group, the mRNA expression levels of *mTOR* and *SLC38A9* were significantly decreased in the Tox group ($P < 0.001$, Figures 6A, B). However, these reductions were mitigated in the Arg+ Tox group ($P < 0.05$). Compared with the Con and Arg groups, the cells exposed to the α toxin alone exhibited lower mRNA expression levels of *4EBP1* ($P < 0.05$, Figures 6C, D) and *S6K* ($P < 0.001$). However, pretreatment with arginine in the α toxin-treated cells led to an increase in the mRNA expression of these genes ($P < 0.05$).

Arginine exhibited no protective effects on IEC-6 cells treated with mTOR and SLC38A9 siRNA

Arginine pretreatment markedly decreased the α toxin-induced cytotoxicity, while mTOR silencing significantly attenuated the effect of arginine ($P < 0.01$, Figures 7A, B). Moreover, arginine-induced upregulation of *4EBP1* and *S6K* mRNA expression downstream of mTORC1 was inhibited by mTOR silencing ($P < 0.001$, Figures 7C, D). The decline in mRNA expression of pro-inflammatory (*IL-6*) and pro-apoptotic (*Caspase-3*) genes due to arginine treatment was abrogated by mTOR silencing ($P < 0.001$, Figures 7E, F).

SLC38A9 silencing significantly increased the cytotoxicity of cells cultured with arginine ($P < 0.001$, Figures 8A, B). Additionally, SLC38A9 silencing abolished the increased *mTOR* mRNA expression caused by arginine treatment in IEC-6 cells exposed to α toxin ($P < 0.001$, Figure 8C). The increase in mRNA expression of

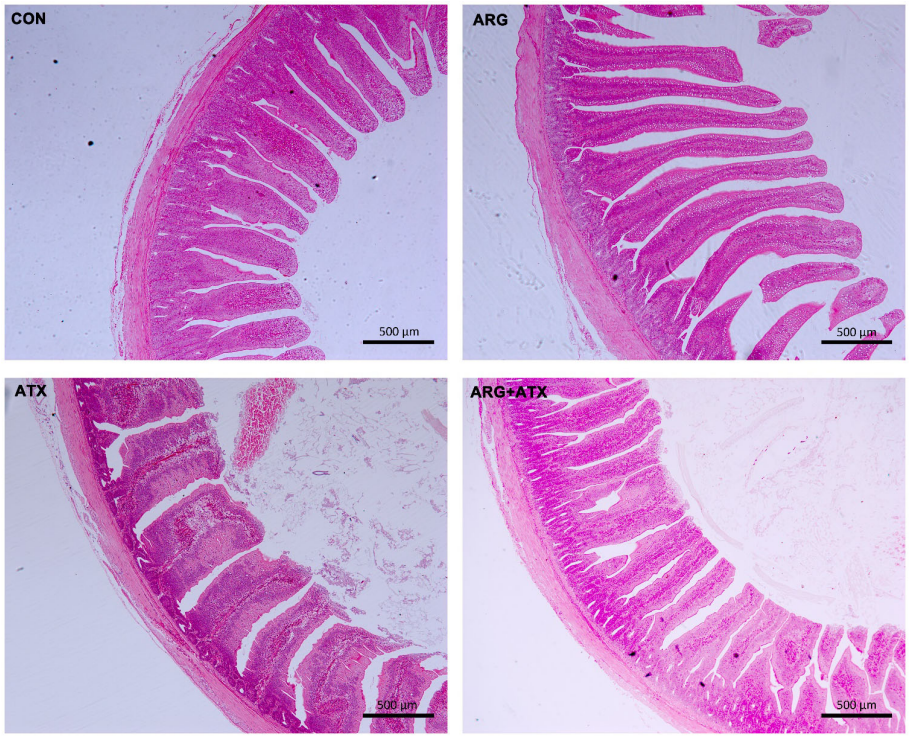

FIGURE 1
Representative histomorphology pictures of jejunum in broiler chickens. CON, birds received a basal diet. ARG, birds received a basal diet supplemented with 0.3% arginine. ATX, birds received a basal diet and subjected to a *C. perfringens* challenge. ARG+ATX, birds received a basal diet supplemented with 0.3% arginine and subjected to a *C. perfringens* challenge. Original magnification, 50×. Scale bar = 500 μm.

4EBP1 and *S6K* mRNA due to arginine treatment was abrogated by SLC38A9 silencing ($P < 0.001$, Figures 8D, E). Knockdown of SLC38A9 also counteracted the decline in mRNA expression of pro-inflammatory (*IL-6*) and pro-apoptotic (*Caspase-3*) genes caused by arginine ($P < 0.001$, Figures 8F, G).

Discussion

Immune stress often results in growth performance impairment and structural gut damage in broiler chickens. In this study, arginine supplementation alleviated the decreased ADFI and final body weight induced by the α toxin challenge, highlighting the importance of exogenous arginine supply during immune stress. Similarly, Liu et al. (27) demonstrated that arginine supplementation decreased body weight loss in pigs challenged with LPS. Arginine may regulate metabolism to promote protein synthesis and reduce protein catabolism during stress and infections (28) by stimulating the secretion of growth hormone, insulin and glucagon (29). Therefore, arginine supplementation alleviated the growth suppression induced by the α toxin challenge, which may be partially due to its influence on protein metabolism. Serum antibody levels are valuable indicators of humoral immunity, with IgA, IgM, and IgG being the main antibodies that selectively bind to antigens (30). In this study, the α toxin challenge lowered the serum IgA and IgG levels of broiler chickens, while arginine supplementation increased these antibody levels. This suggested that arginine pretreatment can enhance Ig levels to counteract the adverse effects of the α toxin challenge in broilers. Similarly, Chen et al. (31) reported that dietary supplementation with 0.3% arginine increased serum IgA and IgG levels in geese.

The morphology of the small intestine, including villus height, crypt depth and VCR, can serve as an indicator of intestinal health and integrity. In this study, arginine supplementation alleviated the increased jejunal crypt depth and decreased VCR of broiler chickens induced by the α toxin, demonstrating its protective effect on intestinal mucosa. Our previous studies also reported that arginine addition reduced the intestinal morphological injury caused by *Salmonella enterica serovar Typhimurium* challenge in broiler chickens (32). In addition, arginine supplementation was found to enhance the jejunal morphology of low-birth-weight piglets (33). Therefore, arginine supplementation alleviated the growth suppression induced by the α toxin challenge partially by alleviating the intestinal mucosa injury. Based on our *in vitro* findings, arginine pretreatment mitigated the increased LDH production and decreased viability of IEC-6 cells induced by α toxin, suggesting that arginine pretreatment could lessen the detrimental effects of α toxin challenge on cells. Furthermore, both flow cytometry and TUNEL staining assays in this study consistently demonstrated that arginine pretreatment effectively prevented the enhanced cell apoptosis generated by the α toxin challenge. The reduction in cytotoxicity and apoptosis in the

TABLE 3 Effects of arginine supplementation on the jejunal morphology of broiler chickens.

Items	Villus height, μm	Crypt depth, μm	VCR
CON	933.64[b]	113.71[b]	8.45[b]
ARG	1149.76[a]	105.92[b]	10.59[a]
ATX	839.76[b]	152.56[a]	5.98[d]
ARG+ATX	868.02[b]	121.11[b]	7.09[c]
SEM	29.563	4.428	0.377
Main effect			
Arg			
–	894.52	131.64	7.10
+	998.05	113.52	8.71
α toxin			
–	1033.39	110.12	9.62
+	856.24	136.84	6.58
P value			
Arg	0.003	0.002	<0.001
α toxin	<0.001	<0.001	<0.001
Interaction	0.019	0.048	0.044

[a-d]Means with no common superscript in the same column indicate significant differences for the interaction effect ($P < 0.05$).
CON, birds received a basal diet. ARG, birds received a basal diet supplemented with 0.3% arginine. ATX, birds received a basal diet and subjected to a C. perfringens challenge. ARG+ATX, birds received a basal diet supplemented with 0.3% arginine and subjected to a C. perfringens challenge. VCR, villus height-to-crypt depth ratio. Eight replicates per group.

intestinal epithelial cells may be the possible mechanism by which arginine supplementation mitigated the damage to intestinal morphology induced by the α toxin challenge *in vivo*.

Cytokines and chemokines have been identified as important regulators of inflammation and infection (34). Intestinal epithelial cells produce various proinflammatory cytokines, such as IL-1β, IL-6, TNF-α and IL-17, which play a critical role in intestinal innate immune defense (35). IL-17 induces inflammation by enhancing the synthesis of essential pro-inflammatory cytokines, primarily IL-1β, IL-6 and TNF-α (36). CXCL10 and CXCL11 are proinflammatory chemokines that recruit immune cells, including monocytes and T cells, to the sites of infection or injury (37). The current study found that arginine supplementation significantly reduced the elevation of mRNA expression levels of *IL-1β*, *IL-6* and *IL-17* induced by α challenge in the jejunum of broiler chickens, as well as the mRNA expression of *IL-6*, *CXCL10* and *CXCL11* in the IEC-6 cells. This result suggested that excessive activation of the inflammatory response was inhibited by arginine. The balance between pro- and anti-inflammatory cytokines is crucial for animal health. In the present study, the administration of arginine to IEC-6 cells resulted in a suppression of the elevated transcription of *TGF-β*, an anti-inflammatory cytokine, because of the α toxin challenge. Consistent with our results, Tan et al. (38) observed that the dietary arginine addition could mitigate the upregulation of mRNA expression of pro- and anti-inflammatory cytokines in broiler chickens challenged with lipopolysaccharide. Our previous *in vivo* and *in vitro* studies indicated that arginine can reduce the increased mRNA expression of pro- and anti-inflammatory cytokines in chickens induced by *C. perfringens* infection (14).

TABLE 4 Effects of arginine supplementation on the mRNA expression of inflammatory cytokines, mTOR and SLC38A9 of broiler chickens.

Items	*IL-1β*	*IL-6*	*IL-8*	*IL-10*	*IL-17*	*TNF-α*	*mTOR*	*SLC38A9*
CON	1.02	1.06	1.34	1.05	0.99	0.97	1.01	1.01[b]
ARG	0.70	0.69	0.94	1.45	0.88	0.87	1.13	1.42[a]
ATX	1.57	1.91	1.92	0.73	1.57	1.19	0.89	0.81[c]
ARG+ATX	0.90	1.21	1.59	1.09	0.97	0.94	1.00	0.68[c]
SEM	0.089	0.132	0.139	0.122	0.091	0.054	0.030	0.058
Main effect								
Arg								
–	1.31	1.48	1.63	0.89	1.28	1.08	0.96	0.91
+	0.80	0.97	1.27	1.27	0.93	0.91	1.07	1.05
α toxin								
–	0.86	0.88	1.14	1.25	0.94	0.92	1.06	1.21
+	1.26	1.53	1.76	0.91	1.25	1.06	0.94	0.75

(Continued)

TABLE 4 Continued

Items	*IL-1β*	*IL-6*	*IL-8*	*IL-10*	*IL-17*	*TNF-α*	*mTOR*	*SLC38A9*
P value								
Arg	0.008	0.004	0.161	0.121	0.043	0.173	0.037	0.007
α toxin	0.001	0.021	0.023	0.160	0.034	0.101	0.030	<0.001
Interaction	0.177	0.445	0.885	0.926	0.129	0.489	0.884	<0.001

[a-c]Means with no common superscript in the same column indicate significant differences for the interaction effect (P < 0.05).
CON, birds received a basal diet. ARG, birds received a basal diet supplemented with 0.3% arginine. ATX, birds received a basal diet and subjected to a C. perfringens challenge. ARG+ATX, birds received a basal diet supplemented with 0.3% arginine and subjected to a C. perfringens challenge. TNF-α, tumor necrosis factor alpha. mTOR, mechanistic target of rapamycin. SLC38A9, solute carrier family 38 member 9. Eight replicates per group.

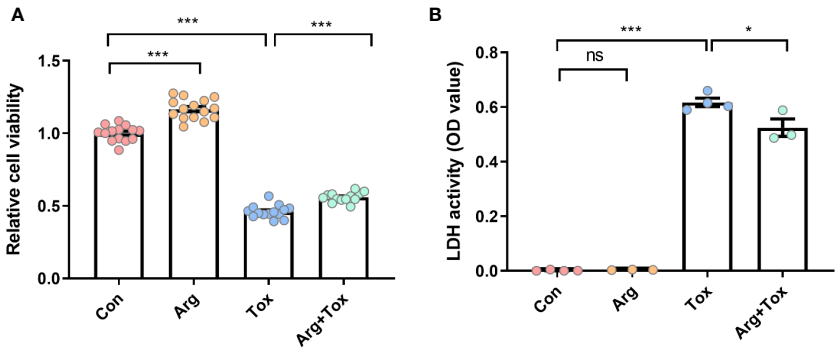

FIGURE 2
Effects of arginine on cell viability and LDH release in IEC-6 cells cultured with α toxin. **(A)** cell viability in IEC-6 cells. **(B)** LDH activity in the culture supernatant of IEC-6 cells. Con, cells cultured in arginine-free DMEM. Arg, cells cultured with 4 mM arginine. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 h. Data are expressed as means ± SEM. Significance was set at $P < 0.05$. *$P < 0.05$, ***$P < 0.001$, and ns means no significant.

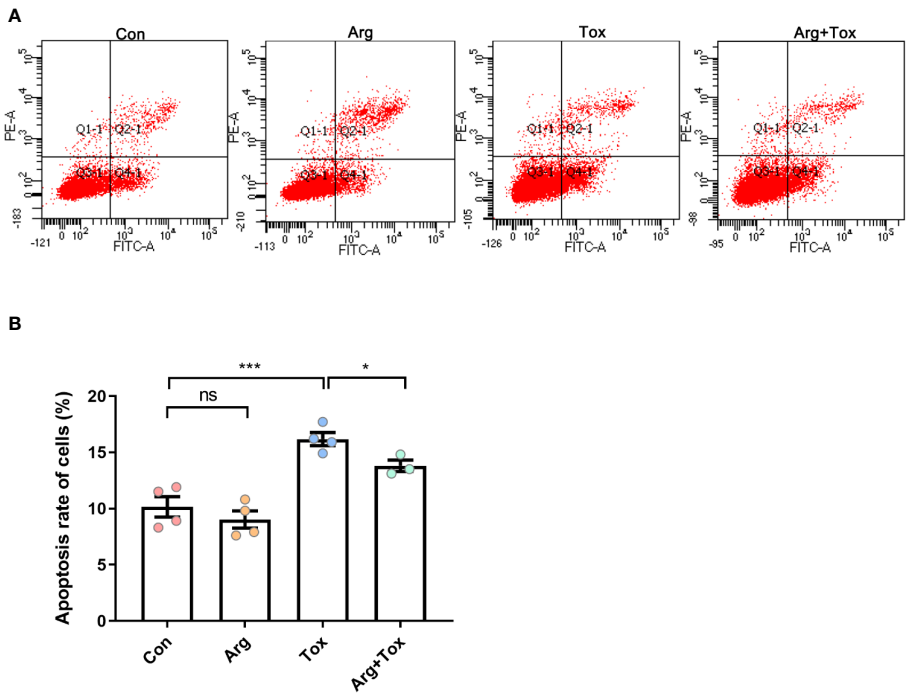

FIGURE 3
Effects of arginine on apoptosis rate in IEC-6 cells cultured with α toxin determined by flow cytometry. **(A)** flow cytometry following FITC-Annexin V and PI double staining. **(B)** apoptosis rate in IEC-6 cells. Con, cells cultured in arginine-free DMEM. Arg, cells cultured with 4 mM arginine. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 h. Data are expressed as means ± SEM. Significance was set at $P < 0.05$. *$P < 0.05$, ***$P < 0.001$, and ns means no significant.

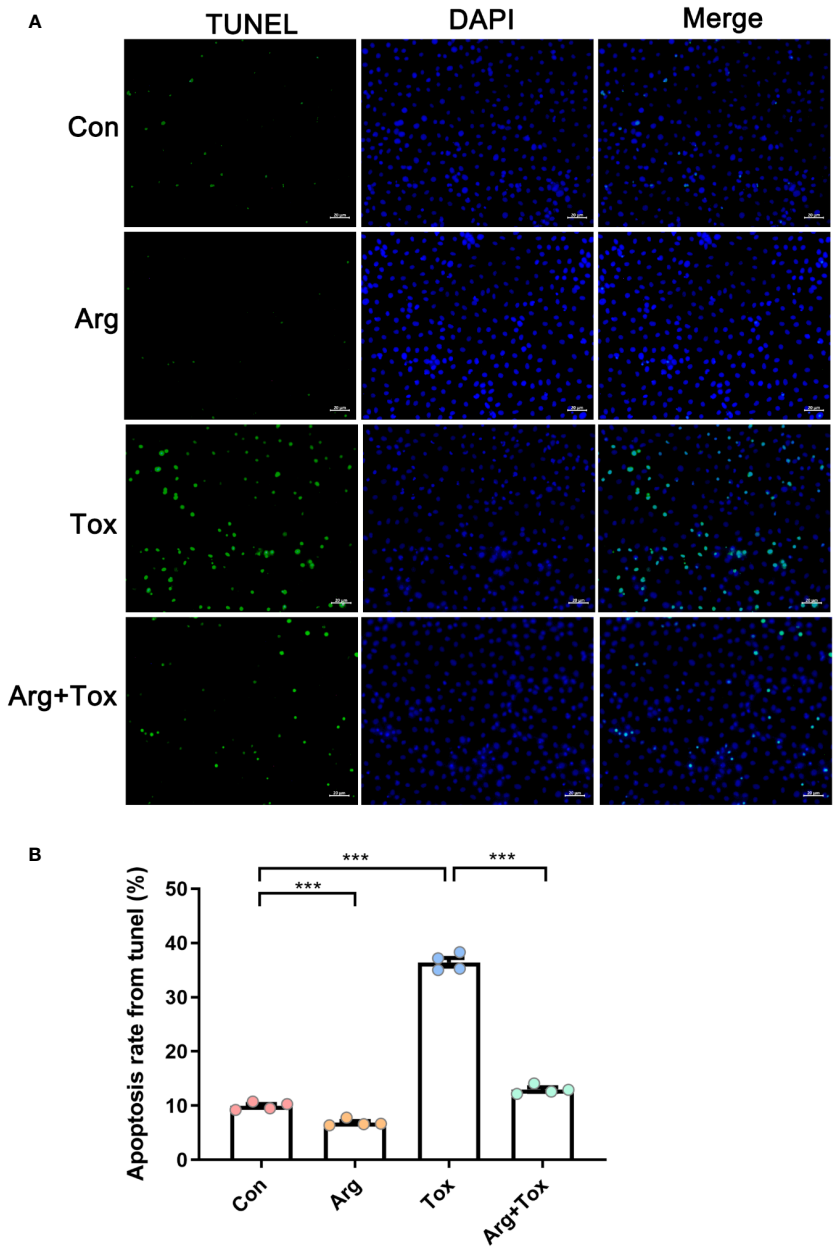

FIGURE 4
Effects of arginine on apoptosis rate in IEC-6 cells cultured with α toxin determined by TUNEL assay. **(A)** immunofluorescence staining of IEC-6 cells. The scale bar represents 20 μm. **(B)** apoptosis rate in IEC-6 cells. Con, cells cultured in arginine-free DMEM. Arg, cells cultured with 4 mM arginine. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 (h) Data are expressed as means ± SEM. Significance was set at $P < 0.05$. ***$P < 0.001$.

Apoptosis is an essential mechanism employed by the immune system to fight infections and eliminate cells with irreversible DNA damage (39). The Bcl-2 family proteins regulate programmed cell death triggered by mitochondrial dysfunction. Some family members, like Bcl-2 and Bcl-XL, inhibit apoptosis, whereas others, like Bax, promote cell death (40). The overexpression of Bax promotes the release of cytochrome C, which subsequently activates the downstream Caspase-3 protease, mediating cell death (41, 42). Bcl-2 and Bcl-XL can heterodimerize with Bax and neutralize the effects of the latter (43). Our results showed that arginine treatment alleviated the elevation of *Bax* and *Caspase-3* mRNA expression caused by α toxin challenge in IEC-6 cells, which indicated that arginine reversed α toxin-induced cell apoptosis. These findings are in line with recent studies demonstrating that arginine effectively suppresses doxorubicin-induced vascular dysfunction by attenuating apoptosis (44). In addition, Ma et al. (45) reported that arginine pretreatment enhanced alveolar integrity and function by reducing LPS-induced apoptosis of alveolar cells, expression of inflammatory cytokines and chemokines, and accumulation of neutrophils and macrophages in the lung tissues of mice.

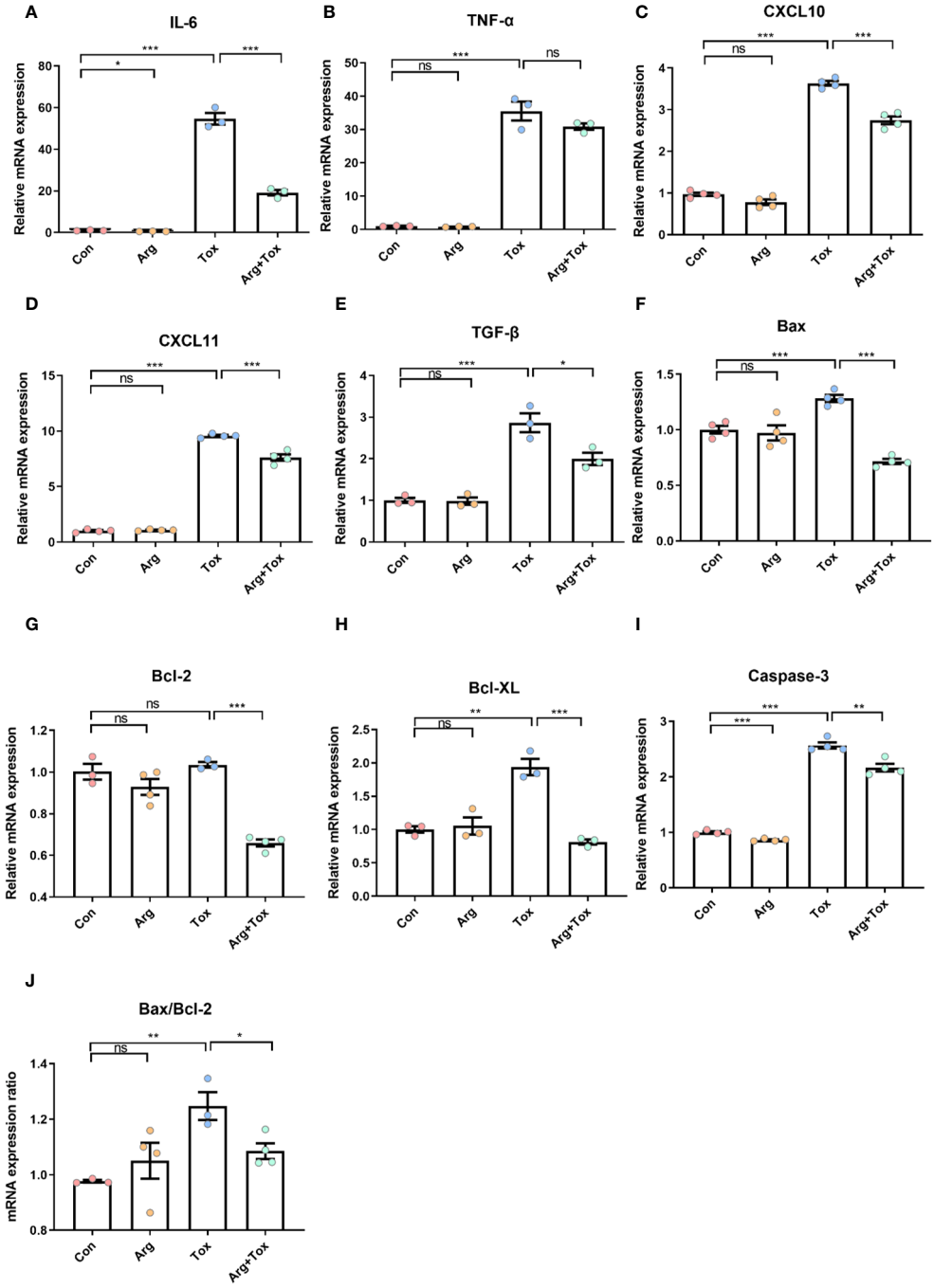

FIGURE 5
Effects of arginine on the mRNA expression of inflammatory and apoptotic genes in IEC-6 cells cultured with α toxin. **(A–E)** mRNA expression of inflammatory genes *IL-6*, *TNF-α*, *CXCL10*, *CXCL11* and *TGF-β* in IEC-6 cells. **(F–I)** mRNA expression of apoptotic genes *Bax*, *Bcl-2*, *Bcl-XL* and *Caspase-3* in IEC-6 cells. **(J)** Bax/Bcl-2 ratio in IEC-6 cells. Con, cells cultured in arginine-free DMEM. Arg, cells cultured with 4 mM arginine. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 h. Data are expressed as means ± SEM. Significance was set at $P < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ns means no significant.

Arginine promotes diverse physiological effects, including immune cell activation in animals, mostly by activating mTORC1 (18–20). This multi-protein complex consists of mTOR, the regulatory associated protein of mTOR (Raptor), and mammalian LST8 homolog (mLst8) as core components (19). mTORC1 can regulate its downstream targets, 4EBP1 and S6K to control several physiological processes (46). The current results showed that arginine alleviated α toxin-induced decreased mRNA expression of *mTORC1*, *4EBP1* and *S6K* in IEC-6 cells. This finding is consistent with previous studies demonstrating that arginine activated mTOR and its downstream targets 4EBP1 and S6K, which play crucial roles in processes such as protein synthesis

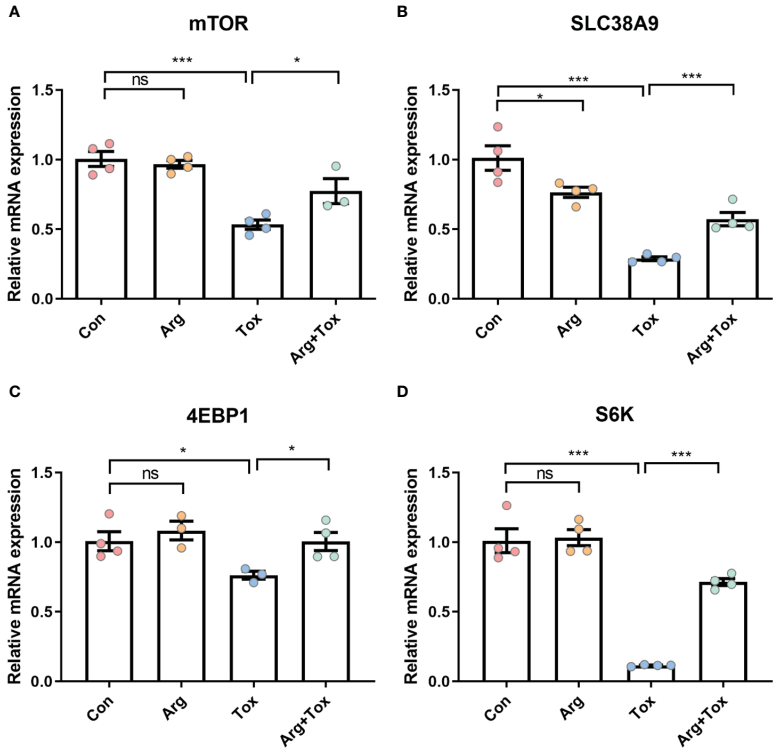

FIGURE 6
Effects of arginine on the mRNA expression of genes in SLC38A9/mTORC1 pathway in IEC-6 cells cultured with α toxin. **(A–D)** mRNA expression of inflammatory-related genes *mTOR*, *SLC38A9*, *4EBP1* and *S6K* in IEC-6 cells. Con, cells cultured in arginine-free DMEM. Arg, cells cultured with 4 mM arginine. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 h. Data are expressed as means ± SEM. Significance was set at $P < 0.05$. *$P < 0.05$, ***$P < 0.001$, and ns means no significant.

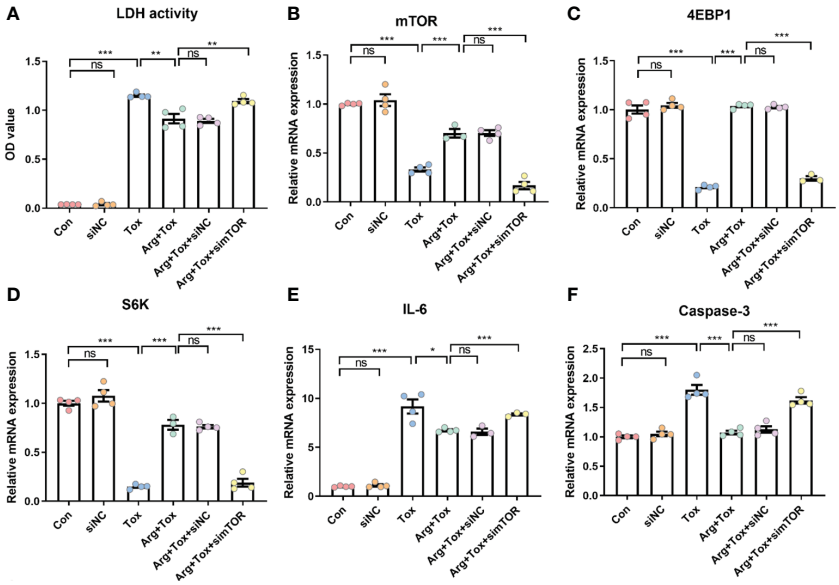

FIGURE 7
Effects of arginine on IEC-6 cells cultured with α toxin after silencing mTOR. **(A)** LDH activity in the culture supernatant of IEC-6 cells. **(B–D)** mRNA expression of *mTOR*, *4EBP1* and *S6K* in IEC-6 cells. **(E, F)** mRNA expression of inflammatory cytokines *IL-6* and apoptotic genes *Caspase-3* in IEC-6 cells. Con, cells cultured in arginine-free DMEM. siNC, cells cultured with NC siRNA. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 h. Arg+Tox+siNC, cells transfected with NC siRNA and then pretreated with arginine for 24 h prior to incubation with both arginine and α toxin for another 4 h. Arg+Tox+simTOR, cells transfected with mTOR siRNA and then pretreated with arginine for 24 h prior to incubation with both arginine and α toxin for another 4 h. Data are expressed as means ± SEM. Significance was set at $P < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ns means no significant.

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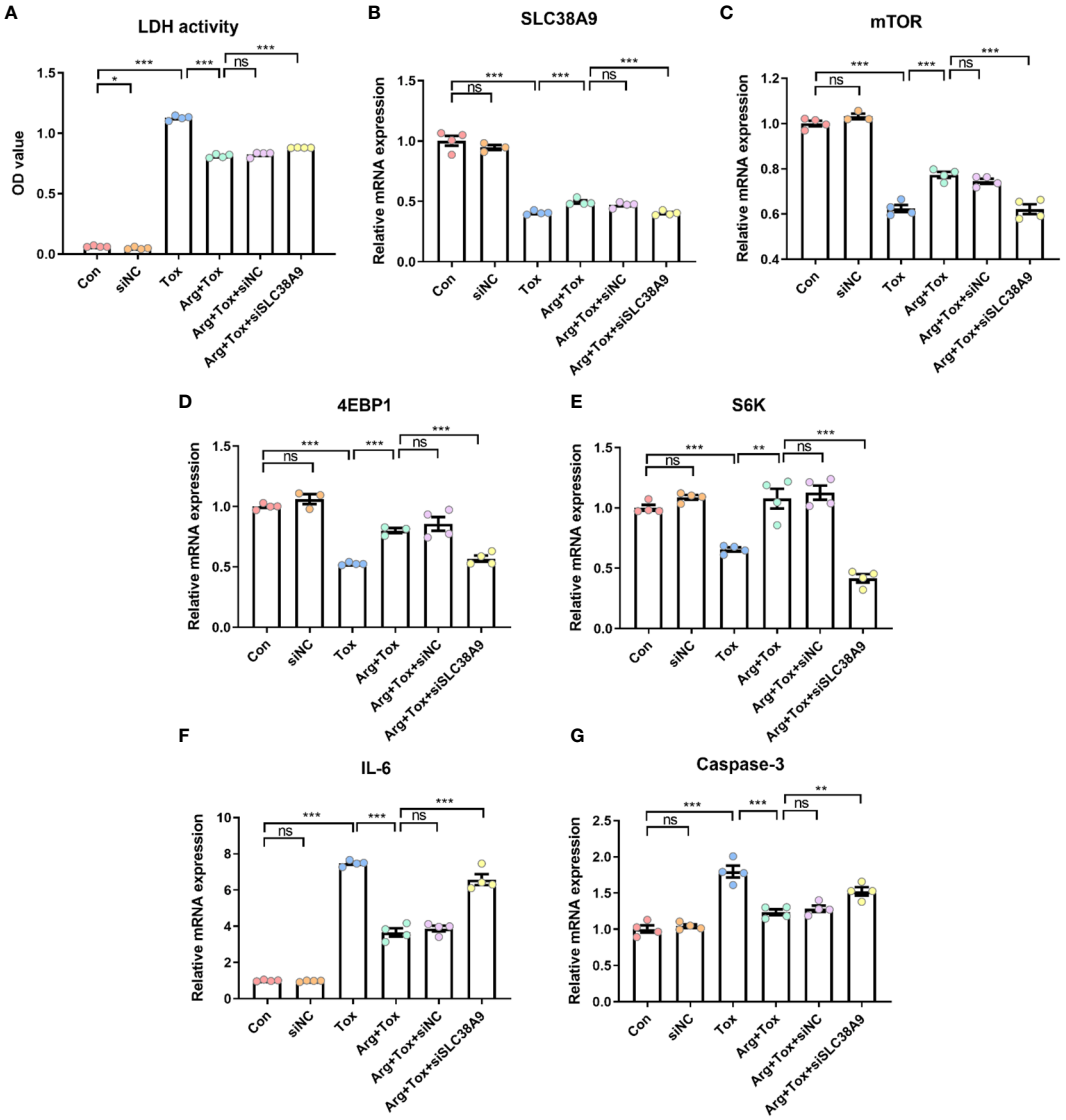

FIGURE 8
Effects of arginine on IEC-6 cells cultured with α toxin after silencing SLC38A9. **(A)** LDH activity in the culture supernatant of IEC-6 cells. **(B–E)** mRNA expression of *SLC38A9*, *mTOR*, *4EBP1* and *S6K* in IEC-6 cells. **(F, G)** mRNA expression of inflammatory cytokines *IL-6* and apoptotic genes *Caspase-3* in IEC-6 cells. Con, cells cultured in arginine-free DMEM. siNC, cells cultured with NC siRNA. Tox, cells cultured with 50 U/L α toxin. Arg+Tox, cells cultured with 4 mM arginine for 24 h and then cultured with both 50 U/L α toxin and 4 mM arginine for another 4 h. Arg+Tox+siNC, cells transfected with NC siRNA and then pretreated with arginine for 24 h prior to incubation with both arginine and α toxin for another 4 h. Arg+Tox+siSLC38A9, cells transfected with SLC38A9 siRNA and then pretreated with arginine for 24 h prior to incubation with both arginine and α toxin for another 4 h. Data are expressed as means ± SEM. Significance was set at $P < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ns means no significant.

(47) and intestinal inflammation (33). Notably, SLC38A9 has been identified as a bona fide arginine sensor for mTORC1 (23). The activation of mTORC1 by arginine requires SLC38A9, which functions to transport and interact with arginine effectively (22, 48). Thus, our results indicated that arginine alleviated the decreased mRNA expression of *SLC38A9* induced by α toxin in IEC-6 cells, indicating the interconnected relationship between arginine, SLC38A9 and mTORC1 signaling pathways within the cellular context.

To further identify the role of SLC38A9 and mTORC1 in the protective effects of arginine supplementation on cells against α toxin challenge, we employed siRNA to knock down SLC38A9 and mTORC1. Our experimental results revealed that the positive effects of arginine on cytotoxicity, inflammation, and apoptosis induced by α toxin challenge were abolished due to siRNA-mediated knockdown of mTOR in IEC-6 cells. Similarly, the protective effects of arginine against α toxin-induced cytotoxicity, inflammation, and apoptosis were abolished by the knockdown of SLC38A9 in IEC-6 cells. In addition, SLC38A9 silencing weakened the increased *mTOR* mRNA expression caused by arginine treatment in α toxin-treated IEC-6 cells. These results indicated that SLC38A9/mTORC1 pathway played an important role in mediating the cytoprotective effects of arginine against α toxin-induced damage in IEC-6 cells.

In conclusion, our study demonstrated that the immunomodulatory potential of arginine administration in

mitigating α toxin-induced challenges both *in vivo* and *in vitro*. Arginine administration effectively attenuated α toxin-induced intestinal injury, which is closely associated with the activation of the SLC38A9/mTORC1 pathway.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Animal Care and Use Committee of Qingdao Agricultural University (No. DKY20220905). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XW: Methodology, Data curation, Writing – original draft, Investigation. TZ: Methodology, Data curation, Writing – original draft, Investigation. WL: Supervision, Project administration, Writing – review & editing. HW: Resources, Methodology, Writing – review & editing. LY: Resources, Investigation, Writing – review & editing. XZ: Software, Methodology, Writing – review & editing. LZ: Software, Methodology, Writing – review & editing. NW: Software, Methodology, Writing – review & editing. BZ: Supervision, Project administration, Investigation, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1357072/full#supplementary-material

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Glossary

mTORC1	mechanistic target of rapamycin complex 1
SLC38A9	solute carrier family 38 member 9
4EBP1	eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
S6K	ribosomal protein S6 kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ADG	average daily gain
ADFI	average daily feed intake
FCR	feed conversion ratio
Ig	immunoglobulins
VCR	villus height-to-crypt depth ratio
DMEM	Dulbecco's Modified Eagle Medium
siRNA	small interfering RNA
CCK-8	Cell Counting Kit-8
LDH	lactic dehydrogenase
PI	propidium iodide
SEM	standard error of the mean
TNF-α	tumor necrosis factor alpha
CXCL10	C-X-C motif chemokine ligand 10
CXCL11	C-X-C motif chemokine ligand 11
TGF-β	transforming growth factor-β
Bax	B-cell lymphoma-2 associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-XL	B-cell lymphoma-extra large
Caspase-3	cysteinyl aspartate specific proteinase 3

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Toenail and blood selenium mediated regulation of thyroid dysfunction through immune cells: a mediation Mendelian randomization analysis

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Purpose: Specific nutrients found in food, such as minerals, antioxidants, and macronutrients, have a significant impact on immune function and human health. However, there is currently limited research exploring the relationship between specific nutrients, immune system function, and thyroid dysfunction commonly observed in autoimmune thyroid diseases, which manifest predominantly as hyperthyroidism or hypothyroidism. Therefore, the objective of this study was to investigate the connections between dietary traits and thyroid dysfunction, as well as the potential mediating role of immune cells, using Mendelian randomization (MR) analysis.

Methods: The two-step MR analysis used single-nucleotide polymorphisms as instruments, with a threshold of $p < 5e{-}08$ for nutrients and thyroid dysfunction, and $p < 5e{-}06$ for immune cells. Data from different GWAS databases and UK Biobank were combined to analyze 8 antioxidants and 7 minerals, while the data for 4 macronutrients came from a cohort of 235,000 individuals of European. The outcome data (hypothyroidism, $N = 3340$; hyperthyroidism, $N = 1840$; free thyroxin [FT4], $N = 49{,}269$; thyroid-stimulating hormone [TSH], $N = 54{,}288$) were source from the ThyroidOmics consortium. Immune trait data, including 731 immune phenotypes, were collected from the GWAS catalog.

Results: The results revealed that nutrient changes, such as lycopene, toenail and blood selenium, and α-tocopherol, impacted the immune system. Immune cells also affected thyroid function, with cDC cells promoting hypothyroidism and median fluorescence intensity (MFI) phenotypes correlating strongly with FT4 levels. Toenail and blood selenium reduce the relative cell counts (RCC) phenotypes of immune cells (CD62L− plasmacytoid DC %DC and transitional B cells %Lymphocyte), thereby diminishing its promoting effect on hypothyroidis. Furthermore, toenail and blood selenium mainly impacted phenotypes in three types of T cells (CD25++ CD8br, CD3 on CD45RA− CD4+, and CD45RA on Terminally Differentiated CD8br), reinforcing the negative regulation of FT4 levels.

Conclusion: The role of immune cells as mediators in the relationship between nutrients and thyroid dysfunction highlights their potential as diagnostic or

therapeutic markers. Toenail and blood selenium levels can indirectly impact hypothyroidism by influencing the RCC levels of two types of immune cells, and can indirectly affect FT4 levels by influencing three types of T cells.

KEYWORDS

selenium, mineral nutrients, antioxidant nutrients, macronutrients, immune cells, Mendelian randomization, thyroid dysfunction, diet and immunity

1 Introduction

Thyroid dysfunction, characterized primarily by hyperthyroidism and hypothyroidism, affects millions globally (1). Autoimmune thyroid diseases (AITD), such as Hashimoto's thyroiditis (HT), and Graves' disease (GD), are the most common causes of thyroid dysfunction (2, 3). Recent scientific research has highlighted the significant connection between nutrition, immune function, and thyroid health. However, there remains a gap in understanding the specific relationship between individual nutrients, the immune system, and thyroid dysfunction. Further investigation in this area is warranted to optimize our understanding and management of thyroid disorders.

Understanding the epidemiology and etiology of thyroid dysfunction, involving genetic predisposition (4–7), environmental influences (8–10), is crucial for effective management. Recognized causes include smoking (11, 12), alcohol (13), drugs (14, 15), and infections (16, 17). With established correlations between nutrients and overall health, iodine is widely acknowledged as a pivotal element that exerts significant influence on thyroid health. Global iodine disparities profoundly influence thyroid dysfunction prevalence (18). However, in iodine-sufficient populations, thyroid autoimmunity, especially in HT, is often considered the primary cause. The specific causal relationship with thyroid function, apart from iodine, remains unclear. Ongoing research examines the cumulative effects of substances like chloride, thiocyanate, nitrate, and iodide found in unhealthy diets, suggesting their impact on sodium-iodide symporter-mediated radioactive iodine uptake (19). Conversely, a plant-based diet is proposed to positively influence thyroid function (20). However, comprehensive research on conventional dietary habits, macronutrients, minerals, and antioxidants in relation to thyroid dysfunction is lacking.

Apart from genetic factors, the most significant pathological mechanism underlying thyroid autoimmune diseases involves the loss of immune tolerance toward autoantigens in the thyroid gland (21). In patients with GD, HT, and postpartum thyroiditis, there is a reduction in CD8+ T cells and an increase in the CD4/CD8 ratio in peripheral blood. The presence of activated T cells expressing HLA-DR is elevated. Within the thyroid tissue, CD4+ and CD8+ T cells infiltrate and remain in an activated state. CD4+ T cells may play a dominant role in HT (22). While the circulating B cell count is normal in AITD, B cells within the thyroid tissue can produce antibodies, serving as a primary source of endogenous autoantibodies. Furthermore, cytokines and chemokines play a crucial role in the pathogenesis of autoimmune thyroiditis and GD. In the thyroid tissue, Th1 lymphocytes may stimulate the production of IFN-γ and TNF-α, triggering thyroid cells to secrete CXCL10, thereby initiating and perpetuating the autoimmune process (2). This results in the immune system attacking the thyroid and other organs, leading to thyroid dysfunction. Implicating circulating immune cells as mediators in the pathogenesis of thyroid dysfunctions. Therefore, it remains unclear whether common nutrients in the environment, including macronutrients, mineral nutrients, and antioxidant nutrients, can also affect thyroid function through specific immune cell interactions.

Mendelian randomization (MR) analysis serves as a formidable tool to evaluate the causal relationship between dietary characteristics, immune cells and thyroid dysfunction. Our objectives were to: (1) investigate the causal impact of nutrients on thyroid dysfunction; (2) determine specific immune cells as mediators in the association between nutrients and thyroid dysfunction, elucidating the proportion of mediation.

2 Materials and methods

2.1 MR analysis

The MR analysis is grounded in three key assumptions (23): (1) establishing a causal link between Single-nucleotide polymorphisms (SNPs) and the factors of exposure; (2) meticulous control for potential confounding factors influencing genetic variation, exposure, and outcome in this study; (3) affirming that genetic variation solely influences the outcome through the exposure, with no involvement of other causative factors. Our study conducted a comprehensive assessment of the causal relationship between nutrients and the risk of thyroid dysfunction via MR analysis. Furthermore, we explored the mediating effects of immune cell traits to understand their indirect influence. The flow diagram, illustrated in **Figure 1**, outlines the procedural steps for the MR analysis.

2.1.1 The calculation method for the proportion of the mediating/indirect effect

The calculation employed the coefficient product method to estimate the mediating effect (**Figure 2**). This involved determining

Abbreviations: AITD, autoimmune thyroid diseases; HT, Hashimoto's thyroiditis; GD, Graves' disease; MR, Mendelian randomization; GWAS, genome-wide association studies; FT4, free thyroxin; TSH, Thyroid-stimulating hormone; SNPs, single-nucleotide polymorphisms; ACC, absolute cell count; RCC, relative cell count; MFI, median fluorescence intensity; MP, morphological parameters; DC, dendritic cells; IVW, inverse variance weighting; IVs, Instrumental variables; OR, odds ratio.

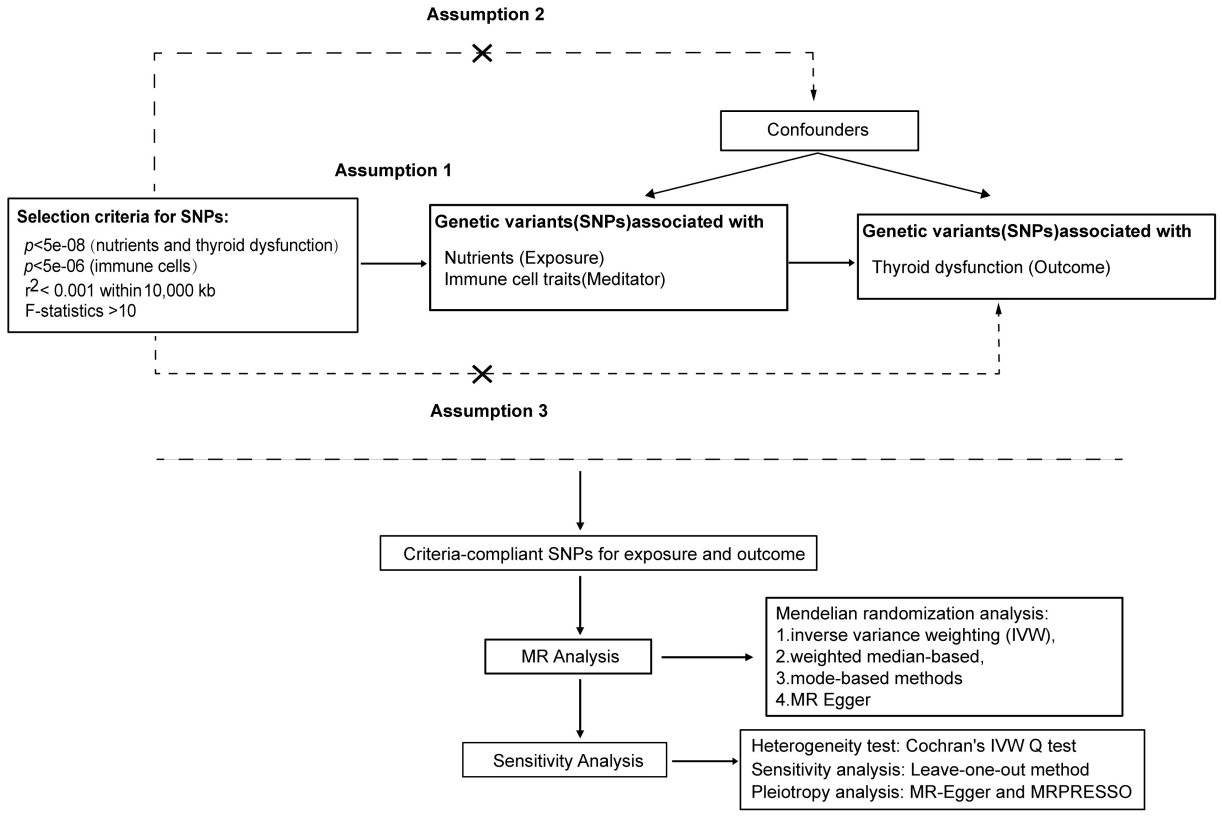

FIGURE 1
MR analysis procedure was shown in the flow chart.

the nutrient's impact on circulating immune cells (β1) and then multiplying it by the effect of the immune cells on thyroid dysfunction (β2). The proportion of the mediating effect (calculated as the mediating effect divided by the total effect [(β1 × β2)/β3]) was then utilized to estimate the overall proportion of the nutrient's impact on thyroid dysfunction that is mediated through circulating immune cells (24).

2.2 Exposure and outcome data sources

2.2.1 Thyroid dysfunction data

The genome-wide association studies (GWAS) data on thyroid diseases and thyroid function are sourced from the ThyroidOmics Consortium (25). This database includes participants of European and non-European ancestry, with exclusions for individuals who have a history of thyroid medication usage or who have undergone thyroid surgery. In each study, only subjects with TSH levels within the cohort-specific reference range were included for the TSH and FT4 analyses. TSH and FT4 were analyzed as continuous variables after inverse normal transformation. Variants with a minor allele frequency of at least 0.5% and an imputation score of at least 0.4 were included in the analysis.

This cohort includes free thyroxin (FT4) from 19 cohorts with 49,269 individuals, and thyroid-stimulating hormone (TSH) from 22 cohorts with a sample size of 54,288 individuals. It also includes two additional groups including hypothyroidism group: 3340 cases with elevated TSH levels, along with a control group of 49,983 cases within the normal reference range, and hyperthyroidism group: 1840/51,823 cases with TSH levels below the reference range. Therefore, these groups included individuals classified as subclinical hypo- or hyperthyroidism cases (25).

2.2.2 Immune trait data

We sourced publicly available GWAS summary data for immune-related traits from the GWAS catalog (GCST0001391 to GCST0002121). The SardiNIA project (26) recruited 6602 volunteers aged 18–102 years (including 57% females and 43%

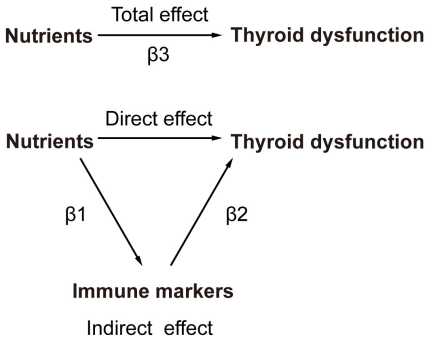

FIGURE 2
The principles of MR Analysis with mediation effects.

males) from Sardinia. And 3,757 of them were immune profiled by collecting peripheral blood and then antibody-stained and processed for flow cytometry. The immune cell panel covered 731 immune phenotypes categorized into absolute cell count (ACC, n = 118), morphological parameters (MP, n = 32), median fluorescence intensity (MFI, n = 389), and relative cell count (RCC, n = 192). MP are typically used to describe the morphological characteristics of cells or tissues. On the other hand, MFI is commonly used to measure the average expression level of a particular marker in cells during flow cytometry. These phenotypes encompassed various immune cell types, such as B cells, T cells, conventional dendritic cells (cDC), myeloid cells, monocytes, TBNK cells, and the Treg panel, distributed across ACC, RCC and MFI features. The MP features included the cDC and TBNK panel. Employing a Sardinian reference panel, a comprehensive analysis was performed on 22 million SNPs.

2.2.3 Nutrients data

Our study investigates three nutrient categories. The summary data for these nutrients were sourced from the total SNPs reported in published papers, which were stored in public GWAS databases and the UK Biobank databases. Antioxidant nutrients include vitamin C (abstrabe), carotene, β-carotene, lycopene, retinol, vitamin E, α-tocopherol, α-tocopherol (metabolite), and γ-tocopherol (27). The data for vitamin C (abstrabe), retinol, and vitamin E are supplemented by multiple databases as additional sources. Mineral nutrients encompass blood selenium, toenail and blood selenium, Ca, Cu, Fe, Mg, and Zn (27, 28). Macronutrients include relative intake of carbohydrates, fat, protein, and sugar, derived from the lead SNPs of the GWAS studied by Meddens et al. (29) (see **Tables 1–3** for detailed information).

2.3 Selection of instrumental variables (IVs)

To prevent the omission of potential causal relationships, we incorporated stronger instrumental variables, allowing for an appropriate relaxation of p-values for immune cells. Instrumental variables were carefully selected using a threshold of $p < 5e{-}08$ for nutrients and thyroid dysfunction, and $p < 5e{-}06$ for immune cells (Using a strict p-value cutoff of 5e−08 led to a small number of SNPs, reducing the representativeness of instrumental variables and weakening statistical power. This threshold also excluded some immune cell variants, hindering the discovery of causal relationships. Therefore, choosing a more lenient p-value threshold like 5e−06 can improve the likelihood of identifying true effects while maintaining error control. This strategy enhances sensitivity, facilitating the detection of significant biological signals). And to ensure data uniformity, a clumping procedure was applied to eliminate variants showing potential linkage disequilibrium (r2 < 0.001 within 10,000 kb). Only SNPs meeting these criteria were included in our MR analysis model. To mitigate bias from weak instrumental variables, SNPs exhibiting F statistics less than 10 were removed from the analysis.

2.4 Statistical analysis

2.4.1 Causal analysis

The methods of inverse variance weighting (IVW) (30), weighted median-based methods (31), weighted mode-based methods (32), and MR Egger (33) have been applied to causal relationship testing using the TwoSampleMR package (30). The results were mainly based on IVW (random effects), followed by sensitivity analysis. A random-effects model was applied when the corresponding p-value was less than 0.05. However, when the p-value exceeded 0.05, an IVW fixed-effect model was employed.

2.4.2 Sensitivity analysis

The selected IVs underwent a heterogeneity test using Cochran's Q statistic (33). To assess the presence of pleiotropy and confirm estimation results, we employed MR-Egger's regression (33, 34). This approach considered an intercept term and excluded SNPs that might influence the outcome through non-exposure pathways. Additionally, the "leave-one-out" method was employed to assess the reliability of this MR results, ensuring they were not influenced by specific SNP results (35). The Causal direction verifies the directionality of each SNP using the Steiger test to ensure the prevention of reverse causality (36).

2.4.3 Statistical tool

The "TwoSampleMR" package (version 0.5.7) within the R software (version 4.3.1) was utilized for causal relationship testing and sensitivity analysis. The "forestploter" package (version 1.1.1) was used for generating forest plots, visualizing the comparison of effect sizes and confidence intervals across multiple results. The code can be found in **Supplementary Material 3**.

3 Results

3.1 Total effect of nutrients on thyroid dysfunction

The IVW assessment reveals statistically significant associations between nutrients and thyroid dysfunction, as illustrated in **Figure 3**. Cu (OR = 1.31, 95% CI = 1.06–1.62, p = 1.12e−02) and ß-carotene (OR = 1.32, 95% CI = 1.05–1.65, p = 1.56e−02) are associated with an increased risk of hyperthyroidism. Lycopene increases the risk of hypothyroidism by 1.21 times, while toenail and blood selenium (OR = 0.85, 95% CI = 0.75–0.96, p = 8.90e−03), α-tocopherol (OR = 0.23, 95% CI = 0.07–0.72, p = 1.16e−02), and ß-carotene (OR = 0.75, 95% CI = 0.63–0.91, p = 3.06e−03) serve as protective factors against hypothyroidism. Toenail and blood selenium (OR = 0.93, 95% CI = 0.91-0.96, p = 3.13e−05) also exhibit a protective effect on FT4 levels. However, Fe (OR = 1.07, 95% CI = 1.01–1.13, p = 2.73e−02), α-tocopherol (OR = 1.40, 95% CI = 1.04–1.88, p = 2.63e−02), and ß-carotene (OR = 1.10, 95% CI = 1.05–1.15, p = 9.64e−05) pose a risk of elevated FT4 levels. Additionally, Ca increases the risk of elevated TSH by 1.30 times. Other mineral nutrients, antioxidants, and genetically predicted carbohydrates, proteins, fats, and carbohydrates are not directly related to thyroid dysfunction.

TABLE 1 Information on instrumental variables and their sources for antioxidant nutrients.

	Antioxidant nutrients[a]	First author (year)	Consortium	Sample size	Sex	Population	PMID/UKBID	IVs (nSNPs)			
								Hyper-	Hypo-	FT4	TSH
1	Vitamin C	Zheng et al. (35)	Mixed[b]	52,018	53.7% female	European	33203707	11	11	11	10
	Vitamin C (UKB)	Elsworth (63)	MRC-IEU	64,979	Mixed[c]	European	ukb-b-19390	22	23	24	24
	Ascorbate	Shine et al. (15)	TwinsUK and KORA	2,085	Mixed[c]	European	24816252	12	12	12	12
2	Carotene (UKB)	Elsworth (63)	MRC-IEU	64,979	Mixed[c]	European	ukb-b-16202	25	27	25	25
3	β-carotene	Ferrucci (64)	Mixed[b]	∼3000	Mixed[c]	European	19185284	5	5	5	5
4	Lycopene	Adamo (65)	HAPI	441	42.4% female	Caucasian	26861389	5	5	5	5
5	Retinol (UKB)	Elsworth (63)	MRC-IEU	62,991	Mixed[c]	European	ukb-b-17406	18	19	20	19
	Retinol	Mondul (66)	ATBC and PLCO	5,006	100% male	Caucasian	21878437	2	2	2	2
6	Vitamin E (UKB)	Elsworth (63)	MRC-IEU	64,979	Mixed[c]	European	ukb-b-6888	28	29	28	27
	α-tocopherol/ Vitamin E	Major (67)	ATBC and PLCO	5,006	100% male	European	21729881	3	3	3	3
7	α-tocopherol (metabolite)	Shine et al. (15)	TwinsUK and KORA	7,725	Mixed[c]	European	24816252	10	10	10	10
8	γ-tocopherol	Shine et al. (15)	TwinsUK and KORA	6,226	Mixed[c]	European	24816252	13	13	13	13

[a]Multiple datasets of a certain antioxidant were used as complementary analyses to the main one.
[b]The data for this study were sourced from more than two Consortiums. The specific information can be found in the original paper.
[c]Information on the sex ratios for the whole sample were not reported or not possible to calculate.
MRC-IEU, The MRC Integrative Epidemiology Unit at the University of Bristol; NHS, The Nurses' Health Study; HAPI, The Heredity and Phenotype Intervention Heart Study; ATBC, The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; PLCO, The Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial; TwinsUK, The Adult UK Twins Study; KORA, The Cooperative Health Research in the Region of Augsburg; Hyper-, Hyperthyroidism; Hypo-, Hypothyroidism; UKB, UK Biobank.

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TABLE 2 Information on instrumental variables and their sources for mineral nutrients.

	Mineral nutrients	First author (year)	Consortium	Sample size	Sex	Population	PMID	IVs (nSNPs)			
								Hyper-	Hypo-	FT4	TSH
1	Blood selenium	Evans (68)	QIMR and ALSPAC	2,603	Mixed[b]	European	23720494	13	13	13	13
2	Toenail and blood selenium	Cornelis (69)	Mixed[a]	4,162	57.4% female	European	25343990	12	12	12	12
3	Ca	O'Seaghdha (70)	Mixed[a]	39,400	Mixed[b]	Mix + European	24068962	6	6	6	6
4	Mg	Meyer (71)	CHARGE	15,366	33.9% male	European	20700443	4	4	4	4
5	Fe	Benyamin (72)	GISC	23,986	Mixed[b]	European	25352340	11	11	11	10
6	Cu	Evans (68)	QIMR	2,603	Mixed[b]	European	23720494	2	2	2	2
7	Zn	Evans (68)	QIMR	2,603	Mixed[b]	European	23720494	2	2	2	2

[a]The data for this study were sourced from more than two Consortiums. The specific information can be found in the original paper.
[b]Information on the sex ratios for the whole sample were not reported or not possible to calculate.
QIMR, The Queensland Institute of Medical Research; ALSPAC, The Avon Longitudinal Study of Parents and Children; CHARGE, The Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium; GISC, The Genetics of Iron Status Consortium; Hyper-, hyperthyroidism; Hypo-, hypothyroidism.

TABLE 3 Information on instrumental variables and their sources for macronutrients.

	Nutrients	First author (year)	Consortium	Sex	Population	PMID	Sample size	IVs (nSNPs)			
								Hyper-	Hypo-	FT4	TSH
1	Relative intake of carbohydrate	Meddens et al. (29)	Mixed[a]	56.7% female	European	32393786	235,391	11	11	11	11
2	Relative intake of fat			60.6% female			268,922	6	6	6	6
3	Relative intake of protein						268,922	7	7	7	7
4	Relative intake of sugar						268,922	4	4	4	4

[a]The data for this study were sourced from more than two Consortiums. The specific information can be found in the original paper.
Hyper-, hyperthyroidism; Hypo-, hypothyroidism.

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Results from other methods, such as MR Egger, weighted median, and weighted mode, can be found in Supplementary Material 1 and Supplementary Tables 1–4.

3.2 Causal effect of immune cells on thyroid dysfunction

Immunomodulatory effects within different immune cell types, encompassing B cells, cDCs, TBNK cells, Tregs, Myeloid cells, maturation stages of T cells, and monocytes, were analyzed in various thyroid dysfunction conditions (Supplementary Material 1 and Supplementary Tables 5–8). Figures 4–7 illustrate several immune cell factors with the potential to influence thyroid dysfunction, specifically hyperthyroidism, hypothyroidism, FT4, and TSH. Notably, all *p*-values associated with these factors are below the threshold of 0.05. Results from other methods, such as MR Egger, weighted median, and weighted mode, can be found in Supplementary Material 1 and Supplementary Tables 5–8.

3.2.1 Causal effect of immune cells on hyperthyroidism

Figure 4 indicates a significant correlation between hyperthyroidism and 58 immune cell phenotypes, with B cells and T cells (TBNK, Treg, maturation stages of T cells) comprising the majority of these associations. Among B cells, the MFI phenotype exhibits the closest correlation with hyperthyroidism. Out of the 28 immune phenotypes, 27 are associated with MFI, with BRFF- being the most prevalent molecular marker (15/27), all of which act as protective factors for hyperthyroidism. The next most common markers are CD25 (7/27) and CD38 (4/27). With the exception of the CD25 on IgD− CD38br phenotype, which decreases the risk of hyperthyroidism, other phenotypes associated with the CD25 molecule increase the risk of developing hyperthyroidism. Additionally, all CD38 phenotypes are negatively correlated with hyperthyroidism.

In the combination of T cell phenotypes, the ACC phenotype is the most common. ACC phenotypes that are negatively correlated with hyperthyroidism include DP (CD4+ CD8+) AC in the TBNK panel, T cell AC, Resting Treg AC in the Treg panel, and Native DN (CD4− CD8−) AC in the maturation stages of T cells. ACC phenotypes positively correlated with hyperthyroidism all belong to the Treg panel, including CD28− CD8dim AC, CD28− CD127− CD25++ CD8br AC, and CD28− CD25++ CD8br AC. Furthermore, CD45RA− CD28− CD8br AC and CD45RA+ CD28− CD8br AC in Treg show no correlation with hyperthyroidism (OR = 1).

In TBNK cells, two SSC-A molecules related to the MP phenotype are associated with hyperthyroidism, while CD3 (2/11) in Treg and HVEM (3/6) in the maturation stages of T cells are negatively correlated with hyperthyroidism in both immune cell types.

All RCC phenotypes in the T cell combination show an increased risk of hyperthyroidism (e.g., CD8dim %T cell, Secreting Treg %CD4, CD28− CD8dim %T cell, CD39+ CD8br %CD8br), while only one RCC phenotype in B cells, IgD+ CD38br %B cell, is associated with a decreased risk of hyperthyroidism.

Only two immune phenotypes involve cDC cells, where the MFI phenotype of CD80 on monocytes plays a protective role, and CD11c on CD62L+ myeloid DC has a promoting effect. The monocyte panel includes three MFI phenotypes and one RCC phenotype associated with hyperthyroidism. Among these, CD14− CD16+ monocyte %monocyte, CD16 on CD14− CD16+ monocyte, and CCR2 on CD14− CD16+ monocyte have a promoting effect, while CD64 on CD14+ CD16+ monocyte has a protective effect.

3.2.2 Causal effect of immune cells on hypothyroidism

Figure 5 illustrates associations between 39 immune phenotypes and hypothyroidism. In B cells, four RCC phenotypes and one MFI phenotype correlate with hypothyroidism. CD20− CD38− %lymphocyte acts as a protective factor, while the remaining four phenotypes (Unsw Mem %lymphocyte, PB/PC %lymphocyte, Transitional %lymphocyte, CD27 on unsw mem) serve as promoting factors.

All five immune phenotypes involving cDC cells contribute to hypothyroidism promotion. This includes CD62L− plasmacytoid DC %DC, as well as MFI phenotypes related to CD123 (2/5) and CD80 (2/5) molecules. TBNK cells often involve HLA DR molecules, where HLA DR+ T cell AC, HLA DR+ T cell %T cell, HLA DR+ T cell %lymphocyte, and HLA DR+ CD4+ AC all promote hypothyroidism, except for HLA DR++ monocyte %monocyte and CD8 on HLA DR+ CD8br, which do not show any correlation.

In Treg cells, Activated & secreting Treg %CD4 Treg and CD28 on CD39+ secreting Treg are negatively correlated with hypothyroidism, while CD28− CD8dim %CD8dim, CD45RA+ CD28− CD8br %T cell, CD3 on CD28+ CD4+, and CD28 on CD28+ CD45RA+ CD8br show positive correlations.

Among the maturation stages of T cells, four RCC phenotypes and two MFI phenotypes are associated with hypothyroidism, with the CD3 molecule showing a positive correlation. Both immune phenotypes involving myeloid cells act as protective factors for hypothyroidism. In monocyte cells, HLA DR on CD14− CD16+ monocyte decreases the risk of hypothyroidism by 0.83 times, while HLA DR on CD14+ CD16+ monocyte increases the risk of hypothyroidism by 1.23 times.

3.2.3 Causal effect of immune cells on FT4 levels

Figure 6 elucidates the impact of immune cell phenotypes on FT4 levels, serving as a direct reflection of changes in thyroid function. It is essential to recognize that FT4 levels can be influenced by various factors, such as those observed in patients with Hashimoto's disease. These individuals may initially exhibit transient increases in FT4 levels, followed by a decline due to autoimmune attacks on the thyroid.

A total of 33 immune phenotypes are associated with variations in FT4 levels, with MFI phenotypes showing the closest relationship in 27 out of 33 instances. Notably, the majority of these immune phenotypes (23/33) act as protective factors for changes in FT4 levels. Within this context, the T cell combination emerges as pivotal, showcasing a significant negative correlation with TSH levels. All immune phenotypes in the TBNK, Treg, and Maturation stages of T cell panels (14/14) contribute to decreasing FT4 levels.

3.2.4 Causal effect of immune cells on TSH levels

Figure 7 delineates 37 immune markers associated with TSH levels, with 31 of them promoting increased TSH levels. Within

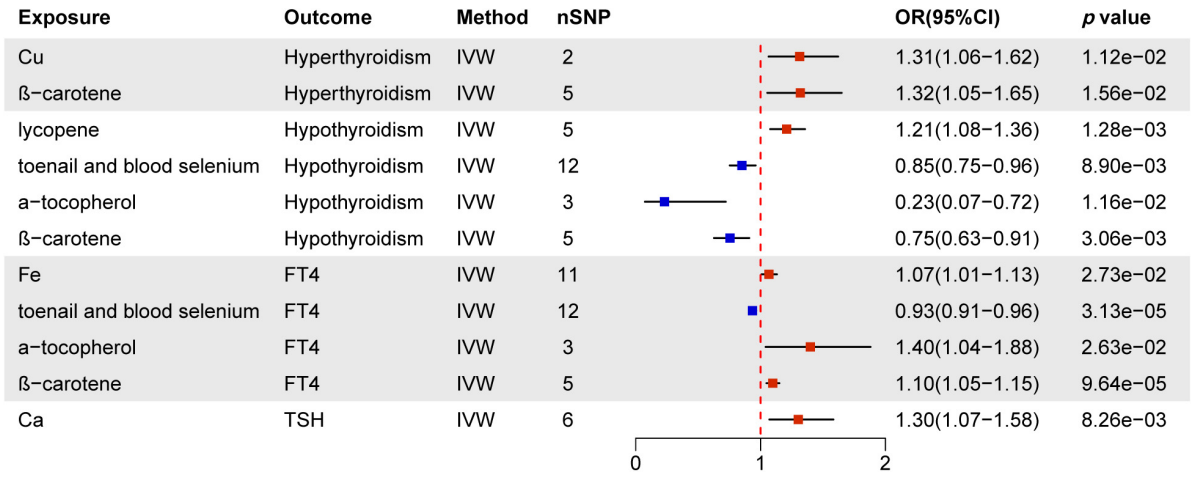

FIGURE 3
MR estimates of the causality between nutrients and thyroid dysfunction. IVW, inverse variance weighting; OR, odds ratio.

B cells, the most prevalent immune cell phenotypes linked to MFI markers are CD19 (4/13) and CD20 (4/13), followed by IgD (2/13). In addition to these mentioned immune cells, CD20− %B cell is associated with decreased TSH levels, while IgD+ CD24− %B cell and CD20− AC promote TSH elevation

Within cDC (FSC-A on monocyte, CD11c on CD62L+ myeloid DC, HLA DR on plasmacytoid DC, HLA DR on DC) and TBNK (HLA DR++ monocyte AC, CD4+ AC, CD3 on HLA DR+ CD8br, CD45 on HLA DR+ T cell), all immune phenotypes are positively correlated with elevated TSH levels. The CD127 molecule, related to MFI phenotypes, shows a negative correlation with TSH levels in two distinct immune marker cells (CD127 on CD45RA− CD4 not Treg, CD127 on CD28+ CD4+). Other factors negatively correlated with TSH levels include CD66b on Gr MDSC in myeloid cells, TD DN (CD4−CD8−) %T cell in maturation stages of T cells, and CD14+ CD16− monocyte %monocyte. All other factors are positively associated with increased TSH levels.

3.3 Causal effect of nutrients and immune cells

Figure 8 provides a comprehensive summary of the significant effects of nutrients on immune cells, highlighting associations with 42 immune cell phenotypes, all supported by statistically significant *p*-values. Notably, lycopene, toenail and blood selenium, and α-tocopherol emerge as influential factors.

Lycopene predominantly influences T cells and monocytes, notably by increasing CD28− CD127− CD25++ CD8br and CD28− CD25++ CD8br T cell Absolute Count. Simultaneously, it decreases FSC-A on monocytes, CD14 on CD14+ CD16− monocytes, CCR2 on monocytes, and SSC-A on HLA DR+ CD4+ T cells.

Toenail and blood selenium exhibit the most substantial impact on immune cells (32/44). This includes 15 B cell subsets, 11 T cell subsets, 4 monocyte subsets, 1 cDC cell (CD62L− plasmacytoid DC %DC), and 1 granulocyte (CD80 on granulocyte). MFI phenotypes demonstrate significant associations, with CD25 (6/32) being the most commonly expressed molecule across different immune cell types. CD38 (2/32), IgD (2/32), HVEM (2/32), CD16 (1/32), and CCR2 (1/32) molecules are negatively regulated by toenail and blood selenium across various immune cell types. Furthermore, toenail and blood selenium positively regulate immune cells associated with MFI, RCC-related phenotypes, ACC-related phenotypes, and MP-related phenotypes.

Additionally, α-tocopherol promotes ACC-related phenotypes, including activated & secreting CD4 Treg %CD4+ T cells, HLA DR+ T cells % T cells, and HLA DR+ T cells % lymphocytes. However, it inhibits the Unsw Mem %lymphocyte phenotype. Results from other methods, such as MR Egger, weighted median, and weighted mode, can be found in **Supplementary Material 1** **Supplementary Table 9**.

3.4 Mediation analysis

Our MR analysis revealed distinct effects of genetically predicted nutrients (Lycopene, toenail and blood selenium, and α-tocopherol and its metabolite) on immune cells. Additionally, we observed a significant association between genetically predicted immune cells and thyroid dysfunction (Table 4). Figure 9 presents the results of our two-step MR analyses, illustrating the proportion of the indirect effect mediated by immune cells in the relationship between genetically predicted nutrients and the likelihood of thyroid dysfunction.

The results revealed that toenail and blood selenium levels influenced the RCC of specific immune cell, such as CD62L− plasmacytoid dendritic cells %DC and transitional B cells% lymphpcyte, mediating the association between selenium levels and hypothyroidism. Furthermore, toenail and blood selenium also impacted T cells, including one trait related to ACC: CD25++ CD8br T cell AC, and two traits related to MFI: CD3 on CD45RA− CD4+ T cell, and CD45RA on Terminally Differentiated CD8br T cell, The CD62L− plasmacytoid DC %DC is positively associated with hypothyroidism (OR = 1.18, 95% CI = 1.05–1.32, p = 4.78e−03). Toenail and blood selenium can

Panel	Exposure	Outcome	Method	nSNP	OR(95%CI)	*p* value
B cell	IgD+ CD38br %B cell	Hyperthyroidism	IVW	12	0.86(0.75−0.98)	2.57e−02
	BAFF−R on CD24+ CD27+	Hyperthyroidism	IVW	10	0.89(0.83−0.96)	1.23e−03
	BAFF−R on IgD+ CD24+	Hyperthyroidism	IVW	9	0.89(0.83−0.96)	1.93e−03
	BAFF−R on IgD+ CD38−	Hyperthyroidism	IVW	9	0.89(0.83−0.96)	2.02e−03
	BAFF−R on IgD+ CD38− unsw mem	Hyperthyroidism	IVW	12	0.91(0.85−0.97)	3.51e−03
	BAFF−R on IgD+ CD38br	Hyperthyroidism	IVW	11	0.92(0.86−0.99)	3.26e−02
	BAFF−R on IgD+ CD38dim	Hyperthyroidism	IVW	11	0.93(0.86−1.00)	4.00e−02
	BAFF−R on IgD− CD24−	Hyperthyroidism	IVW	9	0.89(0.83−0.96)	3.47e−03
	BAFF−R on IgD− CD27−	Hyperthyroidism	IVW	9	0.90(0.83−0.97)	4.64e−03
	BAFF−R on IgD− CD38−	Hyperthyroidism	IVW	6	0.91(0.85−0.98)	1.57e−02
	BAFF−R on IgD− CD38br	Hyperthyroidism	IVW	10	0.84(0.73−0.97)	1.98e−02
	BAFF−R on memory B cell	Hyperthyroidism	IVW	10	0.89(0.83−0.96)	2.25e−03
	BAFF−R on naive−mature B cell	Hyperthyroidism	IVW	11	0.93(0.87−1.00)	4.63e−02
	BAFF−R on unsw mem	Hyperthyroidism	IVW	9	0.89(0.83−0.95)	1.31e−03
	BAFF−R on transitional	Hyperthyroidism	IVW	10	0.90(0.84−0.97)	5.97e−03
	CD20 on IgD+ CD38−	Hyperthyroidism	IVW	11	0.82(0.72−0.94)	3.52e−03
	CD25 on B cell	Hyperthyroidism	IVW	9	1.16(1.01−1.34)	3.51e−02
	CD25 on IgD+ CD24−	Hyperthyroidism	IVW	8	1.23(1.08−1.41)	2.50e−03
	CD25 on IgD+ CD38dim	Hyperthyroidism	IVW	10	1.16(1.01−1.33)	3.59e−02
	CD25 on IgD− CD38−	Hyperthyroidism	IVW	9	1.19(1.04−1.35)	9.20e−03
	CD25 on IgD− CD38br	Hyperthyroidism	IVW	9	0.82(0.68−1.00)	4.55e−02
	CD25 on naive−mature B cell	Hyperthyroidism	IVW	11	1.19(1.05−1.36)	7.25e−03
	CD25 on IgD+	Hyperthyroidism	IVW	8	1.24(1.06−1.45)	7.32e−03
	CD38 on IgD+ CD24−	Hyperthyroidism	IVW	9	0.83(0.70−0.97)	2.18e−02
	CD38 on IgD+ CD38br	Hyperthyroidism	IVW	6	0.76(0.60−0.95)	1.46e−02
	CD38 on IgD+ CD38dim	Hyperthyroidism	IVW	12	0.87(0.77−0.98)	2.21e−02
	CD38 on IgD− CD38dim	Hyperthyroidism	IVW	7	0.83(0.70−0.98)	2.52e−02
	BAFF−R on B cell	Hyperthyroidism	IVW	10	0.91(0.84−0.97)	7.94e−03
cDC	CD80 on monocyte	Hyperthyroidism	IVW	10	0.86(0.77−0.97)	1.15e−02
	CD11c on CD62L+ myeloid DC	Hyperthyroidism	IVW	15	1.14(1.02−1.28)	2.12e−02
TBNK	DP (CD4+CD8+) AC	Hyperthyroidism	IVW	7	0.83(0.71−0.97)	2.13e−02
	CD8dim %T cell	Hyperthyroidism	IVW	5	1.41(1.09−1.82)	9.87e−03
	Lymphocyte AC	Hyperthyroidism	IVW	5	0.63(0.49−0.80)	1.94e−04
	T cell AC	Hyperthyroidism	IVW	6	0.79(0.63−0.99)	3.73e−02
	CD45 on T cell	Hyperthyroidism	IVW	4	1.33(1.02−1.74)	3.55e−02
	SSC−A on NKT	Hyperthyroidism	IVW	10	1.21(1.04−1.40)	1.38e−02
	SSC−A on HLA DR+ CD4+	Hyperthyroidism	IVW	7	0.80(0.65−0.98)	2.76e−02
Treg	Resting Treg AC	Hyperthyroidism	IVW	13	0.87(0.76−0.99)	3.61e−02
	Secreting Treg %CD4	Hyperthyroidism	IVW	12	1.20(1.05−1.37)	6.95e−03
	CD28− CD8dim %T cell	Hyperthyroidism	IVW	13	1.16(1.02−1.32)	2.72e−02
	CD28− CD8dim AC	Hyperthyroidism	IVW	12	1.14(1.01−1.30)	3.72e−02
	CD39+ CD8br %CD8br	Hyperthyroidism	IVW	5	1.16(1.01−1.32)	3.86e−02
	CD28− CD127− CD25++ CD8br AC	Hyperthyroidism	IVW	8	1.27(1.08−1.49)	4.37e−03
	CD28− CD25++ CD8br AC	Hyperthyroidism	IVW	7	1.24(1.04−1.49)	1.65e−02
	CD45RA− CD28− CD8br AC	Hyperthyroidism	IVW	23	1.00(1.00−1.00)	2.21e−07
	CD45RA+ CD28− CD8br AC	Hyperthyroidism	IVW	56	1.00(1.00−1.00)	7.56e−07
	CD3 on CD39+ resting Treg	Hyperthyroidism	IVW	7	0.86(0.75−0.99)	3.90e−02
	CD3 on activated Treg	Hyperthyroidism	IVW	10	0.90(0.83−0.98)	1.69e−02
Maturation stages of T cell	EM CD4+ %T cell	Hyperthyroidism	IVW	12	1.19(1.02−1.39)	3.16e−02
	Naive CD8br %CD8br	Hyperthyroidism	IVW	11	1.24(1.00− 1.54)	4.68e−02
	Naive DN (CD4−CD8−) AC	Hyperthyroidism	IVW	8	1.24(1.01−1.52)	3.69e−02
	HVEM on T cell	Hyperthyroidism	IVW	12	0.89(0.80−0.98)	1.68e−02
	HVEM on TD CD4+	Hyperthyroidism	IVW	8	0.89(0.79−0.99)	3.01e−02
	HVEM on CD8br	Hyperthyroidism	IVW	6	0.85(0.75−0.97)	1.41e−02
Monocyte	CD14− CD16+ monocyte %monocyte	Hyperthyroidism	IVW	7	1.27(1.06−1.53)	1.04e−02
	CD16 on CD14− CD16+ monocyte	Hyperthyroidism	IVW	8	1.13(1.03−1.22)	5.75e−03
	CCR2 on CD14− CD16+ monocyte	Hyperthyroidism	IVW	10	1.16(1.00−1.34)	4.99e−02
	CD64 on CD14+ CD16+ monocyte	Hyperthyroidism	IVW	7	0.83(0.68−1.00)	4.83e−02

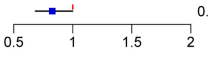

FIGURE 4
MR estimates of the causality between immune cells and hyperthyroidism. IVW, inverse variance weighting; OR, odds ratio; unsw mem, unswitched memory; AC, cell absolute count.

exert a negative regulatory effect on hypothyroidism by reducing the presence of this cell type (OR = −0.14, 95% CI = −0.26 to 0.03, p = 1.63e−02). This finding aligns with the direct protective effect of toenail and blood selenium against hypothyroidism. The proportion of the indirect effect mediated by CD62L− plasmacytoid DC %DC was 14.5%. Similarly, the transitional B cells %Lymphocyte serves as a mediator negatively regulated by toenail and blood selenium, leading to a reduction in the risk of

Panel	Exposure	Outcome	Method	nSNP	OR(95%CI)	*p* value
B cell	Unsw Mem %lymphocyte	Hypothyroidism	IVW	13	1.13(1.00–1.28)	4.54e−02
	PB/PC %lymphocyte	Hypothyroidism	IVW	8	1.27(1.08–1.50)	3.61e−03
	CD20− CD38− %lymphocyte	Hypothyroidism	IVW	8	0.79(0.66–0.95)	1.03e−02
	Transitional %lymphocyte	Hypothyroidism	IVW	14	1.13(1.00–1.27)	4.45e−02
	CD27 on unsw mem	Hypothyroidism	IVW	20	1.08(1.00–1.15)	3.88e−02
cDC	CD62L− plasmacytoid DC %DC	Hypothyroidism	IVW	6	1.18(1.05–1.32)	4.78e−03
	CD123 on plasmacytoid DC	Hypothyroidism	IVW	4	1.20(1.05–1.36)	8.23e−03
	CD123 on CD62L+ plasmacytoid DC	Hypothyroidism	IVW	4	1.20(1.05–1.37)	8.33e−03
	CD80 on CD62L+ myeloid DC	Hypothyroidism	IVW	9	1.14(1.02–1.28)	2.38e−02
	CD80 on plasmacytoid DC	Hypothyroidism	IVW	9	1.13(1.01–1.25)	2.92e−02
TBNK	HLA DR++ monocyte %monocyte	Hypothyroidism	IVW	6	0.74(0.64–0.85)	4.52e−05
	DN (CD4−CD8−) AC	Hypothyroidism	IVW	9	1.21(1.07–1.37)	1.99e−03
	DN (CD4−CD8−) %leukocyte	Hypothyroidism	IVW	9	1.19(1.04–1.37)	1.20e−02
	HLA DR+ T cell AC	Hypothyroidism	IVW	13	1.09(1.00–1.19)	5.00e−02
	HLA DR+ T cell%T cell	Hypothyroidism	IVW	7	1.15(1.05–1.27)	4.08e−03
	HLA DR+ T cell%lymphocyte	Hypothyroidism	IVW	9	1.17(1.08–1.27)	2.10e−04
	HLA DR+ CD4+ AC	Hypothyroidism	IVW	10	1.14(1.01–1.29)	2.83e−02
	CD45 on NKT	Hypothyroidism	IVW	5	0.84(0.72–0.99)	3.33e−02
	FSC−A on CD14+ monocyte	Hypothyroidism	IVW	6	0.76(0.63–0.92)	5.22e−03
	FSC−A on HLA DR+ NK	Hypothyroidism	IVW	8	0.88(0.77–0.99)	3.84e−02
	CD8 on HLA DR+ CD8br	Hypothyroidism	IVW	8	0.88(0.78–0.99)	4.01e−02
Treg	Activated & secreting Treg %CD4 Treg	Hypothyroidism	IVW	11	0.88(0.79–0.98)	2.44e−02
	CD28− CD8dim %CD8dim	Hypothyroidism	IVW	7	1.22(1.03–1.45)	2.03e−02
	CD45RA− CD28− CD8br AC	Hypothyroidism	IVW	23	1.00(1.00–1.00)	8.20e−05
	CD45RA+ CD28− CD8br AC	Hypothyroidism	IVW	57	1.00(1.00–1.00)	3.41e−06
	CD45RA+ CD28− CD8br %T cell	Hypothyroidism	IVW	15	1.02(1.01–1.04)	6.70e−03
	CD3 on CD28+ CD4+	Hypothyroidism	IVW	14	1.08(1.00–1.17)	3.99e−02
	CD28 on CD39+ secreting Treg	Hypothyroidism	IVW	8	0.90(0.81–0.99)	3.39e−02
	CD28 on CD28+ CD45RA+ CD8br	Hypothyroidism	IVW	8	1.14(1.04–1.25)	3.63e−03
Myeloid cell	Im MDSC AC	Hypothyroidism	IVW	10	0.92(0.85–0.99)	3.13e−02
	HLA DR on CD33dim HLA DR+ CD11b−	Hypothyroidism	IVW	8	0.91(0.84–0.99)	2.93e−02
Maturation stages of T cell	EM CD8br %CD8br	Hypothyroidism	IVW	10	0.87(0.76–1.00)	4.88e−02
	TD CD8br %T cell	Hypothyroidism	IVW	5	0.82(0.69–0.97)	2.27e−02
	Naive DN (CD4−CD8−) %DN	Hypothyroidism	IVW	9	1.17(1.00–1.37)	4.77e−02
	EM DN (CD4−CD8−) %T cell	Hypothyroidism	IVW	12	1.15(1.02–1.30)	2.53e−02
	CD3 on CM CD4+	Hypothyroidism	IVW	14	1.10(1.03–1.18)	4.12e−03
	CD3 on Naive CD4+	Hypothyroidism	IVW	17	1.06(1.00–1.12)	4.37e−02
Monocyte	HLA DR on CD14− CD16+ monocyte	Hypothyroidism	IVW	6	0.83(0.74–0.92)	7.16e−04
	HLA DR on CD14+ CD16+ monocyte	Hypothyroidism	IVW	6	1.23(1.04–1.44)	1.26e−02

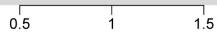

FIGURE 5
MR estimates of the causality between immune cells and hypothyroidism. IVW, inverse variance weighting; OR, odds ratio; unsw mem, unswitched memory; AC, cell absolute count; DC: Dendritic cells.

hypothyroidism. The proportion of the indirect effect mediated by transitional B cells %Lymphocyte is 13.0%.

CD25++ CD8+ T cell Absolute Count (OR = 0.97, 95% CI = 0.95–1.00, p = 3.45e−02), CD3 on CD45RA− CD4+ T cell (OR = 0.98, 95% CI = 0.96–1.00, p = 3.62e−02), and CD45RA on Terminally Differentiated CD8+ T cell (OR = 0.96, 95% CI = 0.92–1.00, p = 3.34e−02) are negative regulatory factors for FT4 levels. Toenail and blood selenium amplify the protective effect of three immune markers on FT4 levels, as indicated by their positive correlations (ORs of 0.18, 0.16, and 0.14, respectively). The proportion of the indirect effect mediated by these factors is 7.3%, 4.4%, and 8.8%, respectively.

3.5 Sensitivity analysis

The sensitivity analysis results are assessed and provided in Supplementary Material 2. The results of heterogeneity and pleiotropy tests ($p > 0.05$) suggest the absence of heterogeneity and pleiotropy in the MR study. Additionally, the "leave-one-out" analysis shows that the overall results remain relatively stable and do not change significantly after excluding each SNP. The results of the Steiger test are all "True". All these results collectively confirm the reliable causal effect of nutrients on immune cells and thyroid function.

4 Discussion

Our MR analysis provides compelling evidence for the role of toenail and blood selenium in genetically reducing the risk of thyroid dysfunction. Our study findings indicate that genetically predicted immune cells may act as mediators, and toenail and blood selenium may offer protection against the occurrence of hypothyroidism and abnormal FT4 levels.

Panel	Exposure	Outcome	Method	nSNP	OR(95%CI)	*p* value
B cell	IgD− CD38dim %B cell	FT4	IVW	10	1.03(1.00−1.06)	3.50e−02
	Sw mem %lymphocyte	FT4	IVW	9	1.05(1.02−1.09)	4.75e−03
	IgD− CD38dim AC	FT4	IVW	9	1.04(1.00−1.09)	4.77e−02
	BAFF−R on IgD+ CD38− unsw mem	FT4	IVW	12	1.01(1.00−1.03)	3.88e−02
	BAFF−R on IgD− CD24−	FT4	IVW	10	1.02(1.00−1.03)	2.57e−02
	BAFF−R on IgD− CD38br	FT4	IVW	10	1.04(1.01−1.07)	8.03e−03
	CD19 on IgD+ CD38− naive	FT4	IVW	7	0.95(0.91−0.98)	9.88e−04
	CD20 on CD24+ CD27+	FT4	IVW	11	0.97(0.94−1.00)	2.81e−02
	CD38 on transitional	FT4	IVW	12	0.97(0.94−1.00)	3.26e−02
	IgD on IgD+ CD38br	FT4	IVW	9	0.95(0.92−0.98)	2.02e−03
	IgD on transitional	FT4	IVW	13	0.96(0.94−0.99)	1.10e−02
cDC	CD62L− HLA DR++ monocyte %monocyte	FT4	IVW	5	1.05(1.00−1.10)	4.35e−02
	CD80 on myeloid DC	FT4	IVW	12	0.97(0.94−1.00)	3.25e−02
	CD80 on granulocyte	FT4	IVW	20	1.02(1.00−1.05)	3.40e−02
TBNK	CD45 on CD8br	FT4	IVW	8	0.96(0.93−1.00)	3.27e−02
	CD4 on HLA DR+ CD4+	FT4	IVW	7	0.96(0.93−1.00)	4.41e−02
Treg	CD4 Treg %T cell	FT4	IVW	8	0.96(0.92−0.99)	2.08e−02
	CD25++ CD8br AC	FT4	IVW	17	0.97(0.95−1.00)	3.45e−02
	CD3 on resting Treg	FT4	IVW	18	0.96(0.94−0.98)	8.34e−04
	CD28 on CD4+	FT4	IVW	15	0.97(0.95−1.00)	2.10e−02
	CD25 on CD39+ activated Treg	FT4	IVW	6	0.96(0.92−1.00)	4.63e−02
	CD4 on CD39+ activated Treg	FT4	IVW	6	0.96(0.92−1.00)	3.62e−02
	CD8 on CD28+ CD45RA+ CD8br	FT4	IVW	6	0.97(0.95−1.00)	1.53e−02
Myeloid cell	CD66b on CD66b++ myeloid cell	FT4	IVW	15	1.03(1.01−1.05)	1.54e−02
	CD45 on CD33br HLA DR+ CD14dim	FT4	IVW	4	0.96(0.93−1.00)	3.06e−02
Maturation stages of T cell	CD3 on CD45RA− CD4+	FT4	IVW	18	0.98(0.96−1.00)	3.62e−02
	HVEM on naive CD8br	FT4	IVW	9	0.97(0.95−1.00)	1.86e−02
	HVEM on naive CD4+	FT4	IVW	6	0.96(0.93−0.98)	1.72e−03
	HVEM on EM CD4+	FT4	IVW	7	0.96(0.93−0.99)	2.74e−03
	CD45RA on TD CD8br	FT4	IVW	6	0.96(0.92−1.00)	3.34e−02
Monocyte	CD14 on CD14+ CD16− monocyte	FT4	IVW	10	0.96(0.93−0.99)	1.97e−02
	CX3CR1 on CD14− CD16−	FT4	IVW	7	1.04(1.00−1.07)	4.42e−02
	CCR2 on monocyte	FT4	IVW	11	0.96(0.93−0.99)	1.73e−02

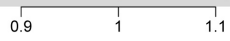

FIGURE 6
MR estimates of the causality between immune cells and FT4 level. IVW, inverse variance weighting; OR, odds ratio; unsw mem, unswitched memory; AC, cell absolute count; DC: dendritic cells.

Several nutrients are well-known for their influence on thyroid function. Iodine, an important element for thyroid hormone synthesis, is associated with hypothyroidism and goiter when deficient (37, 38). Excessive iodine can disrupt thyroid function, primarily through oxidative stress, but it generally affects only a small percentage of individuals susceptible to autoimmune thyroid diseases (39). Selenoproteins play a crucial role in regulating T cell proliferation, differentiation, and redox metabolism, reducing excessive immune responses and chronic inflammation by preventing the overproduction of reactive oxygen species (40). Zinc, functioning as a signaling molecule, antioxidant, and immune modulator (41–43), is also involved in thyroglobulin metabolism, regulating enzyme activities and modifying the structures of transcription factors related to TG synthesis (44). Zinc deficiency is implicated in hypothyroidism development (45). Additionally, copper, calcium, and magnesium impact thyroid function (46, 47).

Selenium, acting as a cofactor for enzymes in thyroid cells and participating in thyroglobulin synthesis (48), plays a vital role in governing thyroid hormone metabolism through three specific selenoproteins, namely iodothyronine deiodinases 1–3 (49). Insufficient selenium levels increases the risk of thyroid dysfunction, including autoimmune thyroid diseases, while selenium supplementation can improve clinical symptoms (50, 51), further supported by our research.

In vivo research indicates that high selenium levels promote the proliferation and differentiation of CD4 Th cells, particularly Th1 (52). Selenium supplements in aged mice enhance the proliferation of cytotoxic T cells induced by mitogens, although limited information is available regarding selenium's impact on cytotoxic CD8 T cells (53). The deletion of the *trsp* gene, crucial for selenoprotein synthesis, influences T cell functionality and antibody secretion by B cells (54). Dietary selenium intake also influences Natural Killer (NK) cells, with serum selenium concentrations in elderly individuals positively correlated with peripheral CD16 NK cells (55). Selenium has been suggested to modulate the migration and phagocytic function of macrophages (56).

Selenium is an important risk factor for AITD (57). Selenium deficiency is often associated with immune dysfunction (58, 59). Studies suggest that Selenium may reduce thyroid antibodies by upregulating activated Treg cells (60). Selenium deficiency may upregulate Th1/Th2 effector molecules and enhance immune

Panel	Exposure	Outcome	Method	nSNP	OR(95%CI)	*p* value
B cell	IgD+ CD24− %B cell	TSH	IVW	7	1.05(1.01−1.10)	2.07e−02
	CD20− %B cell	TSH	IVW	6	0.94(0.90−0.99)	9.59e−03
	CD20− AC	TSH	IVW	10	1.04(1.00−1.07)	4.53e−02
	CD19 on CD24+ CD27+	TSH	IVW	18	1.04(1.01−1.06)	4.54e−03
	CD19 on IgD+ CD24+	TSH	IVW	13	1.03(1.00−1.07)	4.85e−02
	CD19 on IgD− CD38−	TSH	IVW	9	1.04(1.01−1.07)	2.15e−02
	CD19 on unsw mem	TSH	IVW	9	1.05(1.01−1.09)	1.43e−02
	CD20 on CD24+ CD27+	TSH	IVW	11	1.03(1.00−1.06)	3.39e−02
	CD20 on IgD+ CD24+	TSH	IVW	9	1.04(1.01−1.08)	2.15e−02
	CD20 on IgD+ CD38− naive	TSH	IVW	10	1.04(1.02−1.07)	1.39e−03
	CD20 on sw mem	TSH	IVW	15	1.03(1.01−1.05)	6.01e−03
	IgD on IgD+ CD24−	TSH	IVW	11	1.03(1.01−1.06)	1.29e−02
	IgD on IgD+ CD38br	TSH	IVW	8	1.04(1.01−1.07)	1.52e−02
cDC	FSC−A on monocyte	TSH	IVW	10	1.03(1.00−1.06)	4.91e−02
	CD11c on CD62L+ myeloid DC	TSH	IVW	15	1.02(1.00−1.05)	3.41e−02
	HLA DR on plasmacytoid DC	TSH	IVW	7	1.02(1.01−1.04)	3.57e−03
	HLA DR on DC	TSH	IVW	7	1.02(1.01−1.04)	6.77e−03
TBNK	HLA DR++ monocyte AC	TSH	IVW	7	1.04(1.00−1.09)	4.45e−02
	CD4+ AC	TSH	IVW	10	1.07(1.02−1.12)	1.03e−02
	CD3 on HLA DR+ CD8br	TSH	IVW	10	1.03(1.00−1.05)	3.39e−02
	CD45 on HLA DR+ T cell	TSH	IVW	5	1.04(1.00−1.07)	2.55e−02
Treg	CD39+ activated Treg %CD4 Treg	TSH	IVW	10	1.02(1.00−1.04)	3.67e−02
	Secreting Treg %CD4	TSH	IVW	12	1.03(1.01−1.06)	1.16e−02
	Activated & secreting Treg %CD4+	TSH	IVW	11	1.03(1.00−1.06)	3.51e−02
	CD28+ CD45RA+ CD8dim %T cell	TSH	IVW	16	1.03(1.01−1.06)	1.12e−02
	CD127 on CD45RA− CD4 not Treg	TSH	IVW	8	0.96(0.93−1.00)	3.41e−02
	CD127 on CD28+ CD4+	TSH	IVW	10	0.97(0.94−0.99)	4.09e−03
	CD25 on CD39+ CD4 Treg	TSH	IVW	7	1.06(1.03−1.10)	3.53e−04
Myeloid cell	CD33− HLA DR+ AC	TSH	IVW	9	1.04(1.01−1.06)	1.40e−03
	CD66b on Gr MDSC	TSH	IVW	9	0.98(0.96−1.00)	4.10e−02
	CD45 on basophil	TSH	IVW	9	1.02(1.00−1.05)	4.32e−02
Maturation stages of T cell	Naive CD4+ %CD4+	TSH	IVW	13	1.03(1.00−1.06)	2.87e−02
	TD DN (CD4−CD8−) %T cell	TSH	IVW	12	0.97(0.94−1.00)	4.19e−02
	CD3 on CD45RA− CD4+	TSH	IVW	18	1.02(1.01−1.04)	1.06e−02
Monocyte	CD14+ CD16− monocyte %monocyte	TSH	IVW	9	0.96(0.93−1.00)	3.52e−02
	CD16+ monocyte %monocyte	TSH	IVW	10	1.05(1.01−1.08)	1.39e−02
	CD64 on CD14+ CD16+ monocyte	TSH	IVW	7	1.08(1.01−1.14)	2.27e−02

FIGURE 7
MR estimates of the causality between immune cells and TSH level. IVW, inverse variance weighting;OR, odds ratio; AC, cell absolute count; DC: dendritic cells.

responses. Daily supplementation of 100 μg of Selenium has been shown to improve thyroid function and quality of life in patients by reducing interferon-γ levels and increasing interleukin-1β levels (61).

The MR study contributes genetically evidence establishing a causal relationship between nutrient intake (mineral, antioxidant nutrients, and macronutrients) and thyroid dysfunction. We found that an increase of one unit of Cu results in a 1.31-fold increase in the likelihood of hyperthyroidism. Each unit of Fe results in a 1.07-fold increase in FT4 levels, while each unit of Ca causes a 1.30-fold increase in TSH levels. β-carotene promotes hyperthyroidism and increases FT4 levels but exhibits a negative correlation with hypothyroidism. Toenail and blood selenium act as protective factors against hypothyroidism and FT4 levels. Each unit of α-tocopherol can enhance FT4 levels by 1.40 times and reduce the occurrence of hypothyroidism by 0.23 times. Furthermore, lycopene is positively associated with hypothyroidism. However, other constant nutrient intake does not have a direct causal effect on thyroid dysfunction.

Indeed, we have also discovered that a variety of immune cell phenotypes has an impact on thyroid dysfunction. Specifically, in individuals with hyperthyroidism, MFI-related phenotypes in B cells and ACC-related phenotypes in T cells are the most frequently observed. Conversely, in cases of hypothyroidism, phenotypes of all cDC cells tend to promote the occurrence of hypothyroidism. Notably, MFI phenotypes demonstrate the strongest correlation with FT4 levels. Moreover, alterations in TSH levels are associated with multiple markers within the immune system, with the majority of these markers resulting in an elevation in TSH levels.

Changes in nutrients such as lycopene, toenail and blood selenium, and α-tocopherol have distinct effects on the immune environment. For example, lycopene predominantly influences T cells and monocytes, while α-tocopherol promotes ACC-related

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Exposure	Outcome	Method	nSNP	Estimate(95%CI)	*p* value
lycopene	CD28− CD127− CD25++ CD8br AC	IVW	5	0.18(0.07− 0.29)	1.52e−03
	CD28− CD25++ CD8br AC	IVW	5	0.16(0.05− 0.26)	2.85e−03
	FSC−A on monocyte	IVW	5	−0.17(−0.29−−0.05)	6.12e−03
	CD14 on CD14+ CD16− monocyte	IVW	5	−0.14(−0.25−−0.04)	8.09e−03
	CCR2 on monocyte	IVW	5	−0.13(−0.24−−0.03)	1.52e−02
	SSC−A on HLA DR+ CD4+	IVW	5	−0.12(−0.24−−0.01)	3.97e−02
toenail and blood selenium	CD20− %B cell	IVW	12	0.14(0.03− 0.26)	1.59e−02
	IgD+ CD38br %B cell	IVW	12	−0.11(−0.23− 0.00)	4.65e−02
	CD62L− plasmacytoid DC %DC	IVW	12	−0.14(−0.26−−0.03)	1.63e−02
	TD DN (CD4−CD8−) %T cell	IVW	12	0.15(0.03− 0.27)	1.38e−02
	Transitional %lymphocyte	IVW	12	−0.18(−0.29−−0.06)	2.33e−03
	CD14+ CD16− monocyte %monocyte	IVW	12	0.12(0.01− 0.23)	3.99e−02
	CD8dim %T cell	IVW	12	0.17(0.05− 0.28)	4.90e−03
	DN (CD4−CD8−) %leukocyte	IVW	12	0.14(0.03− 0.25)	1.32e−02
	CD25++ CD8br AC	IVW	12	0.18(0.06− 0.30)	4.46e−03
	CD19 on IgD+ CD24+	IVW	12	0.12(0.00− 0.23)	4.68e−02
	CD19 on unsw mem	IVW	12	0.14(0.03− 0.26)	1.57e−02
	CD25 on B cell	IVW	12	−0.17(−0.28−−0.05)	5.43e−03
	CD25 on IgD+ CD24−	IVW	12	−0.17(−0.29−−0.06)	3.51e−03
	CD25 on IgD+ CD38dim	IVW	12	−0.18(−0.29−−0.06)	3.03e−03
	CD25 on IgD− CD38−	IVW	12	−0.17(−0.29−−0.06)	3.72e−03
	CD25 on naive−mature B cell	IVW	12	−0.21(−0.33−−0.10)	3.60e−04
	CD25 on IgD+	IVW	12	−0.14(−0.26−−0.03)	1.58e−02
	CD38 on IgD+ CD24−	IVW	12	−0.22(−0.34−−0.11)	1.67e−04
	CD38 on IgD+ CD38dim	IVW	12	−0.13(−0.24−−0.01)	3.42e−02
	IgD on IgD+ CD38br	IVW	12	−0.25(−0.36−−0.13)	2.86e−05
	IgD on transitional	IVW	12	−0.24(−0.36−−0.12)	5.64e−05
	CD3 on CM CD4+	IVW	12	0.18(0.05− 0.31)	5.35e−03
	CD3 on CD45RA− CD4+	IVW	12	0.16(0.03− 0.28)	1.72e−02
	HVEM on T cell	IVW	12	−0.25(−0.45−−0.06)	1.10e−02
	HVEM on EM CD4+	IVW	12	−0.22(−0.41−−0.03)	2.55e−02
	CD16 on CD14− CD16+ monocyte	IVW	12	−0.15(−0.27−−0.04)	9.46e−03
	CCR2 on CD14− CD16+ monocyte	IVW	12	−0.14(−0.25−−0.02)	2.06e−02
	CD80 on monocyte	IVW	12	−0.15(−0.27−−0.02)	2.73e−02
	CD80 on granulocyte	IVW	12	0.18(0.05− 0.31)	7.28e−03
	CD8 on HLA DR+ CD8br	IVW	12	−0.22(−0.34−−0.10)	5.13e−04
	SSC−A on NKT	IVW	12	−0.14(−0.26−−0.01)	3.40e−02
	CD45RA on TD CD8br	IVW	12	0.14(0.01− 0.26)	3.66e−02
a−tocopherol (metabolite)	Unsw Mem %lymphocyte	IVW	11	−0.95(−1.79−−0.10)	2.75e−02
a−tocopherol	Activated & secreting Treg %CD4+	IVW	3	1.28(0.21− 2.36)	1.94e−02
	HLA DR+ T cell%T cell	IVW	3	1.05(0.08− 2.03)	3.45e−02
	HLA DR+ T cell%lymphocyte	IVW	3	1.10(0.09− 2.10)	3.19e−02

FIGURE 8
MR estimates of the causality between nutrients and immune cells. IVW, inverse variance weighting; OR, odds ratio; unsw mem, unswitched memory; AC, cell absolute count; DC: dendritic cells.

phenotypes. However, toenail and blood selenium demonstrate the most significant impact on immune cells. Importantly, we found that the effects of nutrients on thyroid dysfunction may be mediated through alterations in the immune environment: CD62L− plasmacytoid DC %DC, and transitional B cells %Lymphocytecan serve as mediators between toenail and blood selenium levels and hypothyroidism. Toenail and blood selenium reduce the levels of these two immune cells, thereby diminishing their promoting effect on hypothyroidism. Furthermore, toenail and blood selenium enhance the levels of CD25++ CD8br T cell Absolute Count, CD3 on CD45RA− CD4+ T cell, and CD45RA on Terminally Differentiated CD8br T cell, thus reinforcing the negative correlation between immune cells and FT4 levels. In summary, toenail and blood selenium play a role in regulating various immune cells to counteract thyroid dysfunction. The study by Ran et al. (62) suggests that CD62L− plasmacytoid DCs have a protective effect on chronic obstructive pulmonary disease. There are currently no relevant reports on the other immune phenotypes.

TABLE 4 Information on mediation effects in MR analysis.

Exposure	Outcome	Method	nSNP	OR (95% CI)	*p* value
Toenail and blood selenium	Hypothyroidism	IVW	12	0.85 (0.75–0.96)	0.009
	FT4	IVW	12	0.93 (0.91–0.96)	0.000
Toenail and blood selenium	CD62L− plasmacytoid DC %DC	IVW	12	0.87 (0.77–0.97)	0.016
	Transitional B cell %lymphocyte	IVW	12	0.84 (0.75–0.94)	0.002
	CD25++ CD8+ T cell Absolute Count	IVW	12	1.20 (1.06–1.35)	0.004
	CD3 on CD45RA− CD4+ T cell	IVW	12	1.17 (1.03–1.33)	0.017
	CD45RA on Terminally Differentiated CD8+ T cell	IVW	12	1.15 (1.01–1.30)	0.037
CD62L− plasmacytoid DC %DC	Hypothyroidism	IVW	6	1.18 (1.05–1.32)	0.005
Transitional B cell %lymphocyte		IVW	14	1.13 (1.00–1.27)	0.045
CD25++ CD8+ T cell Absolute Count	FT4	IVW	17	0.97 (0.95–1.00)	0.035
CD3 on CD45RA− CD4+ T cell		IVW	18	0.98 (0.96–1.00)	0.036
CD45RA on Terminally Differentiated CD8+ T cell		IVW	6	0.96 (0.92–1.00)	0.033

IVW, inverse variance weighting; OR, odds ratio.

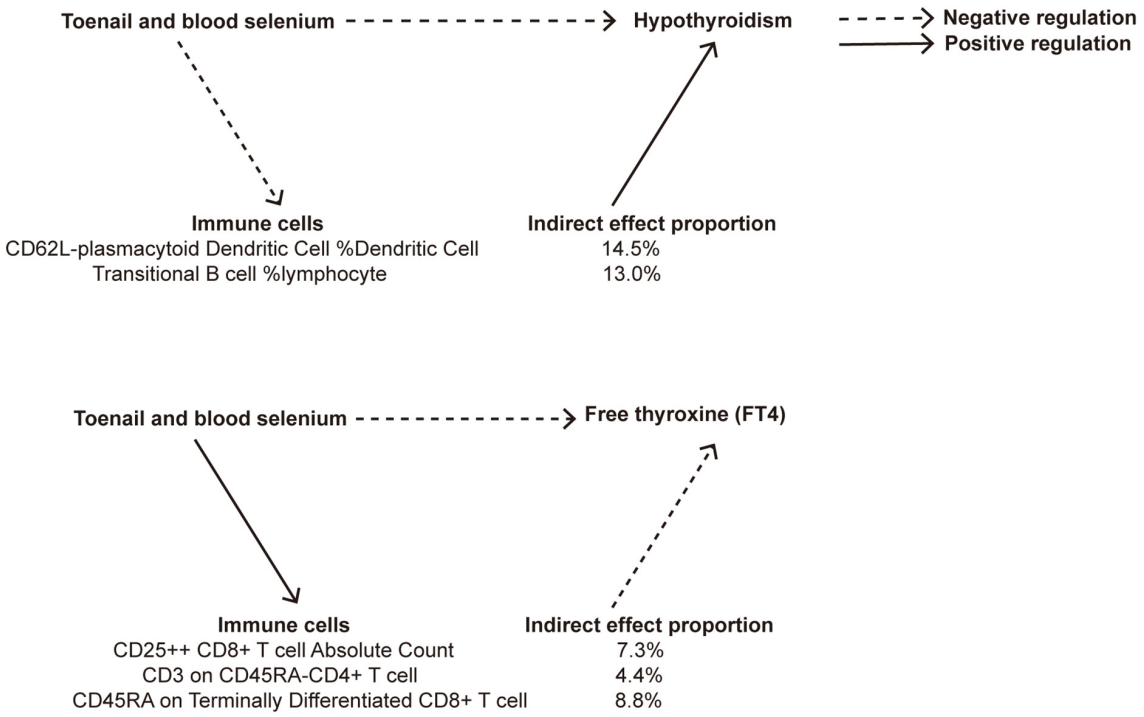

FIGURE 9
A diagram illustrating the indirect effect of nutrients on thyroid dysfunction via immune cells.

Despite these findings, our study has limitations. Firstly, using SNPs as proxies for nutrient levels may have inherent shortcomings, and a more rigorous approach would involve incorporating data from Food Frequency Questionnaire surveys. Secondly, there is an overlap between abnormal FT4 and TSH levels and the definitive diagnosis of hyperthyroidism or hypothyroidism. However, not all cases of abnormal FT4 and TSH levels correspond to clinical hyperthyroidism or hypothyroidism, as some may be in a subclinical state. Nevertheless, to ensure comprehensive results, we included all available data.

5 Conclusion

This study establishes an association between nutrient intake and thyroid dysfunction, revealing that nutrient effects on thyroid function are mediated through immune system alterations. Toenail and blood selenium levels were identified as influential factors on specific immune cells, mediating their connection with hypothyroidism. Furthermore, toenail and blood selenium impacted other immune cell levels, reinforcing the relationship between immune cells and FT4 levels. These findings underscore

the significance of selenium levels and immune modulation in understanding and addressing thyroid dysfunction.

Data availability statement

The original contributions presented in this study are included in this article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

Y-JJ: Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft. Y-QX: Formal analysis, Validation, Visualization, Writing – original draft. TH: Conceptualization, Project administration, Supervision, Writing – review and editing. Y-XX: Conceptualization, Data curation, Methodology, Project administration, Resources, Supervision, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1378969/full#supplementary-material

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Effects of maternal advanced lipoxidation end products diet on the glycolipid metabolism and gut microbiota in offspring mice

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Introduction: Dietary advanced lipoxidation end products (ALEs), which are abundant in heat-processed foods, could induce lipid metabolism disorders. However, limited studies have examined the relationship between maternal ALEs diet and offspring health.

Methods: To investigate the transgenerational effects of ALEs, a cross-generation mouse model was developed. The C57BL/6J mice were fed with dietary ALEs during preconception, pregnancy and lactation. Then, the changes of glycolipid metabolism and gut microbiota of the offspring mice were analyzed.

Results: Maternal ALEs diet not only affected the metabolic homeostasis of dams, but also induced hepatic glycolipid accumulation, abnormal liver function, and disturbance of metabolism parameters in offspring. Furthermore, maternal ALEs diet significantly upregulated the expression of TLR4, TRIF and TNF-α proteins through the AMPK/mTOR/PPARα signaling pathway, leading to dysfunctional glycolipid metabolism in offspring. In addition, 16S rRNA analysis showed that maternal ALEs diet was capable of altered microbiota composition of offspring, and increased the Firmicutes/Bacteroidetes ratio.

Discussion: This study has for the first time demonstrated the transgenerational effects of maternal ALEs diet on the glycolipid metabolism and gut microbiota in offspring mice, and may help to better understand the adverse effects of dietary ALEs.

KEYWORDS

maternal diet, advanced lipoxidation end products, offspring, glycolipid metabolism, gut microbiota

1 Introduction

The heat-processed foods contain high amounts of advanced lipoxidation end products (ALEs), which are crosslinked products of active carbonyl compounds produced by lipid oxidation and nucleophilic groups in proteins (1). In recent decades, overconsumption of heat-processed foods greatly increases the burden of chronic health conditions. The offspring of dams who fed with different types of heat-processed foods had lower birth weight (2) and increased catch-up growth (3) delayed maturation of physical features and impaired glucose homeostasis (4). Therefore, maternal ALEs diet has profound and lasting effects on the long-term health and disease risks of offspring.

It is known that the maternal nutrition and intrauterine environment play a key role in the development of metabolic disease in offspring (5). Recent studies on the underlying mechanisms of this programming effect have found that maternal diets can affect insulin and glucose metabolism, energy balance, cardiovascular function, and obesity in offspring (6, 7). In addition, there is evidence that changes in epigenetic marks are one mechanism that could account for such long-term effects, as they affect gene expression and thus shape the phenotype of the organism (8). Accordingly, the adverse consequences caused by the dietary ALEs are not limited to themselves, but may also have corresponding effects on the health of their offspring, which deserves attention.

ALEs can be formed exogenously or endogenously (9), and endogenous ALEs are closely related to physiological processes, such as oxidative stress, inflammation and liver injury (10). Exogenous ALEs are known as dietary ALEs, which are produced by reaction during food hot processing, and are considered as potential health risk factors. Our previous research has reported that dietary ALEs which are formed by reactive carbonyl compounds modified proteins have been shown not only resulting in alterations in the protein structure and digestibility, but also to cause abnormal liver function and lipid accumulation in mice (11). Dietary ALE is formed by modifying amino acids (lysine, cysteine, arginine and histidine) with active carbonyl compounds (malondialdehyde, 4-hydroxy-2-nonenal, etc.) in food (12). Since the ALEs-modification alters the structure, it changes the protein function. Binding of ALEs to its specific cell surface receptor-receptor for advanced glycation end product (RAGE) initiates downstream pathways leading to the production of reactive oxygen species and inflammatory cytokines, leading to inflammatory or diabetic responses (13, 14). We hypothesized that consumption of an ALEs-rich diet by dams may lead to changes in the metabolism and oxidation status of their offspring. In addition, the glycolipid metabolism of the offspring is closely related to its gut microbiota. To the best of our knowledge, the transgenerational effects of maternal ALEs diet have not been studied so far.

In this study, we continue to investigate the adverse effects of dietary ALEs. A cross-generation mouse model was developed to assess the effects of maternal exposure to dietary ALEs on metabolic disorders in offspring. The results suggested that exposure to maternal ALEs-rich diet might affect offspring's body weight, glucose and lipid metabolism, and gut microbiota. This study investigated the transgenerational effects of maternal ALEs diet on metabolism, and highlighted the regulatory role of maternal ALEs diet on gut microbiota of the offspring.

2 Methods

2.1 Animals and diets

The C57BL/6J (5-week-old, female) mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in the Nankai University Laboratory Animal Center under controlled conditions (temperature, 20–24°C; humidity, 50–60%; 12 h light/dark cycle). The animals and protocols for this study were approved by international ethical guidelines and the Institutional Animal Care and Use Committee of Nankai University.

The dietary ALEs given to the mice were prepared as we previously published (11). Malondialdehyde and myofibrillar proteins were selected to produce dietary ALEs by a heat-processing simulation system. After 1 week of environmental adaptation, all female mice were randomly divided into 3 groups ($n = 8$), as follows: the low dose ALEs (LALEs) group (3% casein in the standard AIN-93G diet was replaced with ALEs), the normal diet control (CON) group (standard AIN-93G diet), and high dose ALEs (HALEs) group (30% casein in the standard AIN-93G diet was replaced with ALEs). The female mice were fed for a total of 11 weeks, including preconception, pregnancy and lactation. The mice were allowed access to food and water *ad libitum* throughout the study, and their daily food consumption was estimated by weighing the remaining food. As previously reported in our research, the dose could reflect the intake of different doses of human dietary ALEs.

The female mice were mated with male mice for 4 days (female: male = 2:1), and the pregnancy was confirmed by postcopulatory plugs. The number of offsprings in each litter was 6–10. To avoid nutrition bias between litters, the offsprings sizes were culled to 6 for each dam at birth (15, 16). The body weight of the dams and offsprings was measured every week. After 3-week of weaning, the offsprings were anesthetized ($n = 8$, one offspring per litter), and sacrificed to collect serum, liver, and fecal samples.

2.2 Glucose tolerance tests in dams and offspring

To avoid stress during pregnancy and lactation, the dams were subjected to intraperitoneal glucose tolerance tests (IPGTTs) after weaning their offsprings. Besides, at 3 weeks of age, IPGTTs were performed on the offsprings after 10 h of fasting. After glucose administration (2 g/kg body weight), blood glucose (BG) levels in the tail vein were monitored using a Contour TS glucometer (Bayer, Beijing, China) before the injection (0 min) and at 30, 60, and 120 min after the injection. The area under the curve (AUC) of IPGTTs was calculated by the trapezoid formula: $AUC = 0.5 \times (BG0 + BG30)/2 + 0.5 \times (BG30 + BG60)/2 + 1 \times (BG60 + BG120)/2$.

2.3 Serum biochemical analysis

The blood samples were collected and centrifuged at 4000 g for 10 min, and the serum was stored at −80°C. The total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) in serum were determined using Nanjing Jiancheng Institute of Bioengineering ELISA kits (Nanjing, China). The level of serum fasting insulin (90,080; Crystal Chem, Downers Grove, United States), leptin (MOB00B, R&D Systems, Minneapolis, United States) were measured by ELISA kits. The insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMO-IR). The HOMO-IR was calculated as the fasting insulin concentration (μU/mL) × fasting glucose concentration (mmol/L)/22.5.

2.4 Histopathological observation

For histological study, the fresh liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin and then cut into 5 μm slices. After deparaffinization and hydration, hematoxylin and eosin (H&E) staining (Servicebio) was performed. For alcian blue periodic

acid–Schiff (AB-PAS) staining (Servicebio), frozen liver samples were first processed using a cryostat, then fixed, and stained. The images of the sections were captured using fluorescent inverted microscope.

2.5 Western blot analysis

Western blotting was applied to determine the expression levels of proteins, including AMPK, mTOR, PPARα, PKM2, IRS-1, TLR4, TRIF and TNF-α in the liver tissue of offspring mice. Total proteins were prepared from frozen liver tissue by Cellytic M cell lysis. The supernatant was loaded on SDS-PAGE gels. After electrophoresis, it was transferred to PVDF membrane (Millipore). The membrane was then incubated at 4°C with the antibodies (Abcam), and β-actin was used as internal load control. After washing, the membrane was incubated with secondary antibodies at room temperature for 1 h. The images were captured by a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, United States).

2.6 Microbial samplings and DNA isolation

High-throughput sequencing of the 16S rRNA gene was used to analyze the distribution of gut microbiota in the feces of maternal and offspring mice. The FastDNA Soil Spin kit (MP Biomedicals, CA, United States) was used to extract microbial genomic DNA from the feces of weaned offspring according to the manufacturer's protocol. Primer pairs were used to amplify the V3-V4 region of the bacterial 16S rRNA gene, followed by purification of PCR products using the AxyPrep DNA Gel Extraction kit (Axygen Biosciences, United States). Quantification was performed using Quantus™ fluorometer (Promega, United States). Purified amplicons were sequenced on an Illumina NovaSeq PE250 (Illumina, United States). The original FASTQ files are multiplexed using internal perl scripts, then quality filtered through Fastp, and merged through FLASH. The classification information was annotated using the Silva database based on the RDP classifier.

The alpha diversity including richness (Chao1 and Ace) and evenness (Simpson and Shannon) was calculated with mothur, and QIIME software was used to determine the community composition of each sample at different classification levels and beta diversity. The Wilcoxon rank sum test was used to determine the distinct species (phylum and genus) between the two groups. In addition, the linear discriminant analysis (LDA) effect size (LEfSe) was used to evaluate the influence of the abundance of each species on the differences between groups. Based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database, phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt 2) was used to predict metagenome function.

2.7 Statistical analysis

Data were analyzed using GraphPad Prism (version 9.0, GraphPad Software, United States). The data were presented as mean ± standard error of the mean (SEM) for normally distributed data. Statistical analyses were performed with the normality test, one-way ANOVA, two-way ANOVA and Tukey *post-hoc* test. Correlation analyses between the relative abundance of bacterial taxa was analyzed by Spearman correlation coefficient test. A p-value <0.05 was considered statistically significant.

3 Results

3.1 The effects of maternal ALEs diet on glucose and lipid metabolism in dams

The female mice were fed with the ALEs diet or standard diet during preconception, pregnancy and lactation. The flowchart of this study is presented in Figure 1A. There was no difference in the amount of food intake between the three groups. After 11 weeks of diet intervention, the HALEs diet negatively impacted body weight, and the LALEs diet did not affect the body weight in dams at weaning (Figure 1C).

To investigate whether the ALEs diet impacted glucose and lipid metabolism, we performed the IPGTTs on dams when they weaned their offspring (Figure 1B). The results of IPGTTs showed that HALEs diet led to significantly higher blood glucose levels in dams at 30 min, 60 min and 120 min, as well as a trend toward the larger area under the curve (AUC) values than those of dams in the LALEs and CON group (Figure 1D). Additionally, the HOMO-IR in the HALEs group was significantly higher than the CON group (Figure 1E).

Then we assessed the serum insulin concentration in dams, and found that HALEs diet significantly increased the serum fasting insulin levels, compared with the CON group (Figure 1F). Meanwhile, there were no significant differences in insulin levels between the LALEs and CON groups. The effects of ALEs diet on serum lipid profiles were also evaluated. The serum levels of TC, TG and LDL-C in dams fed by HALEs diet were higher than those fed the standard diet (Figures 1G–I). Besides, the HALEs diet significantly reduced the HDL-C levels (Figure 1J). However, no significant difference was observed in the lipid profiles between the dams in the LALEs group and in the CON group. Furthermore, the HALEs diet also significantly elevated the serum leptin levels ($p < 0.05$) in dams compared with those from the CON group (Figure 1K). These results suggested that ALEs diet could lead to glucose and lipid metabolism disturbance and even resistance in dams.

3.2 Effects of maternal ALEs diet on glucose and lipid metabolism in offspring

To determine the transgenerational effects of dietary ALEs, we first investigated the phenotype of offsprings. The body weight of offsprings was measured every 3 days from birth to weaning. Our results have shown that no significant differences was observed in the initial body weight (body weight at birth) between the three groups (Figure 2A). Noteworthy, the final body weight (body weight at weaning) of the HALEs group was significantly greater than that of the CON and LALEs groups (Figure 2B). It is observed that the 11-week maternal HALEs diet did affect the body weight of offsprings at weaning.

The IPGTTs was performed on the offspring at weaning. Compared with the CON group, the HALEs group had significantly higher blood glucose levels (Figure 2C). As shown in Figure 2D, the

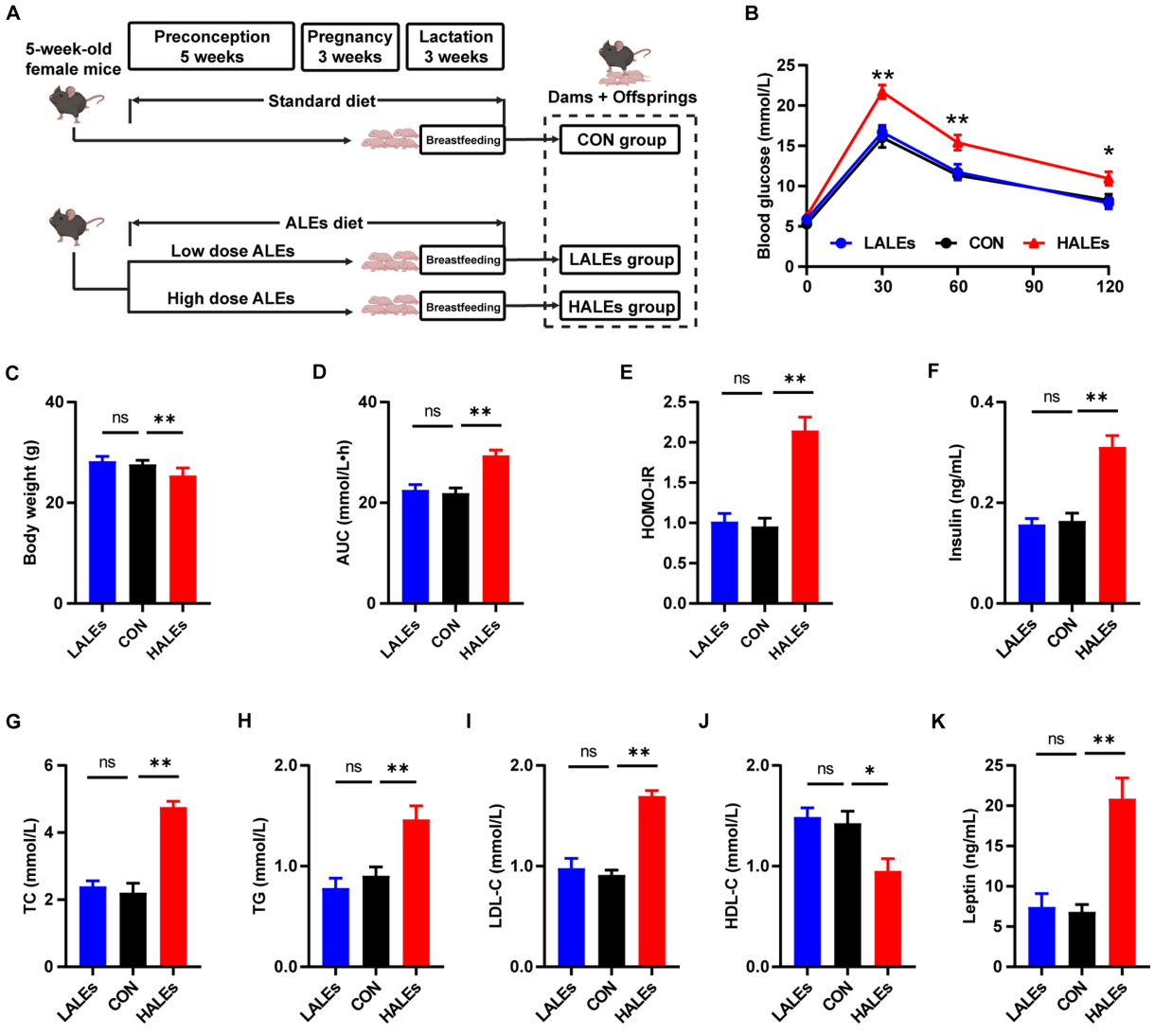

FIGURE 1
Dietary ALEs impaired glycolipid metabolism in dams. **(A)** The flowchart of this study. **(B)** IPGTTs of dams. **(C)** Body weight of dams. **(D)** AUC of dams. **(E)** HOMO-IR of dams. **(F)** Serum insulin level of dams. **(G)** Serum TC level of dams. **(H)** Serum TG level of dams. **(I)** Serum LDL-C level of dams. **(J)** Serum HDL-C level of dams. **(K)** Serum leptin level of dams. $*p < 0.05$, $**p < 0.01$, Data were expressed as the mean ± SEM.

AUC in the HALEs group was significantly larger. There was no difference in the AUC between the LALEs and CON group. In addition, the offspring of the HALEs group had a higher HOMO-IR index compared with the CON group (Figure 2E). To determine whether the HALEs diet reduced insulin sensitivity in the offspring, we assessed the fasting insulin levels. The serum insulin level was significantly higher in the HALEs group (Figure 2F). These results showed that the offspring of the HALEs group exhibited glucose intolerance.

In addition, we evaluated the effects of maternal ALEs diet on serum lipid profiles in offspring. The serum levels of TC (Figure 3A) and TG (Figure 3B) were both significantly increased in the offsprings due to the maternal ALEs diet, compared with the CON group. Furthermore, the offspring of the HALEs group displayed higher LDL-C level (Figure 3C). As shown in Figure 3D, the HDL-C level of offspring were not significantly different between the three groups. However, the offspring of the HALEs group had significantly higher serum leptin levels than the offspring of the LALEs and CON groups (Figure 3E), which suggested that maternal ALEs diet induced lipid metabolism disorder in offspring.

3.3 Effects of maternal ALEs diet on liver function in offspring

To explore the impact of maternal ALEs diet on liver damage in offspring, histopathology profiles were observed by H&E and AB-PAS staining. As shown in Figure 3F, the offspring liver ultrastructure of LALEs and CON group was normal, and there was no steatosis. The hepatocytes were arranged neatly, and their cords were arranged radially around the central vein. However, the offspring liver of the HALEs group was characterized by deformed and compressed hepatocytes, disorganized cords, and cytoplasmic accumulation of lipid droplets with varied size, number and shape. The AB-PAS

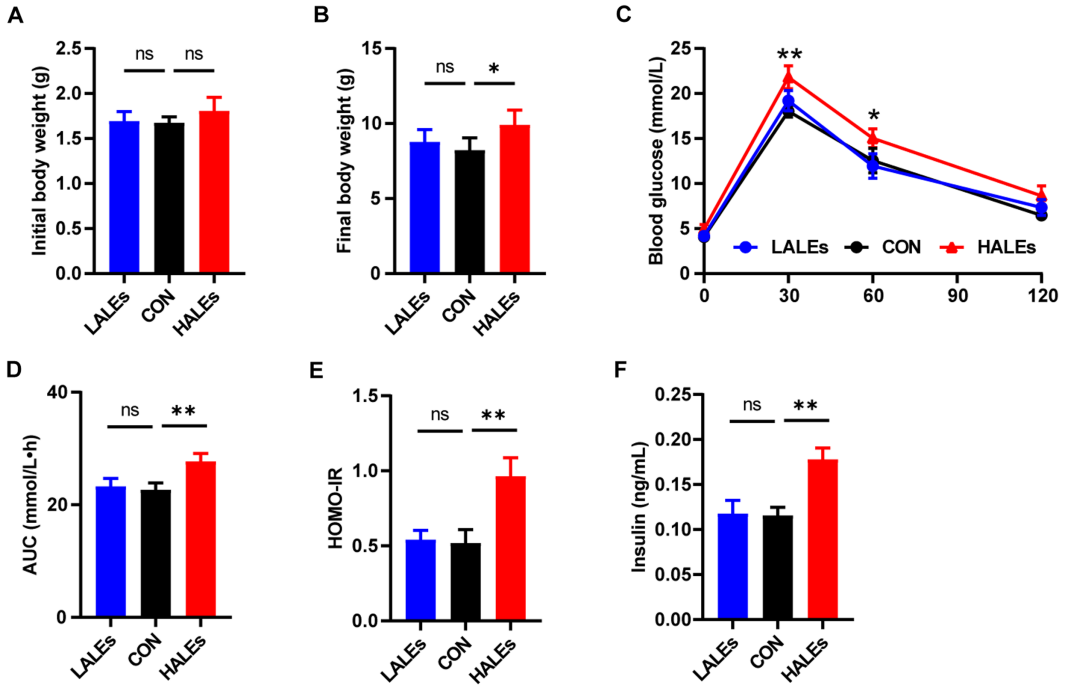

FIGURE 2
Maternal ALEs diet impaired glucose metabolism in offspring. **(A)** Body weight of offspring at birth. **(B)** Body weight of offspring at weaning. **(C)** IPGTTs of offspring. **(D)** AUC of offspring. **(E)** HOMO-IR of offspring. **(F)** Serum insulin level of offspring. *$p < 0.05$, **$p < 0.01$, Data were expressed as the mean ± SEM.

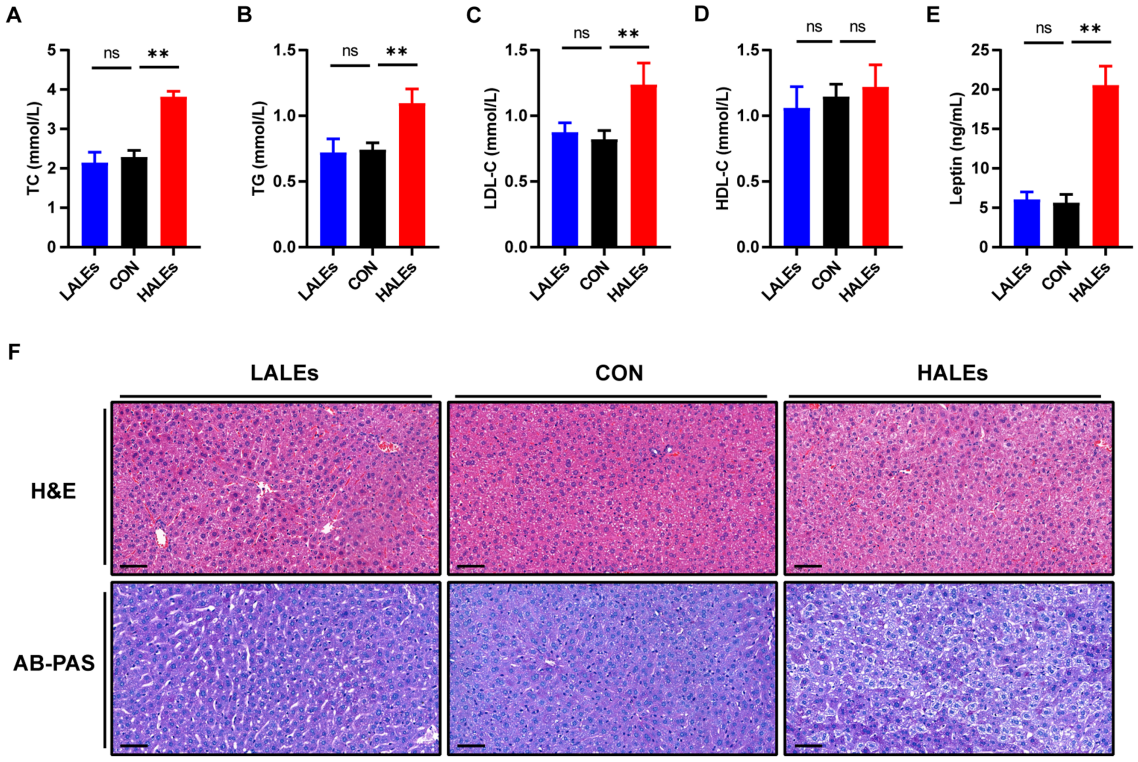

FIGURE 3
Maternal ALEs diet impaired lipid metabolism in offspring. **(A)** Serum TC level of offspring. **(B)** Serum TG level of offspring. **(C)** Serum LDL-C level of offspring. **(D)** Serum HDL-C level of offspring. **(E)** Serum leptin level of offspring. **(F)** Representative images of H&E-stained and AB-PAS stained liver tissue (200×, bar = 50 μm). *$p < 0.05$, **$p < 0.01$, Data were expressed as the mean ± SEM.

staining showed that the glycogen content in the HALEs group was significantly increased compared with the CON group. The results indicated that maternal ALEs diet induced liver damage, liver lipid accumulation, and increased liver glycogen content in offspring.

We further investigated the molecular mechanism of glucose lipid metabolism disorder induced by maternal ALEs diet in offspring. As the liver is the most important organ of metabolism, the expression of proteins involved in glucose and lipid metabolism in the liver was examined by Western blot (Figure 4A). As shown in Figure 4B, the expression of AMPK was significantly increased in the HALEs group compared with the CON group. The expression of mTOR, PPARα, PKM2 and IRS-1 in the HALEs group was significantly lower than those in the CON group (Figures 4B,C). In addition, the maternal HALEs diet significantly increased the expression of TLR4, TRIF, and TNF-α (Figures 5A,B). These results suggested that maternal HALEs diet may affect the expression of AMPK/mTOR/PPARα signaling pathway proteins, and lead to the disturbance of glycolipid metabolism in offspring.

3.4 Effects of maternal ALEs diet on gut microbiota in offspring

To investigate the impact of maternal ALEs diet on gut microbiota in offspring at weaning, we performed 16S rRNA sequencing of the cecal contents and the corresponding biological analysis. By analyzing the OTUs to assess shared and unique germs between the three groups, we found 291 shared OTUs, 10,943 unique OTUs in the CON group, 9,367 unique OTUs in the LALEs group, and 7,491 unique OTUs in the HALEs group (Figure 6A). The maternal HALEs diet had no significant effect on the alpha diversity of bacteria in the offspring (Figure 6B). The beta diversity measures, which characterizes differences between groups, showed significant separation of the gut microbiota between the three groups (Figures 6C,D).

Then, we evaluated the relative abundance of gut microbial composition. The top 10 species at the phylum level were shown in Figure 6E, and the results have shown that Firmicutes was enriched in the HALEs diet offspring, followed by Bacteroidota, Actinobacteria, Verrucomicrobiota, and Proteobacteria, while Bacteroidota was enriched in the LALEs diet offspring, followed by Firmicutes, Verrucomicrobiota, and Proteobacteria. Besides, the top 20 species at the genus level with significant differences was shown in Figure 6F. To examine alteration in microbiota composition, the Wilcoxon rank-sum test was applied to analyze the differential species. Compared with the CON diet offspring, the maternal HALEs diet significantly increased the relative abundance of Firmicutes, and the maternal LALEs diet significantly increased the relative abundance of Patescibacteria at the phylum level. At the genus level, the maternal HALEs diet significantly increased the relative abundance of *Dubosiella*, unclassified_*Anaerovoracaceae*, *[Eubacterium]_nodatum_* group, unclassified_Peptococcaceae, unclassified *[Eubacterium]_coprostanoligenes_*group, and decreased the relative abundance of *Bifidobacterium*, uncultured_Bacteroidales_bacterium (Figure 7C).

To further assess the influence of each species on the differences between groups, we analyzed the gut microbiota using LEfSe (Figure 7A). The phylum Firmicutes, the class Clostridia, the order

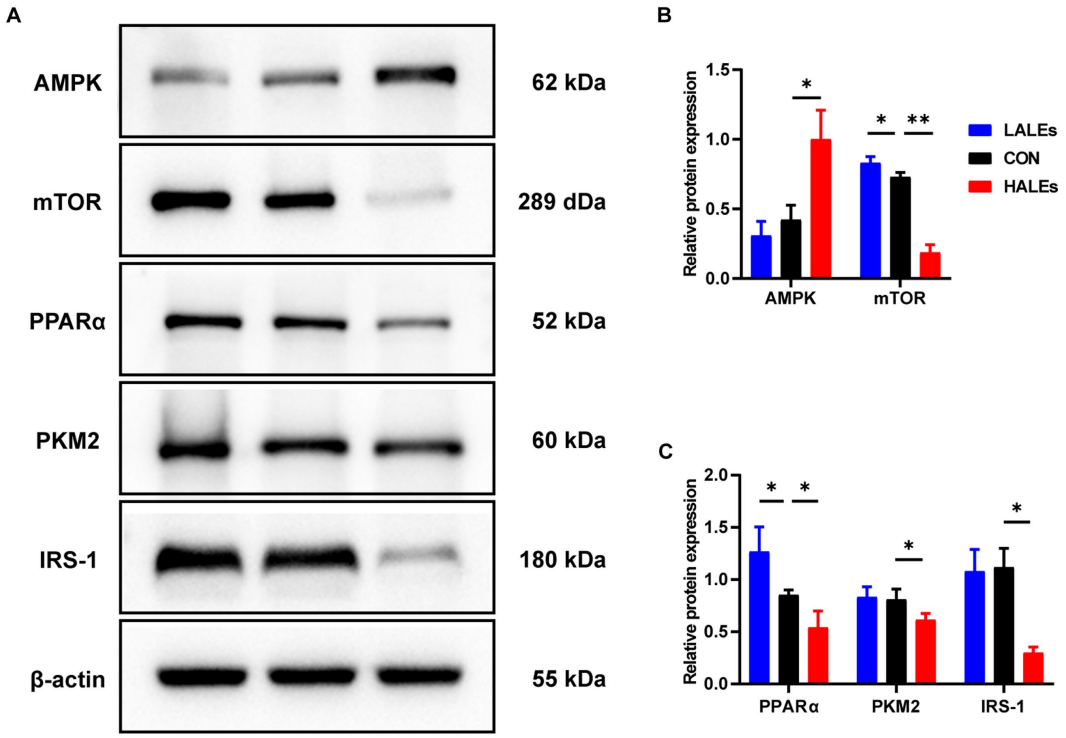

FIGURE 4
Maternal ALEs diet regulated the AMPK/mTOR/PPARα signaling pathway. **(A)** Protein expression measured by Western blot. **(B)** Protein expression of AMPK and mTOR in the liver. **(C)** Protein expression of PPARα, PKM2 and IRS-1 in the liver. *$p < 0.05$, **$p < 0.01$, Data were expressed as the mean ± SEM.

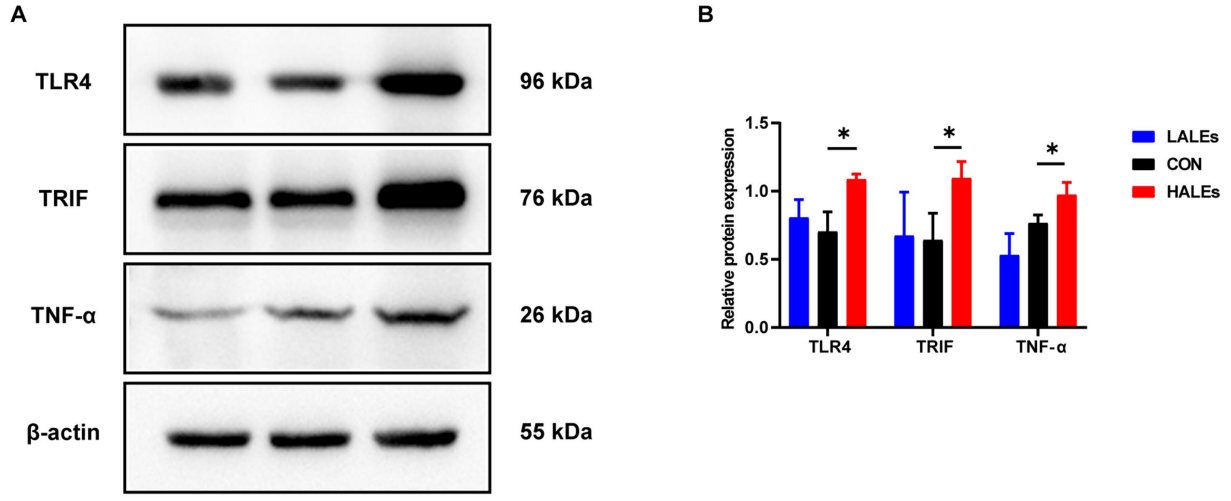

FIGURE 5
Maternal ALEs diet influenced the expression of TLR4/TRIF/TNF-α proteins. **(A)** Protein expression measured by Western blot. **(B)** Protein expression of TLR4, TRIF and TNF-α in the liver. *$p < 0.05$, **$p < 0.01$, Data were expressed as the mean ± SEM.

Lachnospirales, the family lachnospiraceae, and the genus unclassified_Atopobiaceae were the most abundant in the HALEs diet offspring. The species unclassified_*Helicobacter*, the family Deferribacteraceae, the class Deferribacteres, the order Deferribacterales, and the phylum Deferribacterota were the most abundant in the CON group (Figure 7B). In addition, the metabolic pathways of bacterial communities in offspring were analyzed by KEGG database (Figures 8A,B). Analysis of the composition and differences of metabolic pathways can reveal the changes of functional genes in the microbial community. On the level 2 of KEGG, replication and repair, and the carbohydrate metabolism pathway has been observed in HALEs diet offspring. Significantly, the low represented pathways in the HALEs group were mainly in biological processes associated with metabolism compared with the CON group. These pathways included amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, and lipid metabolism (Figure 8A).

4 Discussion

With the increasing proportion of heat-processed foods in global food consumption, the onset age of metabolic diseases such as obesity and diabetes has gradually decreased (17). Chronic metabolic diseases that caused by disorders of glucose and lipid metabolism affect a vast number of individuals worldwide, and their occurrence is fetal (18, 19). Significantly, dietary ALEs are formed by modifying proteins with lipid oxidation products, and heat-processed foods contain a certain amount of ALEs. Our previous studies have shown that dietary ALEs induced liver damage by modulating hepatic lipid metabolism. Nevertheless, the transgenerational effects of dietary ALEs have been less studied. It has been found that the adverse outcomes caused by dietary intake of heat-processed foods are not limited to themselves, but may have a corresponding impact on the health of their offspring (20), revealing the transgenerational effects of harmful substances in heat-processed foods. Therefore, it is important to analyze the effects of dietary ALEs on offspring metabolism.

Dietary ALEs caused by non-enzymatic modification of proteins is a health risk factor in heat-processed foods (21, 22). Recent studies have shown that ALEs diet led to intestinal barrier breakdown, liver dysfunction and lipid accumulation, and ultimately liver injury (11). As the central organ of metabolism, liver not only participates in the synthesis of glycogen and fat, but also secretes regulatory factors that act on many major metabolic tissues to synergistically regulate glycolipid metabolism (23). In this study, the levels of blood glucose and serum TC/TG/LDL-C in dams of HALEs group were significantly higher than in those of CON group. We found that ALEs-rich diet caused glucose and lipid metabolism disturbance and even resistance in dams, which is consistent with previous research. In addition, it is worth noting that the offspring of HALEs group at weaning displayed increased body weight and glucose intolerance. These results suggested that the maternal ALEs diet could cause glucose metabolism disorders, which would help create an adverse developmental environment for offspring *in utero* and early postpartum period.

In terms of the metabolism, we found that maternal ALEs-rich diet increased body weight, blood glucose, and insulin levels of the offspring, and the offspring exhibited glucose intolerance. The insulin concentration was elevated, not at the level of insulin resistance, but it is nonetheless of concern. In addition, higher levels of serum TC, TG, LDL-C, and leptin were observed in the offspring of HALEs group, indicating that maternal ALEs-rich diet induced hyperlipidemia. Leptin is a hormone secreted by fat tissue, and its content in the serum is proportional to the size of the fat tissue. It plays a key role in glucose homeostasis, maintenance of fat tissue, and immune function (24). Leptin resistance and obesity-associated hyperleptinemia are associated with insulin resistance, type 2 diabetes, and diabetic vascular complications (25). Insulin and leptin were reliable biomarker of glycolipid metabolism disorders (26). Furthermore, glycogen is the main intracellular storage form of glucose in the liver, and glycogen levels are thought to be associated

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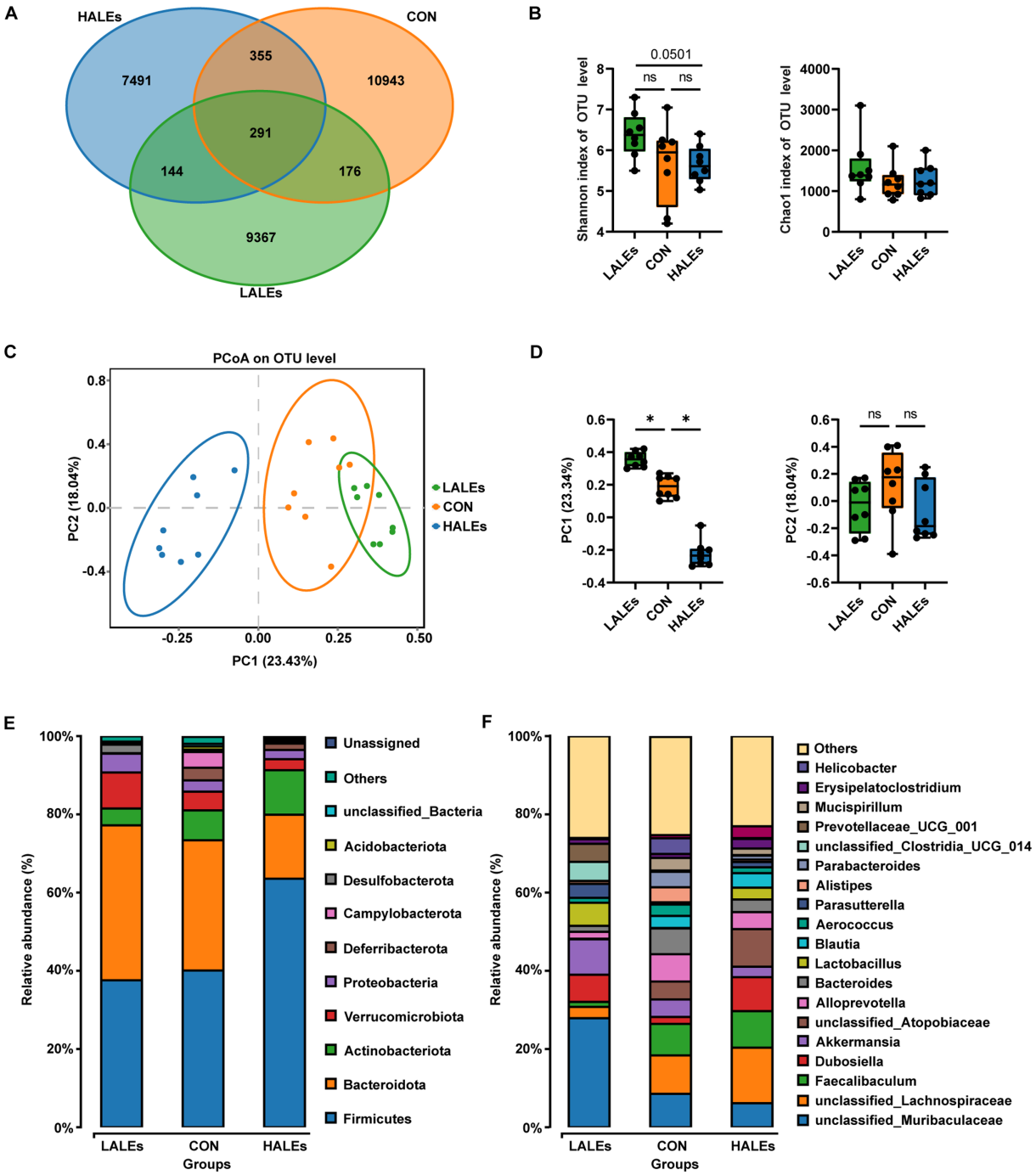

FIGURE 6
Maternal ALEs diet altered structures and composition of gut microbiota in offspring. **(A)** Venn diagram of the OTUs. **(B)** Shannon index of alpha diversity. **(C)** PCoA plots of gut communities. **(D)** PC1 and PC2 index of beta diversity. **(E)** Relative abundance of the bacterial population at the phylum level. **(F)** Relative abundance of the bacterial population at the genus level. *$p < 0.05$.

with insulin resistance in mice (27). The AB-PAS staining in the liver further confirmed the effect of HALEs diet on the hepatic glycogen content of offspring. The insulin and leptin levels of HALEs diet offspring increased significantly, suggesting that maternal ALEs diet adversely affects the glucose and lipid homeostasis of offspring.

Pathological staining of the liver showed that maternal ALEs diet could increase hepatic glycogen and lipid content in offspring, which indicated dietary ALEs might regulate the liver glycolipid metabolism of offspring. To further investigate the molecular mechanism of maternal ALEs diet, the changes of protein expression in the liver tissue of offspring were detected. The results showed that maternal ALEs-rich diet disrupted the glycolipid metabolism and reduced insulin sensitivity of offspring mice via AMPK/mTOR/PPARα pathways. As expected, the glycolipid metabolism of HALEs diet offspring was significantly impaired. Recent studies have demonstrated that the offspring of suboptimal maternal nutrition

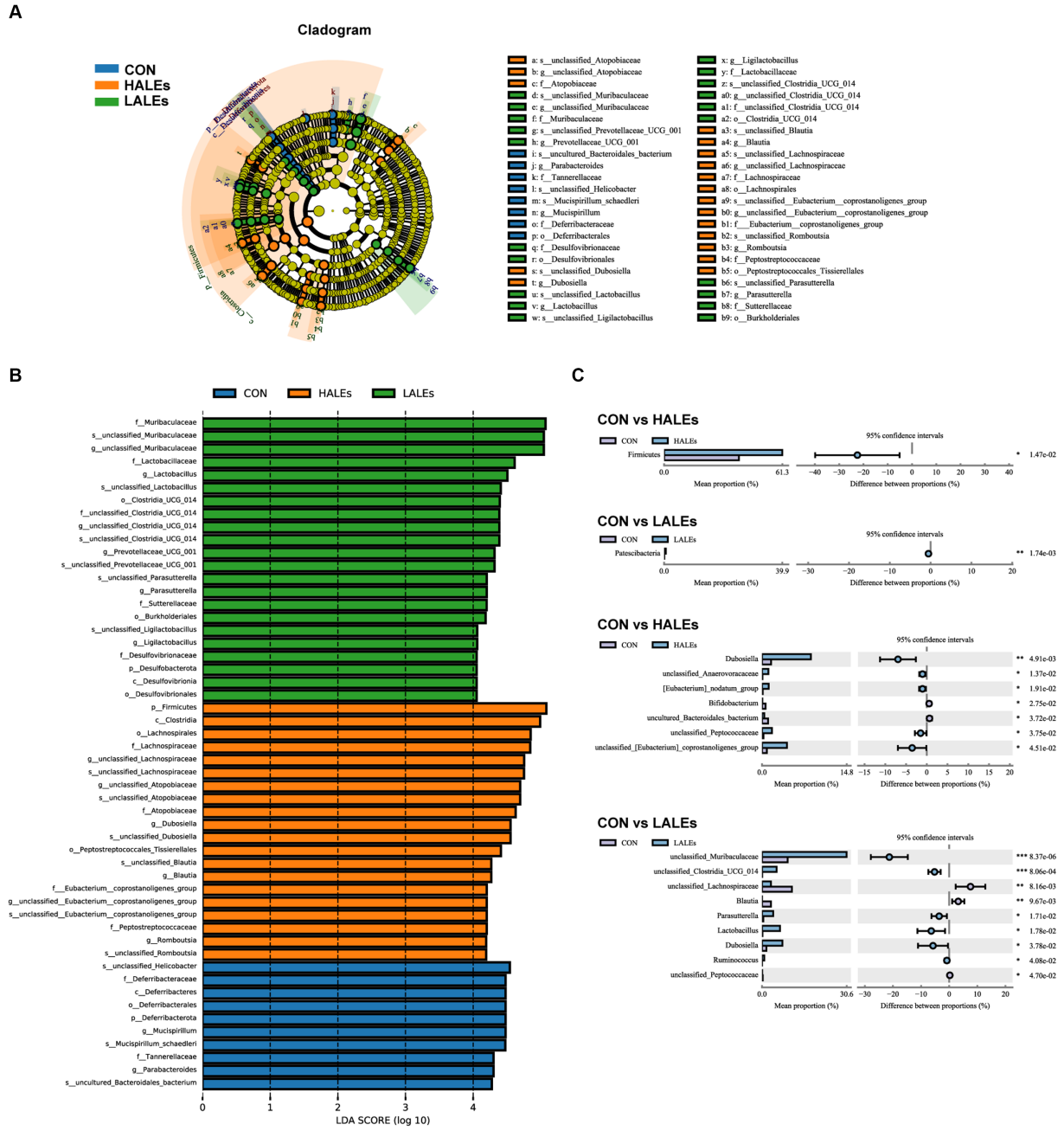

FIGURE 7
Key bacteria altered by the maternal ALEs diet in offspring **(A,B)**. LEfSe analysis of the gut microbiota from the phylum level to the genus level with LDA values of 4.0. **(C)** Wilcoxon rank-sum test bar plot on the phylum and genus level. *$p < 0.05$, **$p < 0.01$.

were obese at weaning, and accompanied by decreased gene expression for PPARα (28). Consistently, we found that the PPARα was significantly decreased in the offspring of HALEs group. Therefore, the liver PPARα pathway is an important signaling pathway for maternal diet to regulate offspring's glycolipid metabolism (29). As a core transcriptional regulator of glycolipid metabolism (30), PPARα could inhibit the release of inflammatory factors, resist oxidative stress, improve insulin resistance and regulate lipid metabolism by inducing transcription of downstream target genes (mainly PKM2/IRS-1) (31, 32). Studies have found that PKM2 is a key regulatory enzyme in glucose metabolism, and IRS-1 mediates the metabolic and growth-promoting functions of insulin, both of which are major factor contributing to impaired glucose transport (33). In addition, activation of TLR4/TRIF signaling pathway has been found to be closely associated with hepatic lipid accumulation (34) and elevated concentrations of TNF-α in liver tissue are thought to be associated with liver inflammation, glucose and insulin disturbances (35). In the present study, maternal ALEs-rich diet induced AMPK activation, followed by mTOR and PPARα inhibition in offspring mice. Our results showed that maternal ALEs diet regulated the glycolipid metabolism of offspring via AMPK/mTOR/PPARα pathways.

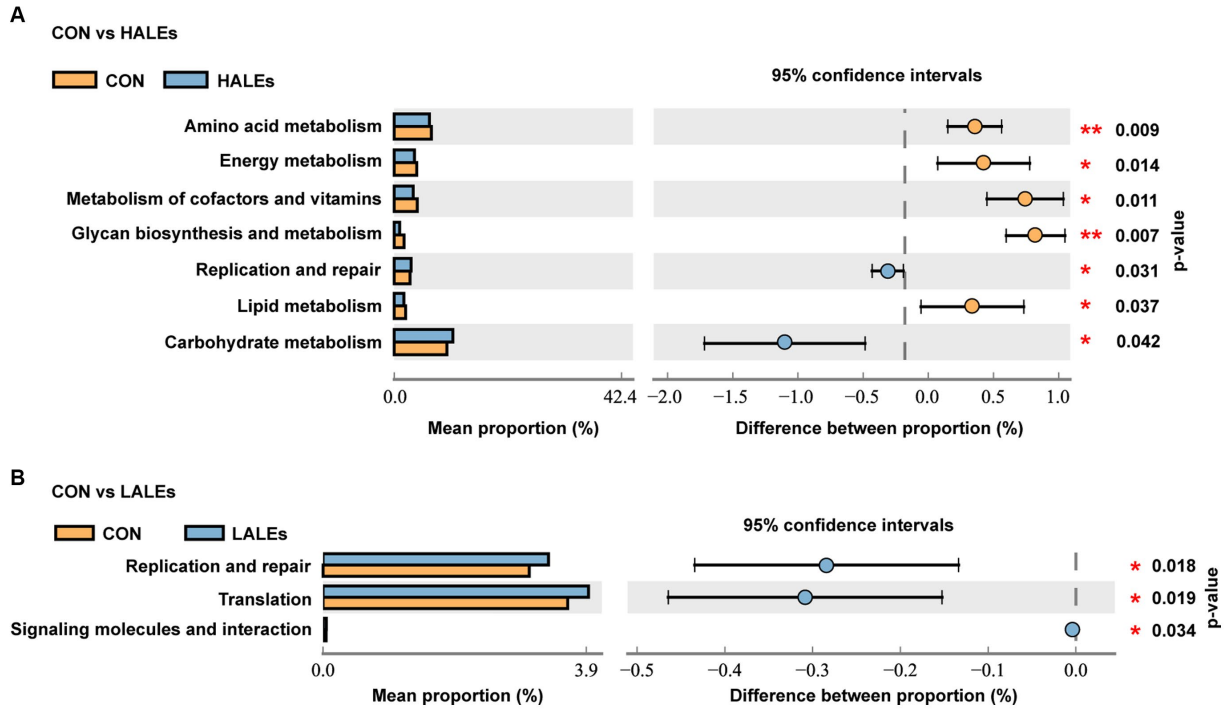

FIGURE 8
Metabolic pathways of bacterial communities by the KEGG pathway database in offspring. **(A)** Comparison of the HALEs and CON groups. **(B)** Comparison of the LALEs and CON groups. *$p < 0.05$, **$p < 0.01$.

A growing body of evidence suggests that the offspring microbiome disorders influenced by maternal diet plays an important role in glycolipid homeostasis (36). Given that the gut microbiota of offspring can be altered by maternal diet (37), we investigated the effects of maternal ALEs diet on the structure and diversity of the gut microbiota in offspring. The study found that maternal HALEs diet had a negative effect on glucose and lipid metabolism in offspring mice and the gut microbiota at weaning. The principal co-ordinates analysis (PCoA) showed that both the HALEs and LALEs groups were significantly different from the CON group. The results indicated that the maternal ALEs diet had a certain effect on the beta diversity of bacteria in the offspring. From the relative abundance of gut microbial composition, it can be observed that maternal HALEs diet resulted in significant changes in the gut microbiota of offspring, characterized by an increased Firmicutes/Bacteroidetes ratio at the phylum level. Previous studies have shown that the relative proportion of Bacteroidetes was reduced in obese mice (38), and the changes in Firmicutes/Bacteroidetes ratios have been closely associated with metabolic disorders, such as obesity and diabetes (39). In this study, the maternal HALEs diet significantly increased the abundance of *Dubosiella,* and *Dubosiella* had significantly increased abundance in high fat diet feeding mice, suggesting its specific role in the obesity-related metabolic phenotype (40). In addition, the relative abundance of *Bifidobacterium* and uncultured_Bacteroidales_ bacterium was significantly decreased in the HALEs group. Claims have been made for positive effects of *Bifidobacterium* on infant growth and health (41). *Bifidobacterium* was important in obesity and type 2 diabetes, and may be involved in the dietary carbohydrate - microbiome - host metabolic axis (42). Bacteroidales has been reported to be a probiotic bacterium, exercising a beneficial effect on the gut microbiota (43). Bacteroidales could stimulate the production of fucosylated glycans in the gut (44). In addition, The bacteria also stimulates angiogenesis in the newborn epithelium (45), which enhances the body's absorption of nutrients. Therefore, the altered gut microbiota of HALEs diet offspring may have a role in the promotion of susceptibility to obesity and diabetes when they encounter metabolic stress in later life. The relationship between intestinal microecological disorders and metabolic disorders may be related to metabolites derived from gut microbiota, which is worthy of further study.

In conclusion, our results showed that maternal ALEs diet negatively affects not only the metabolic homeostasis of dams, but also the glycolipid metabolism and gut microbiota of offspring. The maternal ALEs-rich diet may induce hepatic glycolipid accumulation, abnormal liver function, and disturbance of metabolism parameters in offspring through the AMPK/mTOR/PPARα signaling pathway. However, the causal relationship between maternal ALEs diet, regulation of gut microbiota, and metabolic parameters in offspring remains unclear, and more experiments on fecal microbiota transplantation and antibiotic intervention are needed to investigate the exact association.

Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, accession number PRJNA1119259.

Ethics statement

The animal study was approved by international ethical guidelines and the Institutional Animal Care and Use Committee of Nankai University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WP: Conceptualization, Data curation, Investigation, Writing – original draft. BZ: Formal analysis, Investigation, Writing – original draft. JZ: Methodology, Project administration, Writing – review & editing. TC: Methodology, Visualization, Writing – review & editing. QH: Data curation, Visualization, Writing – review & editing. ZY: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Impact of maternal *Bifidobacterium breve* M-16V and scGOS/lcFOS supplementation during pregnancy and lactation on the maternal immune system and milk composition

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Introduction: Maternal synbiotic supplementation during pregnancy and lactation can significantly influence the immune system. Prebiotics and probiotics have a positive impact on the immune system by preventing or ameliorating among others intestinal disorders. This study focused on the immunomodulatory effects of *B. breve* M-16V and short chain galacto-oligosaccharides (scGOS)/long chain fructo-oligosachairdes (lcFOS), including systemic and mucosal compartments and milk composition.

Methods: Lewis rats were orally administered with the synbiotic or vehicle during pregnancy (21 days) and lactation (21 days). At the weaning day, small intestine (SI), mammary gland (MG), adipose tissue, milk, mesenteric lymph nodes (MLN), salivary gland (SG), feces and cecal content were collected from the mothers.

Results: The immunoglobulinome profile showed increased IgG2c in plasma and milk, as well as elevated sIgA in feces at weaning. The supplementation improved lipid metabolism through enhanced brown adipose tissue activity and reinforced the intestinal barrier by increasing the expression of *Muc3*, *Cldn4*, and *Ocln*. The higher production of short chain fatty acids in the cecum and increased *Bifidobacterium* counts suggest a potential positive impact on the gastrointestinal tract.

Discussion: These findings indicate that maternal synbiotic supplementation during gestation and lactation improves their immunological status and improved milk composition.

KEYWORDS

Bifidobacterium breve M-16V, short chain galacto-oligosaccharides (scGOS), long chain fructo-oligosachairdes (lcFOS), pregnancy, breastfeeding

1 Introduction

Pregnancy and lactation are crucial for a correct development of the infant. These two periods are highly influenced by external stimuli including environmental, nutritional, and lifestyle factors. Nutrition, including the nourishment status and pattern of food intake, impacts on physiological and metabolic responses (1, 2). During gestation the placenta serves as a bridge for nutrients, hormones, cytokines (CK), immunoglobulins (Igs) and other bioactive molecules. In humans, IgG crosses the placenta through an active mechanism due to the presence of the neonatal fragment crystallizable receptor (FcRn) expressed by the cells of the syncytiotrophoblast (3). During lactation breast milk (BM) works as a transference vehicle for nutrients and bioactive compounds including Ig and CK. Its composition is dynamic and changes to supply the nutritional requirements of the infant (4). Thus, during these periods the maternal nutrition influences the composition of the BM (5).

The use of food complements such as probiotics, prebiotics, synbiotics and postbiotics has been growing during the last few years (6) for different purposes such as their anti-infective action. Probiotics reduce the colonization of pathogens, prebiotics promote the growth of the beneficial microbes of the intestine and, synbiotics act as a combination of both taking the advantages of probiotics and prebiotics (6). In 2020, synbiotics were defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (7). Clinical and preclinical studies have verified that the supplementation with these microbial modulators improves the gastrointestinal health and infection resolution (8, 9). In some studies, these effects have been associated with the microbiota and its impact on the immune system (6, 9).

Until now, only a few studies have reported beneficial effects of probiotics and prebiotics supplementation during gestation and lactation on the newborn (10). However, less information is known about the impact of synbiotic supplementation on the maternal immunity. Hence, this study aims to evaluate the impact of a synbiotic mixture, particularly composed of *Bifidobacterium breve* M-16V (10^9 CFU) and short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) at ratio 9:1 during gestation and lactation on the maternal immune system. These probiotic and prebiotic have been linked to immunomodulatory effects in previous studies (11–16).

2 Materials and methods

2.1 Animals

Seven-week-old Lewis rats (16 females and 8 males) were obtained from Janvier Labs (La Plaine Saint Denis Cedex, France). After one week of acclimatization, females were randomly distributed into two groups: Reference (REF, n=8) or Synbiotic (SYN, n=8). At the same day, females were introduced into the male cages for one week, and then separated into individual cages. Since the mating day, the female rats of the SYN group were supplemented daily during gestation (21 days) and lactation (21 days) with the synbiotic mixture, and the REF group received a matched volume of saline solution. Animals were fed with a commercial diet corresponding to the American Institute of Nutrition 93G formulation (17) and water *ad libitum*. Rats were allowed to deliver naturally, and the day of birth was considered as day 1 of pups. Pups had free access to the nipples and rat diet during the entire study. The experiment was finally executed with the 5 dams of the SYN group and 6 dams of the REF group that became pregnant.

Animal room conditions (temperature and humidity) were controlled in a 12 h light – 12 h dark cycle in a negative pressure chamber at Animal Facility of the Diagonal Campus, Faculty of Pharmacy and Food Science, from the University of Barcelona. All the experimental procedures were previously approved by the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona (UB) (Ref 240/19) and from the Catalan Government (Ref.10933).

2.2 Synbiotic supplementation

Daily administration of the synbiotic or vehicle was performed during the gestation and lactation periods always at the same time range of the day. The synbiotic solution was obtained by mixing *Bifidobacterium breve* M-16V (10^9 CFU) with scGOS/lcFOS 9:1 concentration. *Bifidobacterium breve* M-16 V has been purchased

from Morinaga Milk Industry, Tokyo, Japan. GOS/FOS is a mixture of GOS (Vivinal GOS, Borculo Domo, Zwolle, The Netherlands) with a degree of polymerization (dp) of 3–8, as well as long-chain FOS (Raftiline HP, Orafti, Wijchen, The Netherlands; average dp > 23) in a 9:1 ratio. The products had a purity of 47.6% for GOS and 94.5% for FOS. The dose of scGOS/lcFOS was approximately 2% of an established daily food intake of 40 g. The mix was extemporaneously prepared by the mixture of the prebiotic and the probiotic dissolved in physiological saline solution. One mL of the synbiotic (10^9 CFU/rat/day) or saline solution was intragastrically administered through an oral gavage during pregnancy. After birth, dams were separated from the pups for animal handling and the volume administered was increased to 1.5 mL. All supplements were kindly provided by Danone Nutricia Research (Utrecht, The Netherlands). The control group received the same volume of saline with same amount of corn starch as the treated group.

2.3 Sample collection and processing

Animal body weights (BW), and food and water consumption were monitored daily, and feces were collected weekly during the study. The relative humidity and the pH of the feces was monitored in fresh after collection. At the weaning day, dams were isolated from the pups 1 h before milk extraction to allow the milk to accumulate in the mammary gland (MG). Then, dams were anesthetized with 10 mg/100 g of ketamine (Merial Laboratories S.A., Lyon, France) and administered intraperitoneally with 2 Ul of oxytocin (Syntocinon 10 U.I./mL, Alfasigma S.L., Bologna, Italia) thirty min after administering oxytocin, the milking process began by gently and manually stimulating the teat from its base to the top. The milk was collected with an automatic pipette in sterilized tubes, centrifuged (12000 *g*, 5 min, 4°C) and then the lactic serum (LS) was obtained and stored at -80°C.

Finally, dams were re-anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg; Bayer A.G., Leverkusen, Germany). First, blood was obtained by cardiac puncture and collected in heparin tubes for the hematologic analyses. Then, samples were centrifuged (1000 *g*,10 min 4°C). Also, intestinal and adipose tissues, intestinal and cecal contents, salivary glands (SG), mesenteric lymph nodes (MLN), MGand spleen were collected and immediately processed or stored at -20°C or -80°C for future analysis.

BW was monitored daily and at the end point body and tail lengths were measured to calculate the body mass index (BMI) ($weight/length^2$ (g/cm^2)) and the Lee Index ($weight^{0.33}/length$) × *1,000* ($g^{0.33}/cm$). The weight of different organs was recorded including thymus, spleen, liver, heart, kidney, large intestine, and small intestine (SI) (length and wide were also measured).

2.4 Isolation of mesenteric lymph nodes and spleen lymphocytes

MLN and spleen cells were isolated as previously described (18). For splenic cells, an additional step was required to eliminate erythrocytes by an osmotic lysis. Conditions were immediately restored by adding PBS (Phosphate-buffered saline) to avoid lymphocytes death (19). Cell viability and concentration was analyzed by Countess™ Automated Cell Counter (Invitrogen™, ThermoFisher Scientific, Barcelona, Spain) based on Trypan Blue staining.

2.5 Small intestine sampling

SI was processed for diverse analysis. Two portions of 1 cm from the middle part of the intestine were collected for histomorphometry and gene expression analysis. For gene expression analysis the intestine portion was immersed in RNAlater (Ambion, Life technologies, Madrid, Spain), kept at 4°C for 24 h and then stored at -20°C. The remaining proximal part of the SI was opened lengthwise and cut in 0.5 cm pieces and incubated with PBS in a shaker (37°C for 10 min) to recover the gut wash (GW). The content of the distal part of the SI (intestinal content, IC) was collected for microbiota analysis.

2.6 Histology

The SI and adipose tissues (white adipose tissue (WAT) and brown adipose tissue (BAT)) were fixed in 4% buffered formaldehyde for 24 h at room temperature. Then, samples were rinsed in PBS solution for 3 h until dehydrated in graded ethanols (70%, 90% and 100%) and permeated in xylene (Panreac Química SLU, Barcelona, Spain). Afterwards, samples were embedded in melted paraffin (Merck, Madrid, Spain). Paraffin sections (5 μm) were stained using hematoxylin-eosin (HE). Observation of the samples was performed under the microscope (Olympus BX41 and Camera Olympus XC50, Olympus Barcelona, Spain). Representative photos were made for each sample of WAT (20x), BAT (40x) and intestine (10x) and were analyzed using Image J (Image Processing and Analysis in Java, National Institute of Mental Health, Bethesda, MD, USA). In SI, the length and width of microvilli were measured. In WAT, adipocyte area as well as the number of adipocytes per section were quantified. In BAT, the number of nuclei, the area of LDs as well as the number of LDs with a size greater than 50 μm^2 were measured.

2.7 Immunoglobulin quantification

Different tissues and samples were processed for Ig quantification by Enzyme-Linked ImmunoSorbent Assay (ELISA) (Bethyl, Laboratories Inc., Montgomery, TX, USA) and/or ProcartaPlex™ Multiplex immunoassay (eBioscience, San Diego, CA, USA).

Secretory (s)IgA quantification was performed by a sandwich ELISA technique in milk, salivary and mammary glands, MLN, cecal and fecal homogenates. Additionally, sIgA and IgM were evaluated in GW. Both Igs were quantified following the previous described protocol (20), and absorbance results were measured with a microplate photometer (Labsystems Multiskan, Helsinki, Finland)

at 495 nm, and data were analyzed by Multiskan Ascent v2.6 software (Thermo Fisher Scientific SLU, Barcelona, Spain). The lower limits of detection were 1.95 ng/mL for sIgA and IgM.

IgA, IgM, IgG and IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) were quantified in plasma, milk, and SG and MLN homogenates by ProcartaPlex™ Multiplex immunoassay. Briefly, 96 well flat bottom plates were used to prepare samples following manufacturer's instructions, as in previous studies (21). Data were acquired by MAGPIX® analyzer (Luminex Corporation, Austin, TX, USA) at the Cytometry Service of the Scientific and Technological Centers of the University of Barcelona (CCiT-UB). The lower limits of detection were: 0.58 ng/mL for IgA, 1.70 ng/mL for IgG1, 1.73 ng/mL for IgG2a, 2.67 ng/mL for IgG2b, 3.67 ng/mL for IgG2c and 0.2 ng/mL for IgM. The relative abundance of IgG subtypes was analyzed considering total IgG. Thus, Th1 and Th2 responses were evaluated adding the levels of IgG subtypes, IgG2b + IgG2c and IgG1 + IgG2a, respectively.

2.8 Cell subset staining and flow cytometry analysis

Phenotypic population analysis was performed in MLN and splenic cells by flow cytometry analysis using fluorescent mouse anti-rat monoclonal antibodies (mAbs) conjugated to different fluorochromes. All the chosen mAbs were purchased from BD Biosciences (San Diego, CA, USA), Serotec (Kidlington, Oxford, UK) and Caltag (Burlingame, CA, USA): anti-TCR αβ (R73), anti-CD103 (OX-62), anti-NK (10/78), anti-CD62L (OX-85), anti-CD8α (OX-8), anti-CD4 (OX-35), anti-CD45RA (OX-33), anti-TCRγδ (V65). The staining combination and gates strategy is showed in Supplementary Figure 1. The staining technique was performed following the protocol previously described by Marín-Gallen et al. (22). Analyses were performed with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, United States) in the CCiT-UB and data were analyzed by Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA).

2.9 Cecal bacteria and Ig-coated bacterial analysis

The proportion of cecal bacteria and Ig-coated bacteria (Ig-CB) was determined as previously described (23) with slight modifications, only 10 µL of the homogenized cecal sample was used. A Cytek Aurora (Cytek Biosciences, Inc., CA, USA) flow cytometry equipment was used in the CCTi-UB. The acquisition parameters were adjusted to obtain a maximum of 25.000 counts. Data analysis was performed using the FlowJo v.10 software. The total bacterial and the Ig-CB proportions were evaluated as Massot et al. established before (24).

2.10 Gene expression analysis

SI and WAT samples kept in RNAlater were thawed and homogenized for RNA extraction and gene expression analysis. Samples were placed in lysing matrix tubes (MP biomedicals, Illkirch, France) and homogenized using a FastPrep-24 instrument (MP biomedicals, Illkirch, France). RNeasy Mini Kit (Qiagen, Madrid, Spain) was used for RNA extraction following the manufacturer's instructions. RNA purity and concentration was determined with a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, USA) and cDNA obtained using TaqMan Reverse Transcripiton Reagents (Applied Biosystems, AB, Weiterstadt, Germany). Then, Real Time (RT) – PCR for target genes (Supplementary Table 1) was performed with ABI Prism 7900 HT quantitative RT-PCR system (AB). Results were normalized using the housekeeping gene *Gusb* (β-glucuronidase, Rn00566655_m1, I) and analyzed using the 2-ΔΔCt method, as previously described (25). Data is shown as the percentage of expression in each experimental group normalized to the mean value obtained for the REF group, which was set at 100%.

2.11 Detection of *B. breve* M-16V

B. breve M-16V detection was carried out in fecal and mammary gland samples by qPCR technique following the protocol previously described by Gil-Campos et al. (26). Genomic DNA was extracted from ~ 100 mg of fecal or tissue samples using the FastDNA kit (MP biomedicals Inc., Santa Ana, CA, USA) following the manufacturer instructions. The probiotic genome was detected by Taq-Man based PCR assay. The forward, reverse and probe used were previously designed by Phavichitr et al. (27). The PCR was performed with ABI Prism 7900 HT quantitative RT-PCR system (AB) at the CCiT-UB services.

2.12 Microbial profiling of rat samples analysis

Total DNA was isolated from fecal (100 mg) and milk samples (500 µL-1 mL) using an automated assisted method based on magnetic beads (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain) following the manufacturer's instructions with previous treatments to improve the DNA extraction. In brief, samples were treated with lysozyme (20 mg/mL) and mutanolysin (5 U/mL) for 60 min at 37°C and a preliminary step of cell disruption with 3-µm diameter glass beads during 1 min at 6 m/s by a bead beater FastPrep 24–5 G Homogenizer (MP Biomedicals). After the DNA extraction, DNA was purified using the DNA Purificaton Kit (Macherey-Nagel, Duren, Germany) following the recommended protocol and the final DNA concentration measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Microbial profiling was assessed by amplicon V3-V4 variable region of the 16S rRNA gene. Libraries were prepared following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The libraries were then sequenced using 2x300 bp paired-end run on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Negative and positive mock community (Zymobiomics) communities were also included.

Raw reads were then processed with the integrated dada2 method for denoising, amplicon sequence variance (ASV) clustering and chimeral removal. Reads were trimmed at 270 and 210 nucleotides in forward and reverse reads, respectively. Resulted ASV were then taxonomically assigned using Silva v.138. No rarefaction was done and also, samples with less than 4500 reads were removed and data was normalized using Centered-log-ratio (CLR). Beta diversity was based on Bray-Curtis distances and Permutational Analysis of Variance (PERMANOVA) was performed. Alpha-diversity indexes Chao1 and Shannon were also calculated and differences by group were assessed by Mann-Whitney and/or Kruskal-Wallis non-parametric test. Besides this, the Kruskall-Wallis test on the CLR normalized data were also assessed with Benjamini-Hochberg false discovery rate (FDR) correction. Taxa tables at phylum, family and genus level were provided to integrate to the other data obtained in the study. Negative binomial regression as implemented by DESeq2 tool was used for differential abundance analysis in order to estimate the fold-change of genus taxa (28). Plots were generated using MicrobeAnalyst platform v.2 (29).

2.13 Microbial metabolites profiling by SCFA analysis

SCFA analysis was performed using gas chromatography–mass spectrometry (GC-MS), following the method described by Eberhart et al. (30). An internal standard solution (3-Methylvaleric acid) was added to the samples that were processed and finally centrifuged at 4000 rpm for 2 min at 4°C according to the protocol. The final supernatant was collected, filtered-sterilized (0.22 µm PES size filter, Sarstedt SA) and then, injected in the Agilent GC 7890B–5977B GC-MS with a multipurpose sampler (Gerstel MPS, Mülheim, Germany). The GC column used was Agilent DB-FATWAX, 30 m × 0.25 mm × 0.25 µm, operated in split mode (20:1). The oven temperature program was set as follows: 100°C for 3 min, ramped to 100°C at a rate of 5°C min−1, then to 150°C for 1 min, further ramped to 200°C at a rate of 20°C min−1, and finally held at 200°C for 5 min. Helium was used as the carrier gas at a flow rate of 1 mL min−1, with an inlet temperature of 250°C. The injection volume was 2 µL. Standards curves for acetate, butyrate, and propionate were used for quantifying the SCFAs.

2.14 Statistical analysis

SPSS Statistics 22.0 software package (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. To assess normal distribution and homogeneity of variance of the data, Shapiro-Wilk and Levene test were used. Normal and homogeneous results were analyzed by one-way ANOVA. Kruskal-Wallis test was performed when results did not follow a normal and equal distribution to assess significant differences among groups ($p<0.05$). Spearman correlation coefficient was used to search correlation between variables. Non-metric multi- dimensional scaling (NMDS) in Rstudio using the R package vegan (31) was used to search clusters of similarities between samples in terms of immune factor composition. Besides, the function "envfit" assess the association of factors with the ordination of the samples in the NMDS. Differences were considered statistically significant when p value < 0.05.

3 Results

3.1 Animal body weight

Synbiotic supplementation during gestation and lactation did not affect either the body weight gain or the overall daily food and water intake during pregnancy or lactation. Only punctual changes were observed in the food intake at the middle of the gestation (Figure 1). As expected, in all groups the body weight exponentially increased during gestation and showed a sharped decrease on the delivery day (Figure 1A). The overall intake showed a distinct pattern associated to the growth gain during gestation and the higher requirements of food and water during lactation (Figures 1B, C).

3.2 Organ size and growth parameters

At the weaning day, dams were measured and weighted, and different organs were obtained and weighted (Supplementary Table 2). No changes were observed due to the synbiotic supplementation in the body size evaluation. However, an increase in the relative weight of the SI was observed in the SYN dams.

3.3 Adipose tissue

The SYN administration during gestation and lactation provoked some changes in the adiposity of the rats (Figure 2). The nutritional intervention did not modify the relative weight of the adipose tissue from different body locations (Figure 2A). Representative images of histologic sections of WAT and BAT are shown in Figure 2B and Supplementary Figure 2. The number of adipocytes and the adipocyte area of the parametric-WAT were not modified with the supplementation (Figure 2C). In contrast, the BAT of the SYN group showed an increase in the number of the nuclei associated with a reduction of the area of the lipid droplets (LD) and the number of LD bigger than 50 μm^2 (Figure 2D). Additionally, synbiotic supplementation showed a tendency to increase the relative gene expression of the *Ucp-1* gene (p=0.09) in WAT without affecting *Cidea*, *Prdm16*, *Pparγ*, free fatty acid receptor (*Ffar2)* and IL-1β gene expression (Figure 2E), suggesting a higher thermogenesis of the BAT.

3.4 Hematological variables

The last day of suckling, different hematological variables were analyzed in maternal blood samples (Supplementary Table 3).

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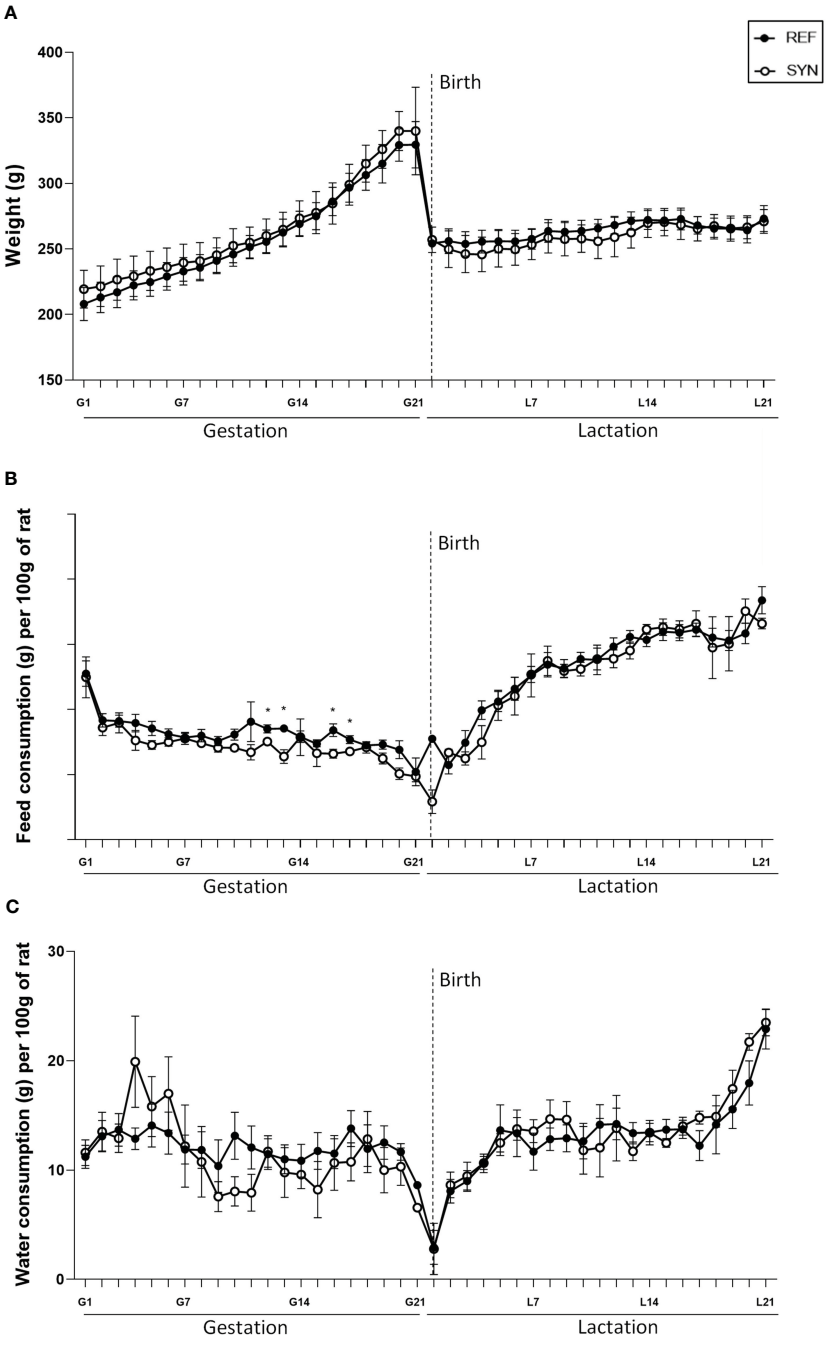

FIGURE 1
Effect of SYN supplementation on dams' growth and daily intake during gestation and lactation. **(A)** Dams' body weight (BW) evolution. **(B)** Dams' chow intake. **(C)** Dams' water intake. Data are expressed as mean ± standard error of the mean (S.E.M.). Statistical differences: *$p < 0.05$ *vs* REF. (n=5–6).

Synbiotic supplementation during gestation and lactation showed no effect on hematological parameters.

3.5 Fecal sample analysis

Feces from the SYN group showed an increase in the total IgA at the end of the lactation (Figure 3A). Regarding to the pH and water content, feces SYN dams had overall lower pH and water content (during pregnancy) compared to REF (Figures 3B, C).

3.6 Immunoglobulin quantification

To evaluate the impact of *B. breve* M-16V and scGOS/lcFOS during gestation and lactation on Ig levels, IgM, IgA and IgG

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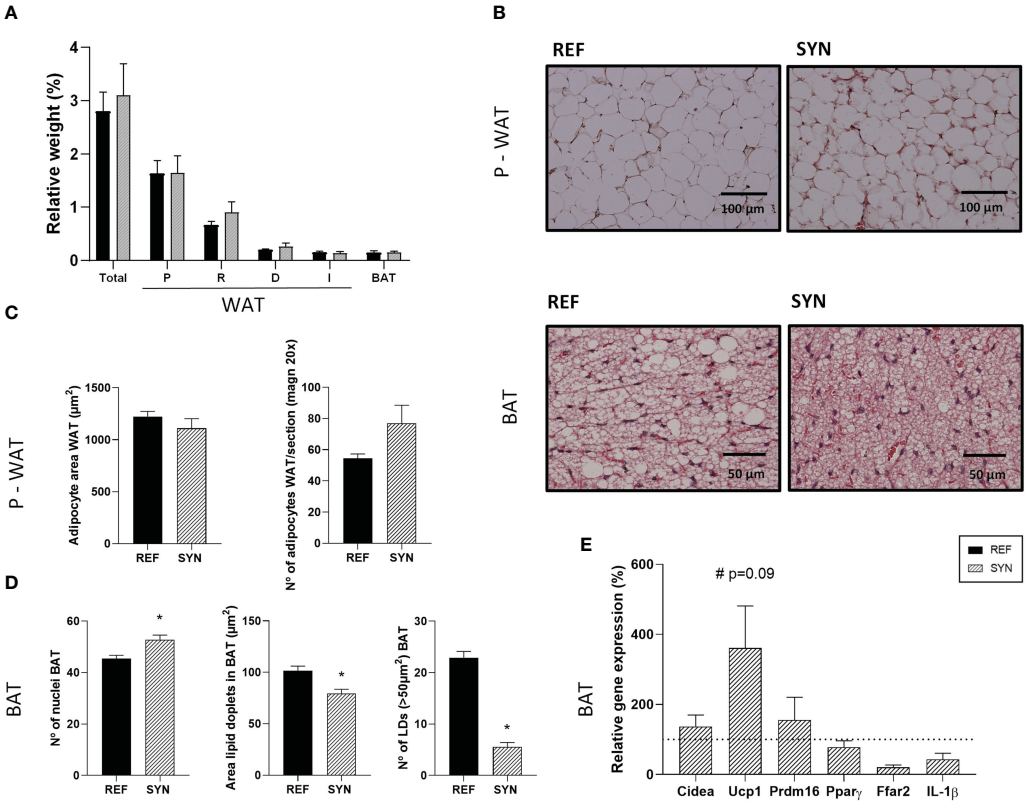

FIGURE 2
Impact of SYN supplementation on the adipose tissue. **(A)** Relative weight of the adipose tissue from different locations. **(B)** Hematoxylin and eosin-stained sections of parametric WAT (P-WAT) and BAT. Images were captured at 200x and 400x magnification, respectively. **(C)** Analysis of P-WAT: adipocyte area and number of adipocytes. **(D)** Analysis of BAT: number of nuclei, area of lipid droplets (LD), and number of LD (>50µm^2). **(E)** Relative gene expression analysis of BAT calculated with respect to REF, which corresponded to 100% of gene expression (represented with a horizontal dotted line). Data **(A, C, D)** are expressed as mean ± S.E.M. Statistical differences: *p< 0.05 *vs* REF. (n=5–6). WAT, white adipose tissue; BAT, brown adipose tissue; P, parametric; R, retroperitoneal; D, dorsal; I, Inguinal.

concentration was determined in plasma, milk, salivary gland and MLN (Figure 4). Both in plasma (Figure 4A) and milk (Figure 4D) the total levels of IgG were increased due to supplementation without changes in IgA or IgM. Conversely, in MLN IgM was higher and IgA tend to be increased without changes in IgG (Figure 4J). The synbiotic supplementation did not influence the Ig levels in the salivary gland (Figure 4G). The relative proportion of IgG subtypes (IgG1, Ig2a, Ig2b, IgG2c) was also evaluated. Synbiotic supplementation modified the IgG subtype profiles in plasma, milk and SG (Figures 4B, E, H, respectively), mainly by increasing the relative proportion of IgG2c without changing the relative proportion of Th1/Th2associated response (Figure 4M). Moreover, non-metric multi-dimensional scaling (NMDS) graphs of each compartment were plotted with the Igs data and, in plasma (Figure 4C) and milk (Figure 4F) samples, different clusters appeared (p<0.01 with the ANOSIM test). Furthermore, plasma

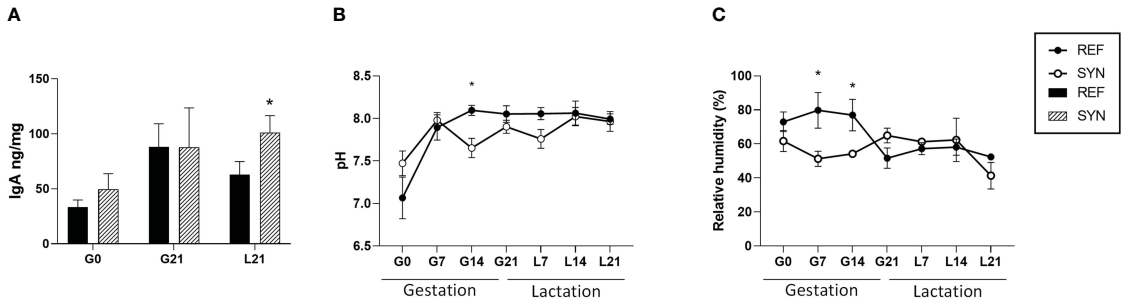

FIGURE 3
Fecal sample analysis throughout gestation and lactation. **(A)** IgA levels. **(B)** pH. **(C)** Relative water content. Data are expressed as mean ± S.E.M. Statistical differences: *p< 0.05 *vs* REF. (n=5–6). G, gestation; L, lactation.

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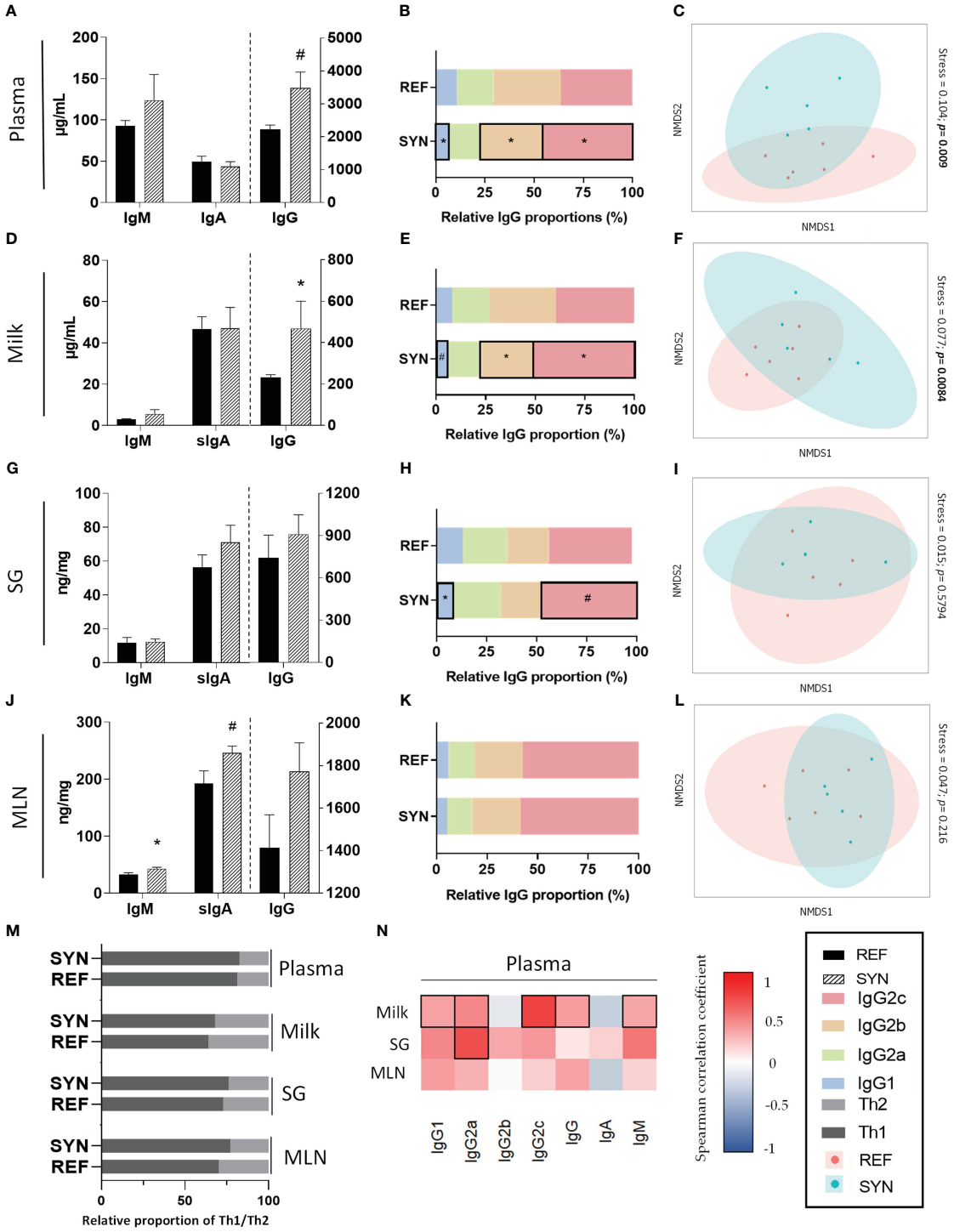

FIGURE 4
Effect of SYN supplementation on the Ig profiles in different compartments. Total Igs levels (IgA, IgM, and IgG) in **(A)** plasma, **(D)** milk, **(G)** SG and **(J)** MLN. Relative proportion of IgG subtypes in **(B)** plasma, **(E)** milk, **(H)** SG and **(K)** MLN. Analysis of non-parametric multidimensional scaling (NMDS) for the Ig profiles based on the Bray-Curtis distance in **(C)** plasma, **(F)** milk, **(I)** SG and **(L)** MLN. **(M)** Analysis of the Th1/Th2 ratio at the end of suckling in the different compartments. **(N)** Correlation between the Ig profile of plasma with respect to the Ig profiles of milk, SG and MLN. Data **(A, D, G, J)** are expressed as mean ± S.E.M. *$p< 0.05$ *vs* REF (by Kruskal Wallis test). (n=5–6). Each point represents an animal in figures **(C, F, I, L)** (n=11) (by ANOSIM test). The Spearman correlation coefficient is represented in the heat map following the color in the legend. Correlations with statistical significance ($p< 0.05$) are shown in a bold frame. SG, salivary gland; MLN, mesenteric lymph nodes.

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levels of IgG2c and IgG2b were highly correlated with the corresponding Ig in milk and in SG, respectively (Figure 4N). Additionally, the sIgA and IgM levels were quantified in the intestinal compartment in GW. Synbiotic supplementation did not affect either the sIgA (REF: 36.56 ± 6.72 µg/g; SYN: 28.98 ± 2.69 µg/g) or the IgM (REF: 0.14 ± 0.02 µg/g; SYN 0.09 ± 0.02 µg/g).

3.7 Phenotypic characterization of MLN and spleen cells

The relative proportion of MLN and spleen cell subsets was analyzed at the end of the study (Table 1). Spleen and MLN lymphocyte populations are rich in T cells with a low proportion of B lymphocytes. In terms of T helper (Th) and T cytotoxic (Tc) cells, Th predominates in both compartments. Regarding the minor populations of NK and NKT cells, the NK subset dominates in the spleen while the NKT subset prevails in the MLN. Synbiotic supplementation did not alter the proportion of B or T lymphocytes either in the spleen or in MLN. Additionally, the expression of CD8 was analyzed in the different subsets of lymphocytes, and only a punctual reduction in the proportion of Tc CD8+ cells occurred in MLN due to the supplementation. Additionally, adhesion molecules important in the intestinal homing such as αE integrin and CD62L were also assessed (Supplementary Figure 3). As expected, in both tissues CD62L was highly expressed and αE integrin was very low expressed. The synbiotic intervention did not modify the pattern expression of any of these molecules in the spleen or in MLN.

3.8 Gene expression analysis

The effect of *B. breve* M-16V and scGOS/lcFOS on gene expression in the intestine and mammary gland was assessed at the end of the lactation period (Figure 5). In the intestinal samples, genes implicated in the activity of the immune system and gut barrier were evaluated. Synbiotic supplementation increased the mRNA levels of *Muc3, Cldn4* and *Ocln*. No changes in TLR gene expression were observed in the intestine. Regarding the mammary gland, the SYN supplementation did not modify the expression of *IgA* or *Ffar2*.

3.9 IgA and Ig-CB cecal analysis

In the cecum, IgA is the most abundant Ig and can be bound to the resident bacteria (Figure 6). Synbiotic supplementation during gestation and lactation did not affect the IgA levels in the cecum (Figure 6A). The proportion of Ig-CB was evaluated in the cecum (Figure 6B), and after the supplementation, neither the total bacteria nor the relative proportion of Ig-coated bacteria were affected. However, the total Ig-CB tended to be increased (p=0.05). Furthermore, the amount of Ig coating of each bacteria

TABLE 1 Effect of *B. breve* M-16V and scGOS/lcFOS on the spleen and MLN immune cells proportion at the end of suckling period.

	SPLEEN		MLN	
%	REF	SYN	REF	SYN
B cells (CD45RA+)	9.13 ± 1.89	9.91 ± 4.09	13.64 ± 5.27	4.44 ± 5.71
T cells (TCRαβ+NK- and TCRgδ+)	72.23 ± 7.38	64.08 ± 8.67	79.90 ± 2.80	70.44 ± 17.04
TCRαβ+ NK-	64.87 ± 8.33	60.40 ± 10.76	77.83 ± 3.27	68.80 ± 17.35
% CD8+	27.83 ± 1.83	27.06 ± 1.52	22.63 ± 1.87	16.51 ± 4.31
TCRgδ+	2.09 ± 0.55	3.68 ± 2.11	2.61 ± 0.69	1.64 ± 0.32
% CD8+	2.56 ± 0.84	1.54 ± 0.35	3.42 ± 2.28	0.72 ± 1.43
CD4+ CD8-	49.15 ± 7.39	41.83 ± 9.31	61.88 ± 2.64	56.11 ± 7.32
CD8+ CD4-	24.72 ± 2.80	24.28 ± 3.68	20.28 ± 0.34	**18.4 ± 0.72***
CD4+ CD8+	2.76 ± 0.59	1.45 ± 0.45	1.33 ± 0.26	1.00 ± 0.25
NK (TCRαβ- NK+)	4.45 ± 2.01	5.33 ± 2.73	0.85 ± 0.16	1.14 ± 0.54
% CD8+	55.86 ± 9.45	40.26 ± 18.52	21.47 ± 7.11	30.42 ± 11.90
NKT (TCRαβ+ NK+)	2.81 ± 0.85	3.77 ± 0.92	1.81 ± 0.44	1.80 ± 0.28
% CD8+	79.16 ± 5.46	86.30 ± 2.00	60.35 ± 7.37	64.80 ± 7.51
αE+	2.72 ± 0.40	4.47 ± 2.20	3.88 ± 0.76	8.04 ± 2.60
CD62L+	77.72 ± 0.33	72.81 ± 8.14	69.07 ± 10.40	52.90 ± 5.86

Data are expressed as mean percentage ± S.E.M. (n=5–6). Statistical differences: *p< 0.05 vs REF. mAbs: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), brilliant violet 421 (BV421), and phycoerythrin-Cyanine 7 (PE-Cγ7).

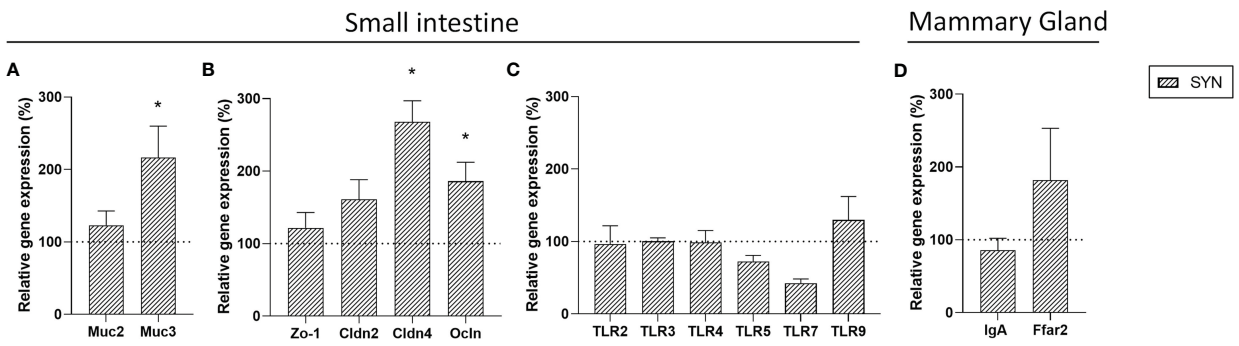

FIGURE 5
Gene expression in small intestine and mammary gland. Relative gene expression analysis in the small intestine of **(A)** mucins, **(B)** TJ proteins and **(C)** Toll-like receptors. **(D)** Relative gene expression of *IgA* and *GPR43* (*Ffar2*) genes in mammary gland. Relative gene expression was calculated with respect to REF, which corresponded to 100% of transcription (represented with a horizontal dotted line). Statistical differences: *$p<0.05$ *vs* REF. (n=5–6). *Muc2*, mucin2; *Muc3*, mucin3; *Zo-1*, Zonula occludens-1; *Cldn2*, claudin 2; *Cldn4*, claudin 4; *Ocln*, occludin; *TLR*, Toll-like receptor; *IgA*, immunoglobulin A; *Ffar2*, free fatty acid receptor 2.

was measured, by means of the Mean Fluorescence Intensity (MFI) of this population which was highly increased after the SYN supplementation (Figure 6C).

3.10 Intestinal histomorphometry

The impact of the synbiotic on the intestinal architecture showed that the synbiotic supplementation during gestation and lactation induced histological changes in the intestinal epithelium (Figure 7; Supplementary Figure 4). Specifically, the villi height and the crypts depth were increased in the SYN group. On the contrary, the villi width was lower in the supplemented animals (Figure 7B). The synbiotic did not change the number of goblet cells responsible for mucus secretion.

3.11 *B. breve* M-16V detection in feces and mammary gland

The presence of *B. breve* M-16V in feces and MG was studied also (Figure 8). In feces, both at the end of the gestation and lactation periods the number of *B. breve* M-16V UFC was increased 1000xin the supplemented dams with respect to the basal levels at the beginning of the intervention represented with the horizontal dotted line. The detection of *B. breve* M-16V in REF animals during

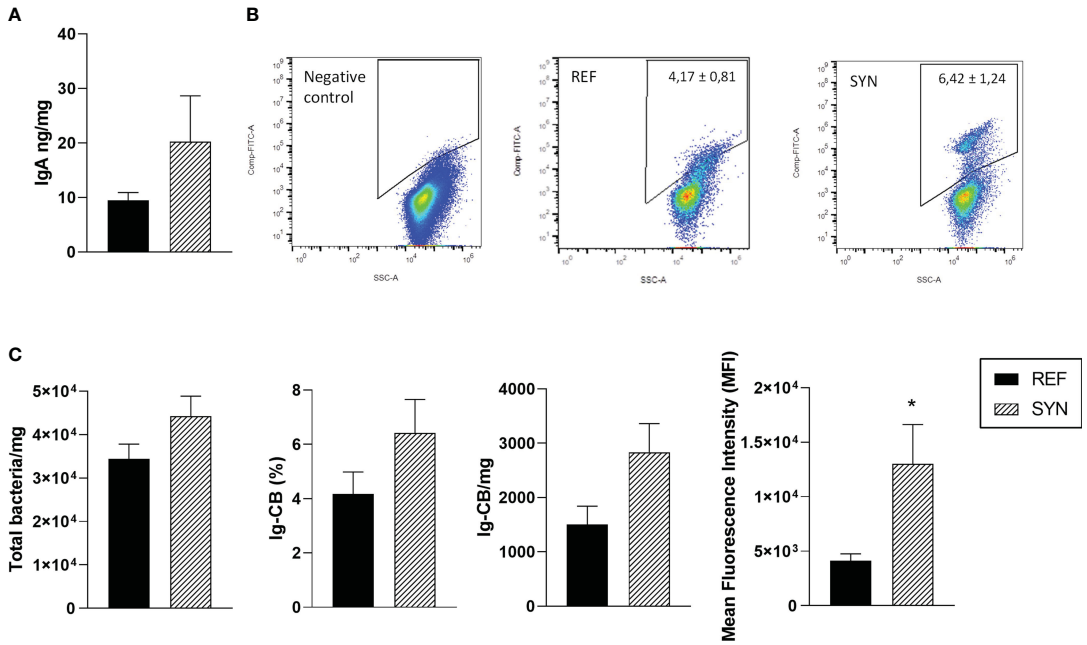

FIGURE 6
Evaluation of the cecal samples. **(A)** IgA concentration in cecum. **(B)** Representative dot-plots of Ig-CB. **(C)** Total bacterial counts in the cecum, proportion of Ig-CB, total Ig-CB and the mean fluorescence intensity (MFI) of the Ig-CB. Data are expressed as mean ± S.E.M. Statistical differences: *$p< 0.05$ *vs* REF. (n=5–6).

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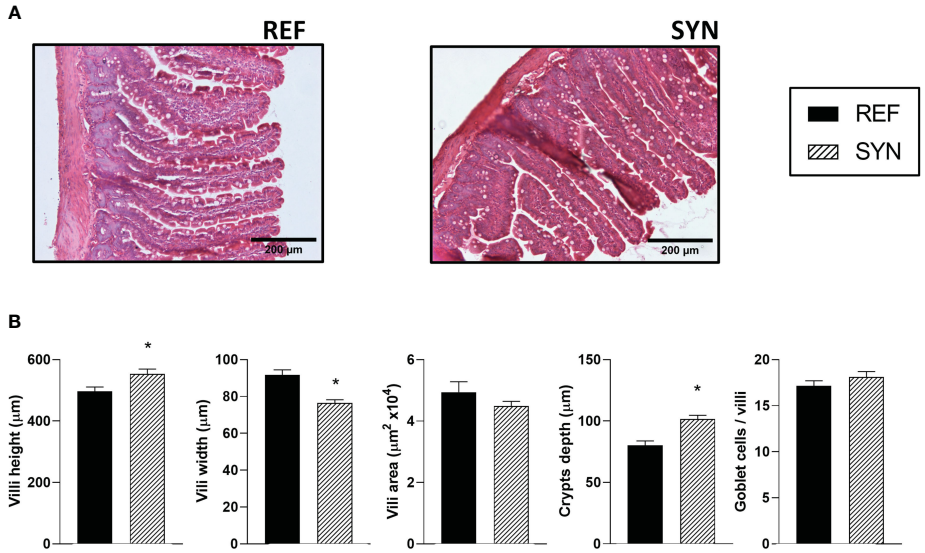

FIGURE 7
Effect of SYN on intestinal architecture. **(A)** Representative images of the small intestine stained with hematoxylin and eosin, 100X. **(B)** Height, width, and area of the intestinal villi. Results are expressed as mean ± S.E.M. Statistical differences: *p< 0.05 *vs* REF. (n=5–6).

gestation and lactation was always at basal levels (˜10EXP3). In order to explore the gut-mammary pathway its presence was also evaluated in MG. However, *B. breve* M-16V was not detected either in the SYN or the REF MG.

3.12 Impact of SYN supplementation on the rat intestinal and milk microbiota

Significant differences between groups and sample types were found (CC, CI and milk samples) as reported in the beta-diversity Bray-Curtis PERMANOVA and also, alpha-diversity indexes as well as taxonomical composition (Supplementary Figure 4).

SYN supplementation had an impact on the CC microbiota profile. Statistically significant differences were detected in the microbiota profiles of REF and SYN (permutational multivariate analysis of variance (PERMANOVA) *Bray-Curtis F-value*=3.1912; *R-squared*=0.26176; p=0.004) (Figure 9A), as well in the alpha-diversity indexes as measured by the Chao1 (p=0.01) and Shannon indices (p=0.662), respectively, (Figures 9B, C).

Regarding the taxonomy of the CC, differences were found in the phylum, family and genera. In the SYN group, the proportion of *Firmicutes* tend (p=0.06) to be reduced while the abundance of *Desulfobacteria* was significantly reduced. The family analysis revealed that *Desulfovibrionaceae* and the *Suterellaceae* families were reduced in the SYN group (Figure 10). Thus, specific microbial genera were significantly present in SYN group including *Bifidobacterium*, *Faecalibaculum* (*Erysipelotrichidae* family) and *Marvinbruantia* (*Lachnospiraceae* family) and other butyrate producers such as *Blautia*, *Ruminoclostridium* and also, other *Lachnospiraceae* (Table 2).

The impact of the SYN supplementation was also observed in the intestinal content (IC) as two distant groups were identified. The beta-diversity analysis with Bray-Curtis distances reported significant differences (PERMANOVA] F-value: 2.6694; R-squared: 0.22875; p-value: 0.007) while no significant differences were observed in alpha-diversity indexes (Figure 9). The taxonomic analysis of the IC showed that the *Firmicutes* phylum was reduced and the *Actinobacteria* phylum tend to be higher in the IC of the SYN supplemented animals (p=0.07). With regard to the family diversity, the *Bifidobacteriaceae* tend to be increased in the SYN group (p=0.07) (Figure 10). The specific genera were overrepresented in SYN compared to REF group, including again the presence of *Bifidobacterium*, *Faecalibaculum* and *Blautia* (Table 3).

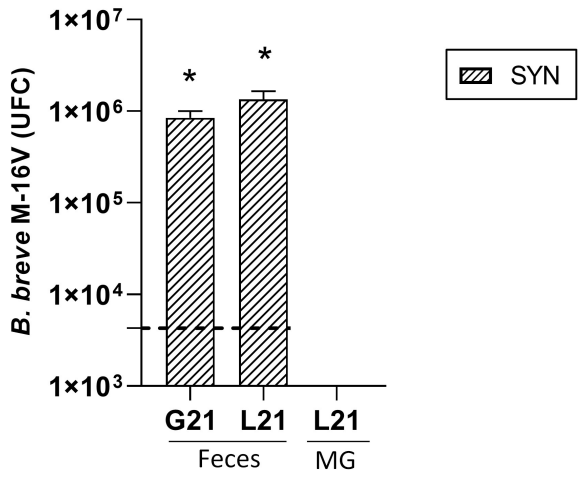

FIGURE 8
Detection of *B. breve* M-16V in fecal samples during gestation (G21) and lactation (L21) and in mammary gland (MG). The horizontal dotted line represents the levels of the REF group, which were considered as basal levels. Results are expressed as mean ± S.E.M. *p< 0.05 *vs* basal levels (n=5–6).

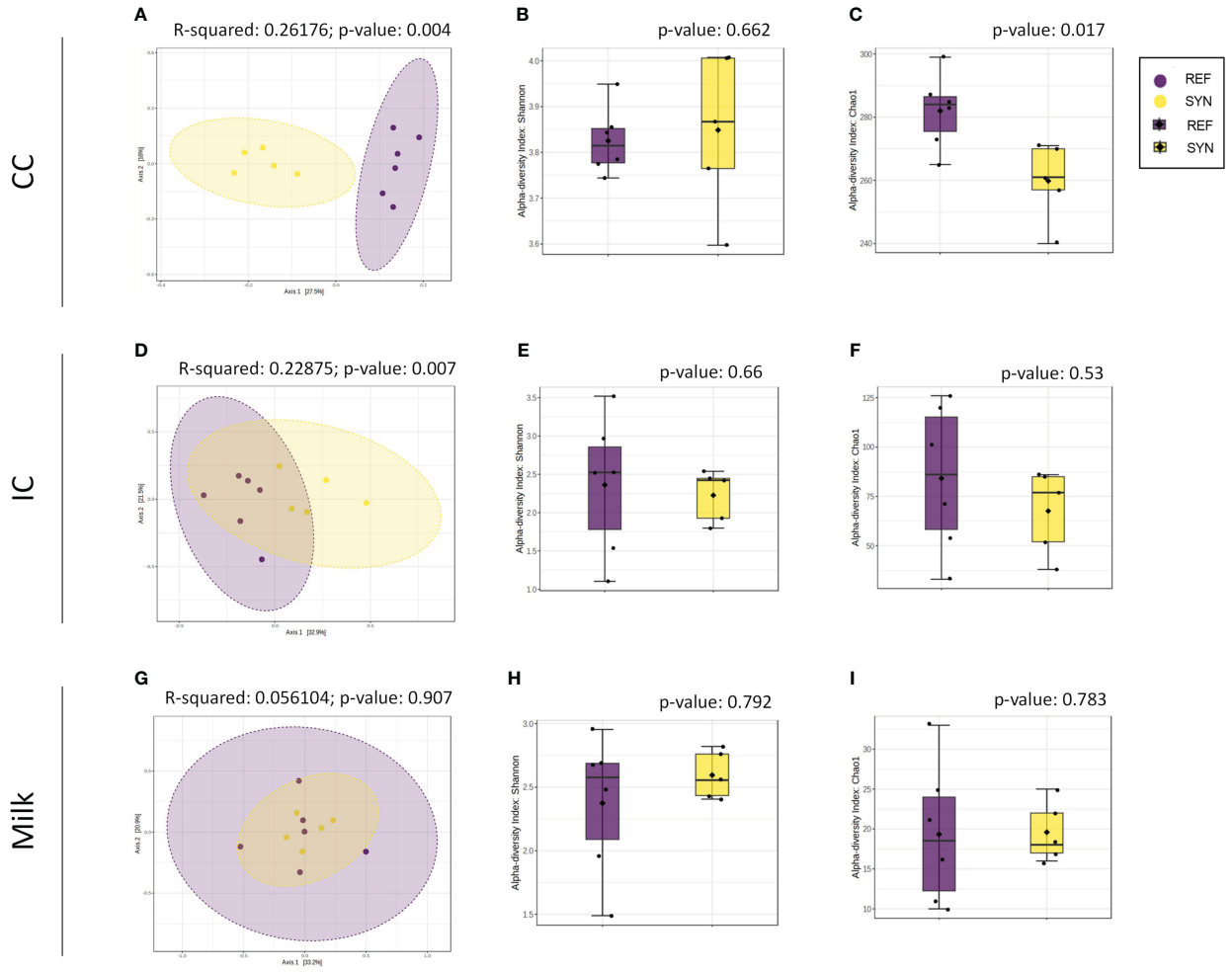

FIGURE 9
Beta- and alpha-diversity in rat microbiota depending on SYN intervention. Beta-diversity analysis using Bray-Curtis distance in CC **(A)**, IC **(D)** and milk samples **(G)**. Alpha-diversity indexes (Shannon index) and richness (Chao1 index) for CC **(B, C)**, IC **(E, F)** and milk **(H, I)** samples. Statistical testing was performed by PERMANOVA using Bray Curtis distances and the Mann-Whitney test was used for alpha-diversity indexes. (n=5–6).

Regarding the milk microbiota profile, the SYN supplementation had no effect on the microbiota profile. No differences were found in beta-diversity and alpha-diversity between SYN and REF groups (Figures 9G–I). In general, milk microbiota in rats is characterized by a higher presence of *Firmicutes*. Our results indicated that the SYN supplementation tend to reduce the proportion of *Firmicutes* and *Actinobacteria* phylums (p=0.084 and p=0.088, respectively). In the family analysis, the *Bifidobacteriaceae* tend to be higher (p=0.07) in the SYN group (Figure 10). Finally, the genus analysis confirmed that SYN group had higher levels of *Bifidobacterium* compared to those observed in the REF group (6% *vs*. 1%, respectively but p-value FDR>0.05).

3.13 Cecal SCFA content

SCFA are the communication channel of the intestinal microbiota and the immune system. For this reason, the main SCFA were evaluated in CC at the end of the study (Figure 11). After the SYN supplementation, the total amount of SCFA was increased in the cecum (Figure 11A). This increase was mainly due to an increase in the total amount of acetic, propanoic, butanoic and isovaleric acids (Figure 11B).

3.14 Correlations between microbiota, SCFA and Igs

Considering the importance of SCFA derived from the microbiota in the overall intestinal health, the correlation between SCFA-microbiota-Ig was evaluated (Figure 12). Acetic, propanoic, and butanoic acids were positively correlated with the cecal *Bifidobacterium* detected by sequencing the 16s gene (0.76, p=0.01; 0.79, p=0.006; 0.84, p=0.002, respectively). Likewise, acetic and propanoic acids were positively correlated with the cecal *Blautia* (0.73, p=0.01; 0.84, p=0.002, respectively). After the microbiota analysis, the correlation between the *Bifidobacterium* and the IgG2c of the plasma, milk, SG and MLN was performed. The cecal *Bifidobacterium* was positively correlated with the plasma and milk IgG2c. Furthermore, the correlation between the cecal

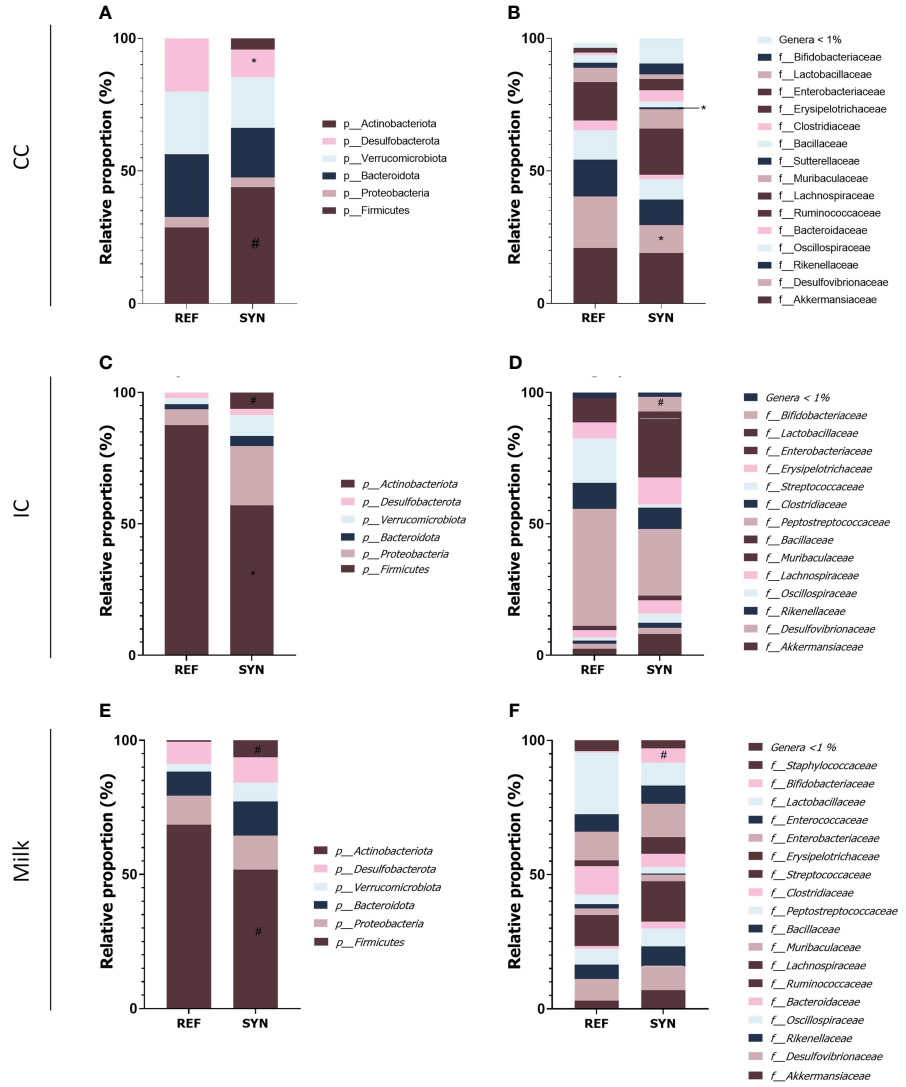

FIGURE 10
Microbiota composition of dams at weaning in cecal content (CC), intestinal content **(IC)** and milk. Relative proportions **(A)** phylum and **(B)** families present in the CC. Relative proportions **(C)** phylum and **(D)** families present in the IC. Relative proportions **(E)** phylum and **(F)** families present in the milk. Results are expressed as relative proportions of population. Statistical differences: $^{*}p< 0.05$ *vs* REF; $^{\#}p< 0.1$ *vs* REF. (n=5–6).

SCFA levels and the concentration of IgG2c in plasma, milk, SG and MLN was assessed. Results suggest that plasmatic and milk IgG2c were positively correlated with the increased acetic and propanoic acids. Additionally, a positive correlation was observed between the IgG2c of the MLN with the valeric acid.

4 Discussion

During gestation and lactation, the maternal immune system undergoes significant changes to adapt to this critical period (32). To protect the fetus, a delicate balance between tolerance and defense is established, mediated by among others a unique Th1/Th2 response equilibrium. In early pregnancy, a shift towards a Th2 dominant immune response occurs, preventing fetal rejection. As gestation progresses, the immune response gradually becomes more balanced between Th1 and Th2, with Th1 primarily defending the fetus against pathogens. Maintaining this equilibrium is vital to ensure immune tolerance towards the fetus while allowing the maternal immune system to combat infections (33). After labor, breastfeeding further supports the infant's immune balance by adjusting its composition based on the infant's needs (34). This bridge between maternal and infant immune systems during gestation and lactation plays a vital role in safeguarding both mother and child's health.

Additionally, maternal immunological status impacts the offspring's development. In the last decades, probiotics, prebiotics and synbiotics have demonstrated beneficial effects in adult individuals (6). The research of synbiotics for immunological health improvement has been focused on prophylactic or complementary treatments for different diseases such as infections or antibiotics-induced diarrhea (9). However, few studies have evaluated the impact of synbiotic supplementation during gestation and lactation on the maternal and newborn immune system (35).

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TABLE 2 Differential microbial genera between REF and SYN in CC.

Genera	log2FC	lfcSE	p-values	FDR
g__*Anaeroplasma*	-23.783	2.4324	0.000	0.000
g__*Bifidobacterium*	8.6122	1.2076	0.000	0.000
g__*Faecalibaculum*	9.2997	1.3103	0.000	0.000
g__*Marvinbryantia*	8.8595	1.3216	0.000	0.000
g__*Eubacterium_ruminantium_group*	-8.72	1.3289	0.000	0.000
g__*Colidextribacter*	-1.2643	0.28954	0.000	0.000
g__*otherMuribaculaceae*	0.98846	0.33762	0.003	0.037
g__*Harryflintia*	1.2993	0.45857	0.005	0.044
g__*Ruminiclostridium*	1.627	0.61146	0.008	0.066
g__*Incertae_Sedis*	1.5033	0.57876	0.009	0.066
g__*Blautia*	4.9847	1.9217	0.009	0.066
g__*Lachnospiraceae_NK4A136_group*	1.2904	0.54075	0.017	0.108

DESeq2 results showing the log2 fold-change values of bacteria at genus level between REF and SYN groups (positive means more represented in SYN vs REF, and negative values means more represented in REF than in SYN).

Here, we have demonstrated that the supplementation with a synbiotic composed of *B. breve* M-16V and scGOS/lcFOS during pregnancy is safe for the dams, since it does not affect the weight gain, food or water intake and the hematological parameters at the weaning day.

Physiologically, the expansion of adipose tissue during pregnancy aims to provide necessary nutrients for the correct development of the fetus (36). Dietary habits during pregnancy play an important role in the offspring health. Maternal high fat diet causes reprogramming of adipose tissue including increasing adipogenic and lipogenic markers in both WAT and BAT (37). The overexpansion of the adipose tissue during gestation may increase the adverse outcomes in the later life of both the mother and the offspring, mainly associated with glucose and insulin metabolism (38–40). In this study, supplementation with scGOS/lcFOS and *B. breve* M-16V during pregnancy, increased the number of nuclei and decreased the area of LDs in BAT. Moreover, the slight increase of *Ucp1* expression may be able to justify the obtained results pointing out the activation of this tissue (38, 41). BAT is specialized in dissipating excess energy into heat (non-shivering thermogenesis) through mitochondrial uncoupling protein 1 (*Ucp1*) (42). It is well documented that bioactive compounds like antioxidants and dietary fiber enhance the expression of thermogenic genes in BAT (43–45). In fact, BAT is involved in heat generation for maintaining the body temperature, and it is diminished in obese individuals. Thus, a higher number of nuclei and a reduction of the area of the LDs indicate a higher activation of the BAT (41). Joining these approaches, our results suggest that synbiotic supplementation influences positively the adipose tissue during gestation and lactation by increasing the BAT activity.

Different samples from the gastrointestinal tract were analyzed such as the SI, the cecum, and feces at weaning. *B. breve* M-16V and scGOS/lcFOS induced macroscopic and microscopic trophic effects on the SI. The relative weight of the SI and the villi and crypts length increased at the end of lactation, suggesting a higher nutrient-absorptive surface that could contribute to a healthier

TABLE 3 Differential microbial genera between REF and SYM in IC.

Genera	log2FC	lfcSE	p-values	FDR
g__*Faecalibaculum*	9.9933	1.7576	4.945E-7	1.3013E-8
g__*Bifidobacterium*	9.6098	1.8423	3.4689E-6	1.8257E-7
g__*Eubacterium_ruminantium_group*	-10.295	2.1721	2.7154E-5	2.1437E-6
g__*Blautia*	7.8482	2.284	0.0050207	5.8997E-4
g__*Colidextribacter*	-6.5453	1.922	0.0050207	6.6061E-4
g__*Lactococcus*	-4.3031	1.5419	0.033301	0.0052581
g__*Ruminococcus_gauvreauii_group*	3.8752	1.8731	0.20928	0.038552
g__*Bacteroides*	-4.3033	2.2519	0.23335	0.056004

DESeq2 results showing the log2 fold-change values of bacteria at genus level between REF and SYN groups (positive means more represented in SYN vs REF, and negative values means more represented in REF than in SYN).

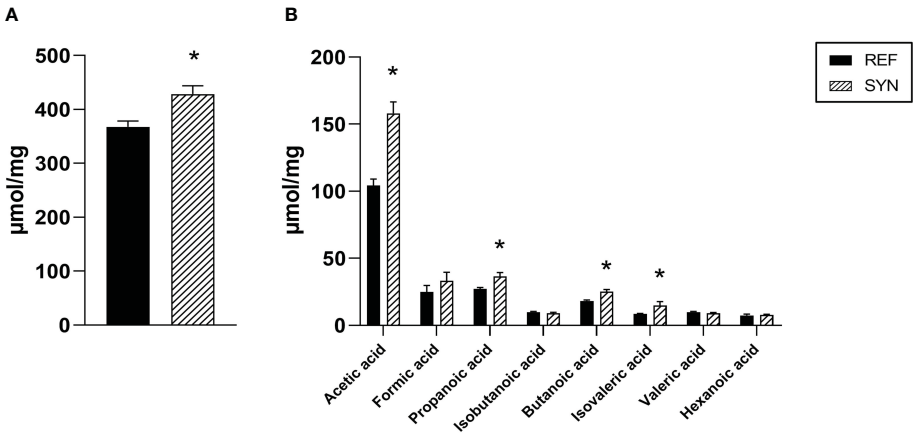

FIGURE 11
Cecal SCFA composition at the end of the weaning. **(A)** Total SCFA. **(B)** Acetic, formic, propionic, isobutanoic, butanoic, isovaleric, valeric and hexanoic acids were quantified by HS-GC-MS. Results are expressed as mean ± S.E.M. Statistical differences: *$p < 0.05$ *vs* REF. (n=5–6).

gastrointesinal tract and overall health (46). In addition to the observed morphologic changes resulting from supplementation, we also examined the immunological maternal status at systemic and gastrointestinal levels. The systemic immunological status was assessed by measuring the concentration of various Igs in different compartments, including plasma and milk. The supplementation of *B. breve* M-16V and scGOS/lcFOS enhanced the presence of IgG2c in both plasma and milk. In addition, a positive correlation between IgG2c levels in plasma and milk was found, suggesting the improvement of the immunological composition of milk. Human milk is known to contain high levels of IgA, which provides protection to the gastrointestinal tract. In contrast, rat milk primarily consists of IgG, which enhances short-term systemic immune response through receptor-mediated endocytosis in the neonatal intestine, facilitating its absorption (47). Similar to our results, previous studies have demonstrated that supplementation with the human milk oligosaccharide 2'-Fucosyllactose increases neonatal plasmatic IgG2c during early life (18) which also correlates in the maternal Ig profile of plasma and milk (48).

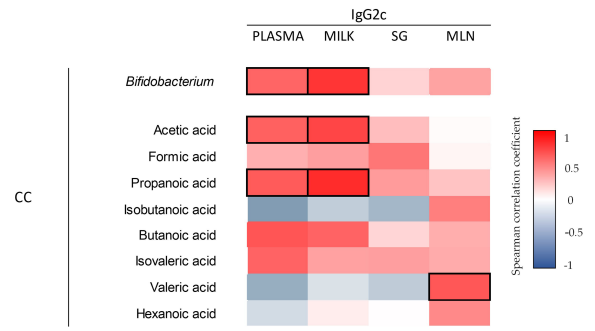

FIGURE 12
Correlation between the levels of Ig2c in plasma, milk, SG, and MLN with respect to the *Bifidobacterium* (16s sequence) and cecal SCFA. The Spearman correlation coefficient is represented in the heat map following the color in the legend. Correlations with statistical significance ($p < 0.05$) are shown in a bold frame. (n=5–6). SG; salivary gland; MLN, mesenteric lymph nodes; CC, cecal content.

After infections IgGs are the main contributors to long-term immunity (49). Little is known about the role of dams IgG2c. However, in mice IgG3 (analog of rat IgG2c) (47) has been linked to regulatory responses in the neonate intestine to translocate microbes and might be involved in long-term immunity (50). Considering the increase of IgG2c in plasma and milk, we suggest that maternal synbiotic supplementation induces the IgG2c isotype switching to promote the long-term passive immunization to the infant through the breastfeeding.

The study of the gastrointestinal tract revealed that the synbiotic supplementation not only modified the microstructures of the SI but also the gene expression levels. The synbiotic supplementation increased *Muc3, Cldn4* and *Ocln* intestinal expression at the weaning day. *Muc3* is a transmembrane mucin that exerts protective roles in inflammatory bowel conditions (51). *Cldn4* and *Ocln* are epithelial tight junction (TJ) proteins involved in the passage of ions and macromolecules across the intestinal epithelium (52). The effects of the probiotic *B. breve* M-16V or the prebiotic scGOS/lcFOS have been widely studied by separate, suggesting anti-inflammatory and protective roles, respectively (53, 54). However, little information is known about the combination of both. Overall, these results indicate a direct relationship between the synbiotic supplementation and the improvement of the gut barrier function.

IgA is the most abundant Ig in the mucosal compartment and contributes to the development of the immune response (55). The analysis of Ig-CB has been controversial due to its imbalance in healthy and disease conditions (56). In heathy people, IgA-coated microbiome plays a homeostatic role in the gut favoring host-microbiome symbiosis while in inflamed gut it may exacerbate the inflammation (57). In our model in healthy conditions, the synbiotic supplementation, did not impact the sIgA, however it increased the Ig-CB in cecum, corroborating previous results that suggest the participation in the maintenance of the gut homeostasis (55, 58–61). The production of cecal IgA can be induced by endogenous or pathogenic bacteria, pathogen-induced IgA is

considered to have high-affinity and specificity (62–64). This fact suggests that even though the IgA is not increased, the increased Ig-CB can be linked to the higher affinity induced by pathogenic species, facilitating its elimination.

Although there is limited information about the impact of synbiotics on cecal and fecal features during gestation and lactation, the effect of probiotic supplementation has been studied during these periods (10, 55). In our case, synbiotic supplementation reduced the fecal pH during gestation. Probiotics have been linked to a reduction of the intestinal pH due to the production of organic acids (61). As the cecum is characterized by the presence of high amounts of SCFA, and the synbiotic supplementation led to an overall increase in SCFA levels, primarily attributed to the rise in acetic, propanoic, butanoic, and isovaleric acids, both facts could be connected. In addition, numerous studies have shown that SCFAs have beneficial effects on energy metabolism, intestinal structure and integrity, as well as immunological regulation of anti-inflammatory activities (65). Overall, our study suggests that synbiotic supplementation enhance the production of SCFA which exert a reduction of the fecal pH.

Previously, it has been demonstrated that a maternal supplementation with a probiotic is able to influence the milk composition (66). The exact mechanisms involved are not yet fully understood, but both a direct and indirect effect could be participating. Regarding the direct effect of the probiotic, it could be due to its arrival to the breast milk by the entero-mammary pathway (67). This pathway enables the movement of commensal bacteria from the intestine to the mammary gland and milk, and consequently microorganisms are able to reach the infant intestine and therefore influencing the colonization of the newborn's microbiota (66). This is not the case in our study, where we did not observe the presence of *B. breve* M-16V in the mammary gland.

Pregnancy leads to dramatic changes in the gut microbiome. In the last decades it has been demonstrated that during pregnancy a healthy microbiota undergoes a shift to a more dysbiotic one (68). In general, pregnancy reduces the alpha diversity and increases the beta diversity (69). Considering our results of the cecal microbiota, the synbiotic supplementation modified the beta diversity and also, the Chao1 diversity index and the cluster aggrupation of the supplemented group was different compared to the non-supplemented one. The SYN intervention also influenced the beta-diversity but not the alpha-diversity indexes in the IC. It is also relevant that SYN intervention did not influence the milk microbiota profile. However, the supplementation with the synbiotic influences the phylum, family and genus composition of the CC, IC and milk. In general, during gestation *Faecalibacterium* is reduced in the CC and IC (70). It has to be noted that *Faecalibacterium* is one of the main producers of butyrate (71). Our results indicated that the maternal synbiotic supplementation is able to counteract this reduction during this period, which can be confirmed with the levels of SCFA, as butyrate was increased in the SYN group. In addition, one of the most remarkable microbiota changes is the increase of the *Bifidobacteriaceae* and *Bifidobacterium* in the milk, suggesting that the maternal supplementation may be able to modify the milk composition through the entero-mammary route (67), as this change is also found in the CC. The importance of *Bifidobacterium* is due to the positive impact on the infant development, improving the nutrients absorption and regulating the immune system (72, 73). These findings confirm that this particular maternal synbiotic supplementation reduces microbiota alterations during gestation while enriches milk composition. These facts will contribute to the infant colonization and immune system maturation through the breastfeeding.

The obtained results point out that maternal nutritional intervention with *B. breve* M-16V and scGOS/lcFOS modulates maternal immune system during pregnancy and lactation. Synbiotic supplementation not only modulates the immunological profile of dams, but also the immune components of the milk composition which targets to encourage the infant development. Although the beneficial results of the study are clear, there are still some limitations and gaps that require further investigation. In this sense, although animal models have been highly useful in understanding the physiological changes induced after conception, there are significant differences between humans and animals, in this case rats, regarding pregnancy features. As consequence, integrating pre-clinical results into clinical knowledge should be performed to ensure translation. Besides this, to complete the study, further research is needed to determine whether supplementation during pregnancy or breastfeeding has a greater impact on modulating the maternal immune system and the composition of breast milk. Additionally, it is important to analyze the impact of *B. breve* M-16V and scGOS/lcFOS separately to evaluate if any of them interacts more with the maternal immune system. Finally, the last step required would be to analyze the impact of these maternal interventions on their offspring and study the transmission of the bioactive components from the mother to the infants.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona (UB). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. GK: Formal analysis, Investigation, Methodology, Writing – review & editing. BG: Formal Analysis, Investigation, Writing – review & editing. MM: Formal

analysis, Investigation, Writing – review & editing. MB: Formal analysis, Investigation, Writing – review & editing. KK: Resources, Visualization, Writing – review & editing. JG: Resources, Visualization, Writing – review & editing. RB: Resources, Writing – review & editing. MC: Supervision, Writing – review & editing. MR: Conceptualization, Supervision, Writing – review & editing. MC: Conceptualization, Funding acquisition, Writing – review & editing. FP: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

JG is part time employee/scientific advisor of Danone Nutricia Research. RB-S is employee of Danone Nutricia Research, and KK was also at time of the experiment.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1418594/full#supplementary-material

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Dietary supplementation with Macleaya cordata extract alleviates intestinal injury in broiler chickens challenged with lipopolysaccharide by regulating gut microbiota and plasma metabolites

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Introduction: The prevention and mitigation of intestinal immune challenge is crucial for poultry production. This study investigated the effects of dietary Macleaya cordata extract (MCE) supplementation on the prevention of intestinal injury in broiler chickens challenged with lipopolysaccharide (LPS).

Methods: A total of 256 one-day-old male Arbor Acres broilers were randomly divided into 4 treatment groups using a 2×2 factorial design with 2 MCE supplemental levels (0 and 400 mg/kg) and 2 LPS challenge levels (0 and 1 mg/kg body weight). The experiment lasted for 21 d.

Results and discussion: The results showed that MCE supplementation increased the average daily feed intake during days 0-14. MCE supplementation and LPS challenge have an interaction on the average daily gain during days 15-21. MCE supplementation significantly alleviated the decreased average daily gain of broiler chickens induced by LPS. MCE supplementation increased the total antioxidant capacity and the activity of catalase and reduced the level of malondialdehyde in jejunal mucosa. MCE addition elevated the villus height and the ratio of villus height to crypt depth of the ileum. MCE supplementation decreased the mRNA expression of pro-

Abbreviations: MCE, Macleaya cordata extract; LPS, lipopolysaccharide; ADG, average daily gain; ADFI, average daily feed intake; F/G, the ratio of feed to gain; T-AOC, total antioxidant capacity; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase; VCR, villus height-to-crypt depth ratio; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard error of the mean; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; QC, quality control; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis; TXB2, thromboxane B2; ROS, reactive oxygen species; NF-κB, nuclear factor kappa B; PLD, phospholipase D; GABA, precursor of γ-aminobutyric acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; M/Z, mass to charge ratio; FC, fold change; VIP, variable important in projection; 20-Hydroxy-PGF2a, 20-hydroxy Prostaglandin F2a.

inflammatory cytokines interleukin (*IL*)-*6* and *IL-8* in the jejunum. MCE addition mitigated LPS-induced mRNA up-expression of pro-inflammatory factors *IL-1β* and *IL-17* in the jejunum. MCE supplementation increased the abundance of probiotic bacteria (such as *Lactobacillus* and *Blautia*) and reduced the abundance of pathogenic bacteria (such as *Actinobacteriota*, *Peptostretococcaceae*, and *Rhodococcus*), leading to alterations in gut microbiota composition. MCE addition altered several metabolic pathways such as Amino acid metabolism, Nucleotide metabolism, Energy metabolism, Carbohydrate metabolism, and Lipid metabolism in broilers. In these pathways, MCE supplementation increased the levels of L-aspartic acid, L-Glutamate, L-serine, etc., and reduced the levels of phosphatidylcholine, phosphatidylethanolamine, thromboxane B2, 13-(S)-HODPE, etc. In conclusion, dietary supplementation of 400 mg/kg MCE effectively improved the growth performance and intestinal function in LPS-challenged broiler chickens, probably due to the modulation of gut microbiota and plasma metabolites.

KEYWORDS

Macleaya cordata extract, lipopolysaccharide, intestinal injury, gut microbiota, plasma metabolites, broiler chicken

Introduction

The intestine is a key site for nutrient absorption and immune response in broiler chickens. However, many factors such as pathogens, mycotoxins and heat stress can damage the intestines of broiler chickens. Lipopolysaccharide (LPS), a structural part of the outer membrane of Gram-negative bacteria, often results in intestinal immune challenge, thereby compromising intestinal integrity, nutrient transport and utilization (1, 2). LPS is recognized by the LPS binding protein, which launches an intercellular cascade response, eventually leading to the release of pro-inflammatory cytokines. This process induces intestinal injury in animals (1, 3) and immune stress in cells (4). LPS is often used to establish the model of intestinal injury in various animals, such as mice (5), chickens (6) and pigs (1). Previous studies have reported that the negative effects induced by LPS in broilers can be mitigated by nutritional interventions, such as probiotics (7), polysaccharides (8), and natural plant extracts (9).

Macleaya cordata, a medicinal plant belonging to the Papaveraceae family, is traditional Chinese medicine and has been widely used as a natural feed additive in animal husbandry in China, Europe, and North America (10, 11). Numerous studies have shown that Macleaya cordata extract (MCE) decreases inflammation, increases antioxidant capacity, and improves intestinal health, thereby increasing the growth performance of livestock and poultry (10, 12, 13). The main biologically active components of MCE are sanguinarine and chelerythrine, both of which belong to the quaternary benzo[c]phen-anthridine (14). Sanguinarine and chelerythrine are reported to have physiological effects, including anti-inflammatory, anti-oxidative, antimicrobial and antiviral properties (15, 16). It has been reported that MCE increases the body weight gain, average daily gain (ADG), and average daily feed intake (ADFI) and decreases the feed-to-gain ratio (F/G) in broiler chickens (10, 12, 17). In addition, MCE enhances the antioxidant function of mice challenged with enterotoxigenic Escherichia coli, as indicated by elevated activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and decreased levels of malondialdehyde (MDA) (18). Moreover, supplementation of MCE increases the villus height and the villus height to crypt height ratio (VCR) and decreases the crypt depth in the jejunum of broiler chickens with necrotic enteritis (19). MCE supplementation regulates serum immunological indices, such as decreasing the serum TNF-α and IL-6 levels of laying hens (20), and increasing the serum IL-10, TGF-β and IgG levels of neonatal piglets (21). However, it is unclear whether MCE can relieve the detrimental effects of LPS on the intestinal function of broiler chickens.

The gastrointestinal tract is not only the largest immunological organ but also the primary site of digestion and absorption for animals (22). The microbes in the gut can be influenced by pathogens, inflammatory cytokines, diet, exercise, gastrointestinal peptides, and so on (23). There is a dynamic balance in the gut microbiota, and when the balance is broken, it will lead to dysbiosis, which can influence the host immunity, the development of the gastrointestinal tract, and the health status of animals (24, 25). It has been reported that MCE changes the composition of gut microbiota, increases the abundance of *Lactobacillus*, and decreases the abundance of *Salmonella* and *Corynebacterium* (26, 27). At the same time, MCE can change the metabolic pathways of amino acids, vitamins and lipids, and increase the contents of

butyrate and secondary cholic acid, thereby improving the growth performance and health status of broiler chickens (27). However, it remains unknown how MCE affects gut microbes and plasma metabolites in broilers challenged with LPS.

Therefore, the purpose of this study was conducted to investigate the effects of dietary MCE supplementation on growth performance, intestinal antioxidant function, intestinal morphology, and inflammation in broiler chickens challenged with LPS, and further to detect gut microbiota and plasma metabolites to reveal the underlying mechanism. Our study will be of significance for a more comprehensive understanding of the anti-immune stress mechanism of MCE in broiler chickens.

Materials and methods

Experimental design and animals

All experimental procedures were approved by the Animal Care and Use Committee of Qingdao Agricultural University. A total of 256 one-day-old male Arbor Acres broilers were obtained from a local commercial hatchery. The broilers were weighed and randomly assigned into 4 groups, including the CON group, MCE group, LPS group, and MCE+LPS group. Each group consisted of 8 replicates, with 8 broiler chickens per replicate. There was no significant difference in initial body weights among the groups. Chickens in the MCE and MCE+LPS groups were fed diets supplemented with MCE (400 mg/kg) from day 0 to day 21. Chickens in the LPS and MCE+LPS groups were intraperitoneally injected with LPS (1 mg/kg body weight) on days 15, 17, 19, and 21. Chickens in the CON and MCE groups were intraperitoneally injected with an equal volume of sterile saline. MCE was provided by Hunan Micolta Biological Resources Co., Ltd. The contents of two active ingredients, sanguinarine and chelerythrine, are 1.5% and 0.075%, respectively. LPS was purchased from Sigma (L-2880, *E. coli* serotype O55:B5). The broilers were reared in wire-floored cages under a 23-h photoperiod and had free access to feed and water. The room temperature was 34°C for the first week and then reduced by 2°C each week. The diet composition and nutritional levels are listed in Table 1.

TABLE 1 Composition and nutrient levels of basal diet (air-dry basis).

Ingredients	Contents, %	Nutrition level[c]	
Corn	55.69	ME, MJ/kg	12.58
Soybean meal, 46% CP	36.74	CP, %	21.53
Soybean oil	3.40	Ca, %	1.01
Limestone	1.15	Non-phytate phosphorus, %	0.46
Calcium hydrophosphate	2.10	Lys, %	1.23
NaCl	0.30	Met, %	0.52
D, L-Met, 98%	0.18	Thr, %	0.84
Vitamin premix[a]	0.10	Arg, %	1.47
Mineral premix[b]	0.15		
Choline chloride, 70%	0.18		
Antioxidant	0.01		
Total	100.00		

[a]Provided per kilogram of complete diet: vitamin A, 8,000 IU; vitamin D_3, 2500 IU; vitamin E, 20 IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 4.5mg; pantothenic acid, 12 mg; vitamin B_{12}, 0.02 mg; niacin, 50 mg; folic acid, 1 mg and biotin, 0.15 mg.
[b]Provided per kilogram of complete diet: Mn, 100 mg; Fe, 80 mg; Zn, 75 mg; Cu, 8 mg; I, 0.4 mg; and Se, 0.3 mg
[c]Calculated value.

Sample collection

Chickens (one bird per replicate) with average body weight were randomly selected and slaughtered at 3 h after LPS injection on day 21. Blood samples were collected using sterile blood collection tubes containing sodium heparin anticoagulant before slaughtering. Heparin plasma was collected after centrifugation at 3000 rpm for 10 min at 4°C, aliquoted, and stored at -20°C until analysis. After blood collection, the broilers were sacrificed by exsanguination. The ileum was collected and fixed in 4% paraformaldehyde for intestinal morphology analysis. The jejunum was collected, immediately frozen in liquid nitrogen, and stored at -80°C for gene expression analysis. Jejunal mucosa was scraped by glass slides, rapidly frozen in liquid nitrogen, and stored at -20°C for antioxidant assays.

Growth performance

Body weight and feed consumption for each replicate cage were recorded on days 14 and 21. ADG, ADFI and F/G during days 0-14 and days 15-21 were calculated.

Antioxidant capacity

The activities of CAT (A007-1-1), GSH-Px (A005-1-2) and SOD (A001-1-1) and the levels of MDA (A003-1-1) and T-AOC (A015-1-2) in intestinal mucosa were determined using commercial kits according to the manufacturer's protocols (Nanjing jiancheng Bioengineering Institute, Nanjing, China). Mucosal homogenates (10%) were prepared by homogenizing intestinal mucosa in ice-cold sterile saline. The homogenates were centrifuged at 3000 rpm for 10 min at 4°C, and the supernatants were then collected for further assays. The protein contents in the supernatants were quantified using a Bicinchoninic acid (BCA) protein assay kit (Cwbio, Beijing, China). Antioxidant indices in intestinal mucosa were expressed as units per milligram of protein in the sample.

Intestinal morphology

The intestinal tissues were dehydrated stepwise with ethanol, embedded in paraffin, and sectioned (5 μm). The tissue slices were

stained with hematoxylin and eosin and sealed with gum. Images were captured using a light microscope (DM2000 LED, Leica, Germany) and analyzed using ZEISS ZEN 2011 (Blue version). Villus height was determined from the tip of the villus to the villus-crypt junction, and crypt depth was defined as the depth of the invagination between adjacent villi. and the VCR was calculated by dividing the villus height by the crypt depth. For each section, ten pairs of villi and crypts were observed, and the average was calculated as the final value.

Real-time quantitative PCR

Total RNA was extracted from the jejunum using Trizol reagent (Invitrogen, Carlsbad, CA). The purity and concentration of total RNA were determined with a nanophotometer (Nano Photometer NP80, Implen, Germany). Reverse transcription into cDNA was performed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Osaka, Japan). Quantitative real-time PCR was performed using TB Green® Premix Ex TaqTM (Takara, Osaka, Japan) on a CFX96 Real-Time PCR Detection System (Bio-rad, USA). The reaction mixture (25 μL) contained 12.5 μL of TB Green Premix Ex Taq (Tli RNaseH Plus) (2X), 0.5 μL of forward and reverse primers (10 μmol/L), 2 μL of cDNA, and 9.5 μL of sterile nuclease-free water. The PCR cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The specificity of the PCR products was assessed using a melting curve. The primer sequences for the target and reference genes are listed in Table 2. The relative mRNA expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, and the housekeeping gene *GAPDH* was used as an internal control.

Gut microbiome

DNA extraction and high-throughput sequencing

Total microbial genomic DNA was extracted from ileal digesta using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') on an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). PCR amplification was performed using TransStart® FastPfu DNA Polymerase kits (TransGen AP221-02) under the cycling conditions: 95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 10 min. All samples

TABLE 2 Sequences of the primers used for quantitative real-time PCR.

Gene name[a]	Accession number	Primer sequence[b] (5′ to 3′)	Product size, bp
IL-1β	XM_015297469.1	F: ACTGGGCATCAAGGGCTA	131
		R: GGTAGAAGATGAAGCGGGTC	
IL-6	XM_015281283.1	F: CGCCCAGAAATCCCTCCTC	152
		R: AGGCACTGAAACTCCTGGTC	
IL-8	XM_015301388.1	F: ATGAACGGCAAGCTTGGAGCTG	233
		R: TCCAAGCACACCTCTCTTCCATCC	
IL-10	NM_001004414.2	F: CGCTGTCACCGCTTCTTCA	88
		R: TCCCGTTCTCATCCATCTTCTC	
IL-17	NM_204460.1	F: CTCCGATCCCTTATTCTCCTC	292
		R: AAGCGGTTGTGGTCCTCAT	
		R: GCCAAGGTGTAGGTGCGATTCC	
TNF-α	XM_046927265.1	F: GAGCGTTGACTTGGCTGTC	64
		R: AAGCAACAACCAGCTATGCAC	
IFN-γ	NM_205149.2	F: AGCTGACGGTGGACCTATTATT	259
		R: GGCTTTGCGCTGGATTC	
iNOS	NM_204961.2	F: TGGGTGGAAGCCGAAATA	241
		R: GTACCAGCCGTTGAAAGGAC	
GAPDH	NM_204305.1	F: TGCTGCCCAGAACATCATCC	142
		R: ACGGCAGGTCAGGTCAACAA	

[a]IL, interleukin; TNF-α, tumor necrosis factor alpha, IFN-γ, interferon γ, iNOS, inducible nitric oxide synthase, and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[b]F, forward; R, reverse.

were amplified in triplicate. The PCR product was extracted from 2% agarose gel, purified using an AxyPrep DNA Gel Extraction kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and quantified using the Quantus™ Fluorometer (Promega, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA).

Sequence Processing and Bioinformatics Analysis

Raw FASTQ files were de-multiplexed using an in-house perl script, quality-filtered using fastp version 0.19.6, and merged using FLASH version 1.2.7. The optimized sequences were clustered into operational taxonomic units (OTUs) using UPARSE 7.1 at a 97% sequence similarity level. The taxonomy of each OTU representative sequence was analyzed using RDP Classifier version 2.2 against the 16S rRNA gene database (Silva v138) at a confidence threshold of 0.7. The α-diversity was analyzed using Mothur (1.30.2). The β-diversity and community barplot were analyzed using Qiime (1.9.1). The microbiota with linear discriminant analysis (LDA) scores > 2 were identified using the linear discriminant analysis effect size (LEfSe) method.

Detection of plasma metabolite composition by non-targeted metabolomics

To extract metabolites, 100 μL of plasma sample was mixed with 400 μL of extraction solution (acetonitrile: methanol = 1: 1, v/v) containing 0.02 mg/mL L-2-chlorophenylalanine as an internal standard, followed by vortexing for 30 s and sonication (40 kHz) for 30 min at 4°C. The mixture was incubated at -20°C for 30 min to precipitate the proteins. The mixture was centrifuged for 15 min (4°C, 13000 g). The supernatant was collected and blown dry under nitrogen. The samples were then re-dissolved in 100 μL of aqueous acetonitrile (acetonitrile: water = 1: 1, v/v) and sonicated (40 kHz)) for 5 min at 5°C, followed by centrifugation (13000 g, 4°C) for 10 min. The supernatant was collected and subjected to LC-MS/MS analysis. Quality control (QC) samples were analyzed at regular intervals (every 12 samples).

The LC-MS/MS analysis was conducted on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm, i.d., 1.8 μm; Waters, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The mobile phases consisted of 0.1% formic acid in Solvent A (water: acetonitrile = 95: 5, v/v) and 0.1% formic acid in Solvent B (acetonitrile: isopropanol: water = 47.5: 47.5: 5, v/v/v). The flow rate was 0.40 mL/min, and the column temperature was 40°C. The injection volume was 3 μL. The LC-MS/MS data were acquired using a Thermo UHPLC-Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operated in both positive and negative ion modes. The optimal conditions were set as follows: source temperature at 425°C; sheath gas flow rate at 50 arb; Aux gas flow rate at 13 arb; ion-spray voltage floating (ISVF) at -3 500 V in the negative ion mode and 3 500 V in the positive ion mode, respectively; Normalized collision energy, 20-40-60 V rolling for MS/MS. Full MS resolution was 60000, and MS/MS resolution was 7500. Data acquisition was performed in the Data-Dependent Acquisition mode. The detection was carried out over a mass range of 70-1 050 m/z.

LC/MS raw data were preprocessed using Progenesis QI (Waters Corporation, Milford, USA) and analyzed on the Majorbio cloud platform (Majorbio Biotech, Shanghai, China). The principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed using the R package "ropls" (version 1.6.2), and 7-cycle interactive validation was used to evaluate the stability of the model. Differential metabolites in volcano plots were identified based on P values ($P < 0.05$, t-test) and VIP values (VIP > 1). Differential metabolites among the two groups were mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on the KEGG database (http://www.genome.jp/kegg/). Python package "scipy.stats" (https://docs.scipy.org/doc/scipy/) was used to perform enrichment analysis.

Statistical analysis

Data are analyzed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA) and expressed as mean ± SEM. Differences between groups were analyzed using a two-way analysis of variance (ANOVA), followed by Duncan's multiple comparison test. The graphs were created using GraphPad Prism 8 Software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant when $P < 0.05$.

Results

Growth performance

As shown in Table 3, MCE supplementation significantly increased the ADFI ($P < 0.05$) in broiler chickens, but did not affect the ADG and F/G ($P > 0.05$) during days 0-14. During days 15-21, there was an interaction between the LPS challenge and MCE supplementation on the ADG ($P < 0.05$). The LPS group significantly decreased the ADG in broiler chickens compared with the CON group, while the MCE+LPS group significantly suppressed the LPS-induced decrease in the ADG ($P < 0.01$). LPS challenge significantly decreased the ADFI in broiler chickens ($P < 0.001$) regardless of MCE supplementation. In addition, MCE supplementation, LPS challenge and their interaction had no effects on the F/G ($P > 0.05$) during days 0-21.

Jejunal mucosal antioxidant status

As shown in Table 4, LPS challenge significantly decreased the T-AOC ($P < 0.001$) and the activities of CAT ($P < 0.01$) and SOD ($P < 0.01$) and increased the level of MDA ($P < 0.05$) in broiler chickens. MCE supplementation significantly increased the T-AOC and the activity of CAT and reduced the level of MDA in broiler chickens ($P < 0.05$). MCE supplementation, LPS challenge and their interaction have no effects on GSH-Px activity ($P > 0.05$).

TABLE 3 Effects of Macleaya cordata extract (MCE) on growth performance in broiler chickens challenged with LPS.

Items		0-14d			15-21d		
		ADG, g	ADFI, g	F/G	ADG, g	ADFI, g	F/G
CON		21.58	29.88	1.39	48.06[a]	68.02	1.43
MCE		22.31	31.37	1.41	46.32[a]	69.37	1.50
LPS		21.04	29.50	1.40	39.72[c]	58.84	1.48
MCE+LPS		21.58	30.92	1.44	42.42[b]	61.40	1.45
SEM		0.274	0.356	0.014	0.704	1.158	0.017
Main effect							
MCE	–	21.31	29.69	1.39	43.89	63.43	1.46
	+	21.94	31.15	1.42	44.37	65.39	1.47
LPS	–	21.95	30.63	1.40	47.19	68.70	1.47
	+	21.31	30.21	1.42	41.07	60.12	1.47
P value							
MCE		0.261	0.044	0.300	0.534	0.266	0.668
LPS		0.258	0.549	0.487	<0.001	<0.001	0.973
Interaction		0.862	0.964	0.862	0.008	0.727	0.140

[a-c]Mean values in the same column with different lowercase letters indicate significant difference (P < 0.05).
CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge; ADG, average daily gain; ADFI, average daily feed intake; F/G: feed to gain ratio. N = 8 in each group.

Ileal morphology

Hematoxylin and eosin staining results revealed nearly normal intestinal morphology in the CON and MCE groups. However, the LPS group exhibited significant intestinal mucosa damage, including damaged villus tips and villi atrophy. Nevertheless, intestinal mucosa damage was reduced in the MCE+LPS group (Figure 1). As shown in Table 5, LPS challenge significantly decreased the villus height in broiler chickens ($P < 0.01$), while MCE supplementation significantly increased the villus height ($P < 0.05$). LPS challenge significantly decreased the VCR in broiler chickens ($P < 0.01$), while MCE supplementation significantly increased the VCR ($P < 0.05$). MCE supplementation and LPS challenge have no effects on crypt depth ($P > 0.05$).

Relative mRNA expression of jejunal inflammatory genes

As shown in Table 6, MCE supplementation and LPS challenge have interactions on the mRNA expression of *IL-1β* and *IL-17*. Compared with the CON group, the LPS group significantly increased the mRNA expression of *IL-1β* and *IL-17*, while the MCE +LPS group significantly suppressed the increase of mRNA expression of *IL-1β* and *IL-17* induced by LPS challenge ($P < 0.01$). LPS challenge significantly increased the mRNA expression of *IL-8* ($P < 0.01$), *IFN-γ* ($P < 0.05$), and *iNOS* ($P < 0.05$). MCE supplementation significantly decreased the mRNA expression of *IL-6* and *IL-8* ($P < 0.05$).

Gut microbiome

α-diversity and β-diversity

As shown in Figures 2A–E, LPS challenge significantly decreased the abundance-based coverage estimator (ACE) index ($P < 0.05$), Chao index ($P < 0.01$) and Shannon index ($P < 0.05$), while MCE supplementation significantly increased the ACE index ($P < 0.05$). MCE supplementation significantly elevated the Simpson index ($P < 0.05$) regardless of LPS challenge. There is an interaction on the Sobs index between MCE supplementation and LPS challenge ($P < 0.05$). The Sobs index was significantly higher in the MCE group than in other groups ($P < 0.01$). PCA and PCoA were used to examine the similarities and differences of gut microbiota between groups in broiler chickens and there was no distinct separation between groups (Figures 2F, G).

The composition of gut microbiota and LEfSe analysis

As illustrated in the Venn diagram, a total of 180 OTUs were identified in all groups, with 36, 26, 47 and 10 particular OTUs in the CON, MCE, LPS, and MCE+LPS groups, respectively (Figure 3A). The dominant microbiota in all groups mainly included *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, *Patescibacteria* at the phylum level (Figure 3B), and *Lactobacillus*, *Peptostreptococcaceae*, *Bacillus* and *Romboutsia* at the genus level (Figure 3C). The LDA scores were used to identify taxonomic biomarkers contributing to the differences between groups (Figure 3D). *Blautia*, *GCA-900066575*, *Anaerostipes butyraticus*, *Anaerostipes*, and *Clostridium_sensu_stricto_1* had higher LDA scores in the MCE group. *Actinobacteriota*, *Staphylococcus_sciuri*, *Micrococcales*, *Streptococcus*, *Alphaproteobacteria*, *Rhizobiales*, *Microbacterium*, *Rhizobiaceae*, *Christensenellaceae_R-7_group*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* had higher LDA scores in the LPS group. *Kurthia* had a higher LDA score in the MCE+LPS group.

The relative abundance of gut microbiota at the phylum and genus levels

As depicted in Figures 4A, B, at the phylum level, MCE supplementation significantly increased the relative abundance of *Firmicutes* ($P < 0.05$). Compared with the CON group, the LPS group significantly increased the relative abundance of *Actinobacteriota*, while the MCE+LPS group significantly suppressed the increase induced by LPS challenge ($P < 0.05$). As shown in Figures 4C–F, at the genus level, MCE supplementation significantly increased the relative abundance of *Lactobacillus* ($P < 0.05$). Interactions have been observed in the relative abundance of

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TABLE 4 Effects of Macleaya cordata extract (MCE) on the antioxidant ability of jejunum in broiler chickens challenged with LPS.

Items		T-AOC, U/mgprot	CAT, U/mgprot	GSH-Px, U/mgprot	SOD, U/mgprot	MDA, nmol/mgprot
CON		0.15	2.05	10.07	484.78	0.33
MCE		0.17	2.60	10.86	524.99	0.25
LPS		0.09	1.56	12.00	363.23	0.42
MCE+LPS		0.12	1.76	13.12	431.83	0.30
SEM		0.008	0.110	0.741	18.117	0.021
Main effect						
MCE	–	0.12	1.81	11.04	424.01	0.37
	+	0.14	2.18	11.99	478.41	0.28
LPS	–	0.16	2.33	10.47	504.89	0.29
	+	0.11	1.66	12.56	397.53	0.36
P-value						
MCE		0.039	0.036	0.531	0.064	0.012
LPS		<0.001	0.001	0.176	0.001	0.046
Interaction		0.918	0.300	0.913	0.616	0.616

CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge; T-AOC, total antioxidant capacity; CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde. N = 8 in each group.

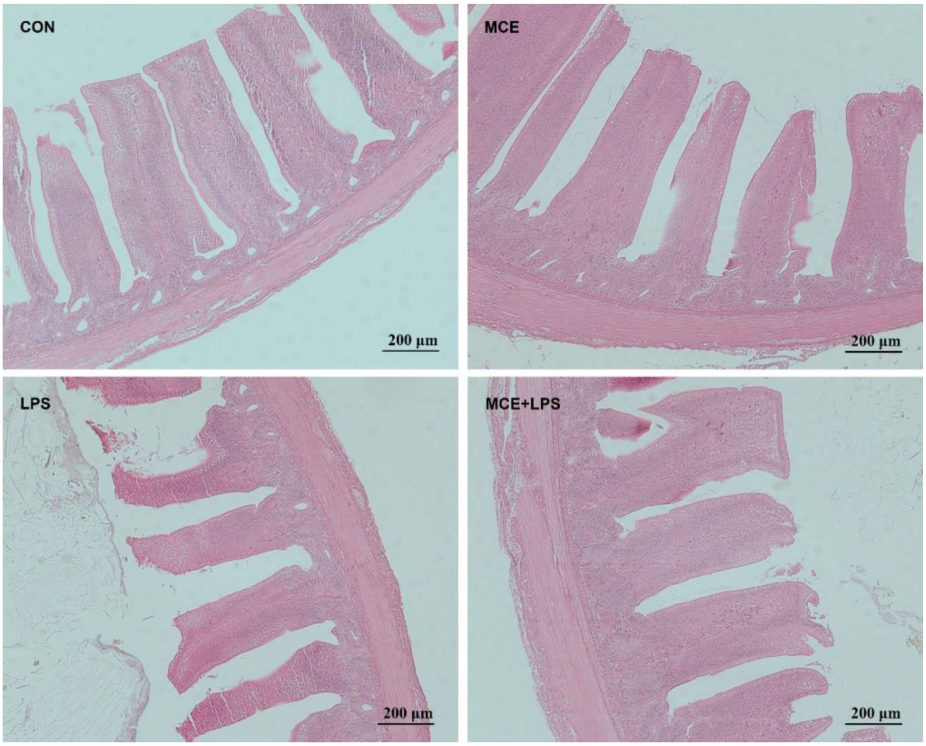

FIGURE 1
The representative images of ileal morphology by hematoxylin and eosin staining. Original magnification 100×, scale bar 200 μm. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge.

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TABLE 5 Effects of Macleaya cordata extract (MCE) on the ileal morphology in broiler chickens challenged with LPS.

Items		Villus height, μm	Crypt depth, μm	VCR
CON		700.09	146.98	4.83
MCE		731.88	151.49	5.10
LPS		651.27	168.00	4.06
MCE+LPS		681.00	148.14	4.64
SEM		8.816	4.410	0.117
Main effect				
MCE	–	675.68	157.49	4.45
	+	706.44	149.82	4.87
LPS	–	715.98	149.23	4.96
	+	666.13	158.07	4.35
P-value				
MCE		0.046	0.389	0.046
LPS		0.002	0.322	0.005
Interaction		0.945	0.176	0.453

CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge; VCR, villus height to crypt depth ratio. N = 8 in each group.

Peptostretococcaceae, *Rhodococcus* and *Blautia* between MCE supplementation and LPS challenge ($P < 0.05$). Compared with the CON group, the LPS group significantly increased the relative abundance of *Peptostretococcaceae* and *Rhodococcus*, while the MCE+LPS group suppressed the increase induced by LPS challenge ($P < 0.05$). *Blautia* had a higher relative abundance in the MCE group than in other groups ($P < 0.05$).

Non-targeted metabolomics in plasma

PCA and PLS-DA plots

There were 551 metabolites identified in the positive mode and 217 metabolites identified in the negative mode in all groups. The differences in metabolites between groups are shown in the PCA and PLS-DA plots (Figure 5). There was a distinct separation between the CON group and the LPS group in both positive and negative modes. A clear separation was also observed in the negative mode between the LPS and MCE+LPS groups. There was a clear separation between the CON and MCE groups in PLS-DA score plots in the positive mode.

Alteration in plasma metabolic composition

The significantly differential metabolites between groups are shown in the volcano maps (Figure 6). As shown in Figure 6A, in

TABLE 6 Effects of Macleaya cordata extract (MCE) on the jejunal relative mRNA expression of inflammatory genes in broiler chickens challenged with LPS.

Items		*IL-1β*	*IL-6*	*IL-8*	*IL-10*	*IL-17*	*TNF-α*	*IFN-γ*	*iNOS*
CON		1.01^{c}	1.08	1.04	1.01	1.01^{c}	1.01	1.06	1.03
MCE		1.14^{c}	0.77	0.94	1.01	1.15^{c}	0.89	0.98	1.03
LPS		2.50^{a}	1.10	1.69	0.99	3.11^{a}	1.04	1.39	1.27
MCE+LPS		1.74^{b}	0.78	1.21	0.93	2.23^{b}	1.01	1.59	1.12
SEM		0.141	0.064	0.080	0.035	0.177	0.031	0.097	0.039
Main effect									
MCE	–	1.76	1.09	1.36	1.00	2.06	1.03	1.22	1.15
	+	1.44	0.77	1.08	0.97	1.69	0.95	1.29	1.08
LPS	–	1.07	0.93	0.99	1.01	1.08	0.95	1.02	1.03
	+	2.12	0.94	1.45	0.96	2.67	1.02	1.49	1.19
P-value									
MCE		0.042	0.012	0.029	0.699	0.008	0.220	0.732	0.337
LPS		<0.001	0.896	0.001	0.503	<0.001	0.252	0.016	0.035
Interaction		0.007	0.965	0.134	0.716	0.001	0.512	0.436	0.279

$^{a-c}$Mean values in the same column with different lowercase letters indicate significant difference ($P < 0.05$).
CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthase. N = 8 in each group.

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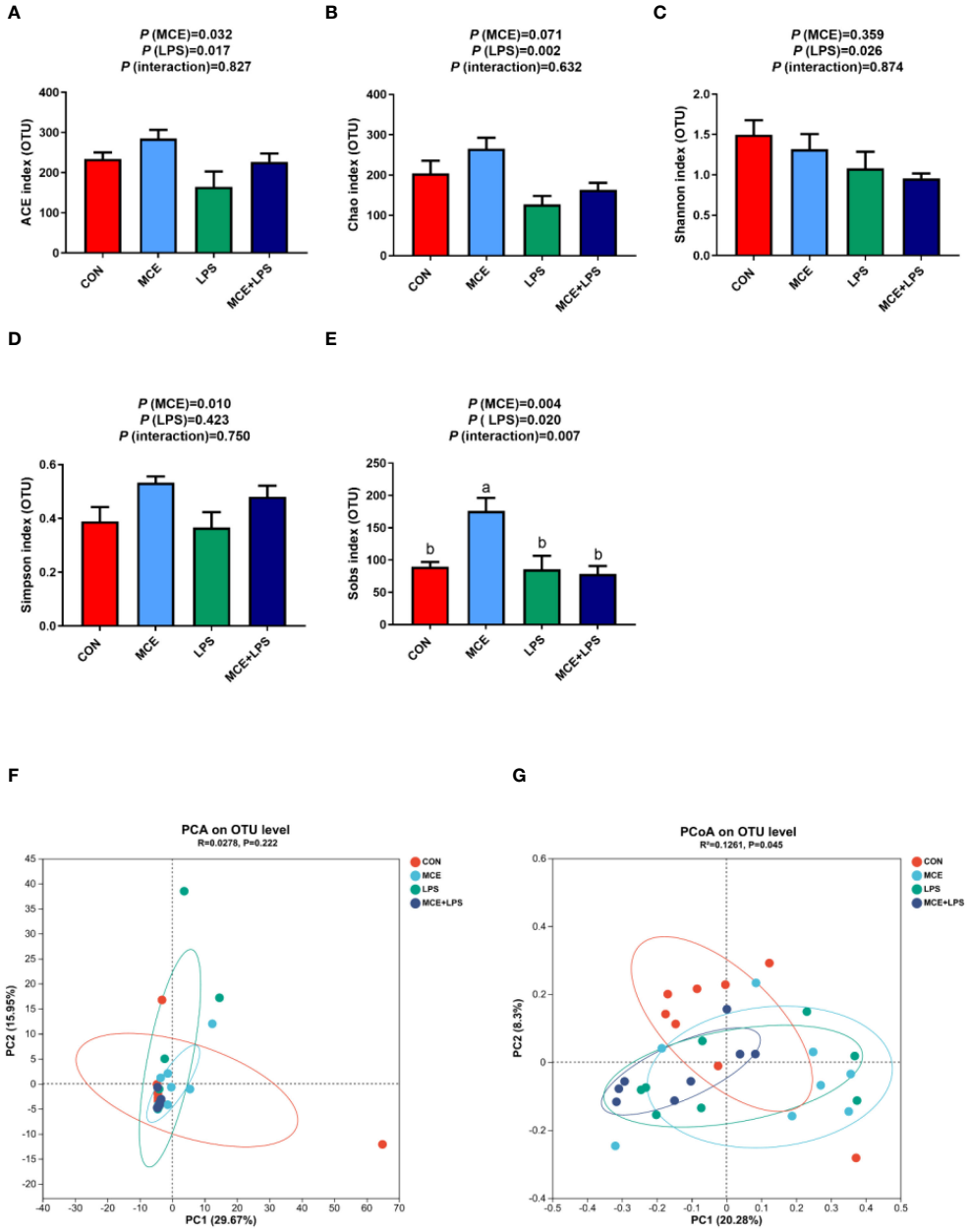

FIGURE 2
Effects of Macleaya cordata extract (MCE) on α-diversity and β-diversity of gut microbiota in broiler chickens challenged with LPS. Changes in α-diversity: **(A)** ACE index, **(B)** Chao index, **(C)** Shannon index, **(D)** Simpson index, and **(E)** Sobs index α-diversity. **(F)** PCA and **(G)** PCoA of the β-diversity. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. [a, b] Mean values with no common lowercase letters indicate significant difference ($P < 0.05$). N = 8 in each group.

the negative mode, compared with the CON group, the MCE group had 6 up-regulated metabolites (2,3-Dihydroxybenzoic acid, (25R)-3beta-hydroxycholest-5-en-7-one-26-oate, Ganoderic acid C1, L-Ascorbic acid, Myo-Inositol, and Pyrocatechol sulfate) and 29 down-regulated metabolites (such as Uridine, 13(S)-HODPE, Xanthosine, Xanthine, and Pseudouridine). As shown in Figure 6B, in the positive mode, compared with the CON group, the MCE group had 16 up-regulated metabolites, (such as Thr-Thr-Lys-Phe, Capsianoside III, Beta-Alanine, 5-Aminolevulinic acid, and Pro-Tyr-Gly) and 45 down-regulated metabolites (such as Hypoxanthine, Octadecenoylcarnitine, 13Z-Docosenamide, Tranexamic acid, and Uracil).

As shown in Figure 6C, in the negative mode, compared with the CON group, LPS challenge increased the levels of 12 metabolites (such as Citramalic acid, Ketosantalic acid, Ribothymidine, Pyrocatechol sulfate, and Glutaric acid) and decreased the levels of 66 metabolites, (such as Inosine, Stearic acid, Pseudouridine, L-Glutamate, and Threoninyl-Tyrosine). As shown in Figure 6D, in

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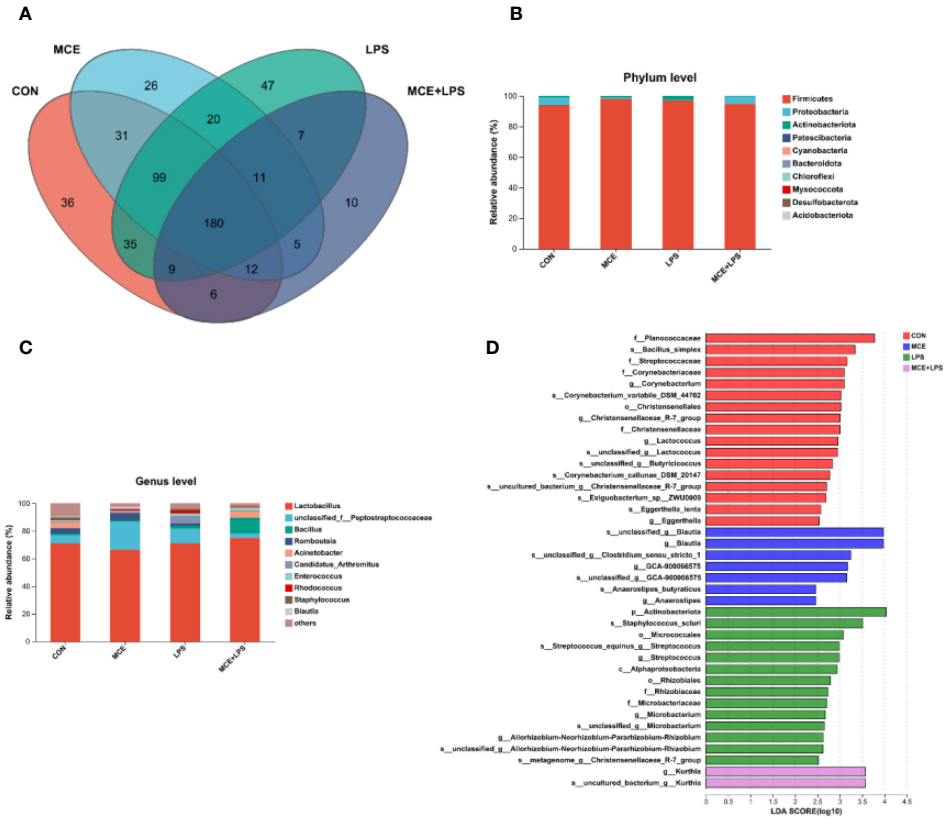

FIGURE 3
Effects of Macleaya cordata extract (MCE) on the composition of gut microbiota in broiler chickens challenged with LPS. **(A)** the Venn diagram of gut microbiota, **(B)** the composition of gut microbiota at the phylum level, **(C)** the composition of gut microbiota at the genus level, and **(D)** the LEfSe analysis of gut microbiota. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE +LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. N = 8 in each group.

the positive mode, compared with the CON group, the LPS group had 35 up-regulated metabolites (such as (E)-2-Methylglutaconic acid, Deoxypyridinoline, 2-Naphthylamine, Stigmastanol, and 2-Deoxybrassinolide) and 108 down-regulated metabolites (such as Pantothenic Acid, Asperagenin, Octadecenoylcarnitine, Acetylcarnitine, and Val Glu Val).

As presented in Figure 6E and Table 7, in the negative mode, compared with the LPS group, the MCE+LPS group had 10 up-regulated metabolites (Gamma-Glutamylthreonine, D-altro-D-manno-Heptose, N-acetylaspartate, (1R,2S,3S,4R)-p-Menthane-2,3-diol, Stearic acid, Threoninyl-Tyrosine, L-Ribulose, L-Aspartic Acid, L-Glutamate and Myo-Inositol) and 5 down-regulated metabolites (TXB2, Lamivudine sulfoxide, Ribothymidine, 6-(3-ethenylphenoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid and 5-Methoxynoracronycine). As presented in Figure 6F and Table 7, in the positive mode, compared with the LPS group, the MCE+LPS group had 5 up-regulated metabolites (Cucurbic acid, L-Serine, Corchorifatty acid A, Cyclohex-2-enone and Vinylacetylglycine) and 7 down-regulated metabolites (Sphingosine-1-phosphate, 1-Methyladenine, Oryzalexin E, Sphinganine-phosphate, Cycloalliin, (8alpha,10beta,11beta)-3-Hydroxy-4,15-dinor-1(5)-xanthen-12,8-olide and 20-Hydroxy-PGF2a).

KEGG enrichment of differential metabolites

As presented in Figure 7, the KEGG metabolic pathway enrichment analysis demonstrated that significantly differential metabolites between the MCE and CON groups were enriched in Nucleotide metabolism, Lipid metabolism, Metabolism of cofactors and vitamins, Metabolism of other amino acids, Carbohydrate metabolism, Amino acid metabolism and others. MCE supplementation altered the pathways of Pyrimidine metabolism, ABC transporters, Caffeine metabolism, Purine metabolism, Pantothenate and CoA biosynthesis, beta-Alanine metabolism and others.

The significantly differential metabolites between the LPS and CON groups were enriched in Lipid metabolism, Nucleotide metabolism, Amino acid metabolism, Metabolism of cofactors and vitamins, Metabolism of other amino acids, Carbohydrate metabolism and others. Compared with the CON group, LPS challenge altered the pathways of Glycerophospholipid metabolism, Sphingolipid metabolism, Linoleic acid metabolism, Pyrimidine metabolism, Purine metabolism, Neuroactive ligand-receptor interaction, Pantothenate and CoA biosynthesis and others.

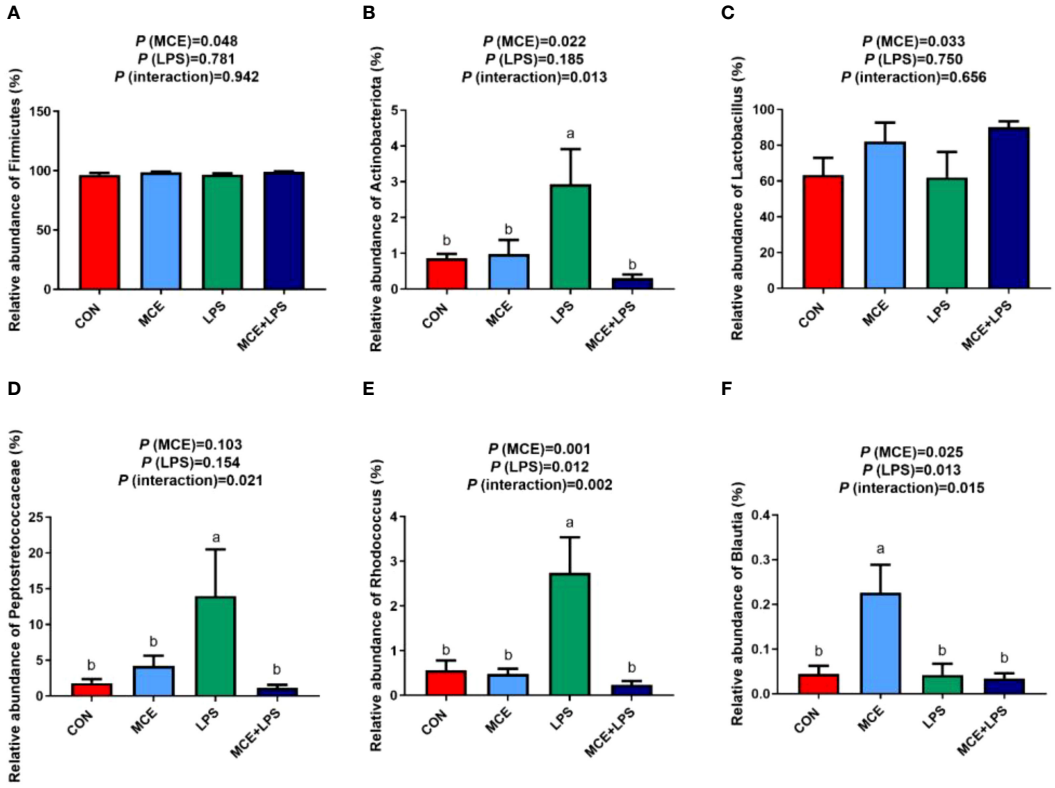

FIGURE 4
Effects of Macleaya cordata extract (MCE) on the relative abundance of bacteria at the phylum and genus levels in broiler chickens challenged with LPS. **(A)** *Firmicutes* and **(B)** *Actinobacteriota* at the phylum level. **(C)** *Lactobacillus*, **(D)** *Peptostretococcaceae*, **(E)** *Rhodococcus*, and **(F)** *Blautia* at the genus level. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. [a, b] Mean values with no common lowercase letters indicate significant difference ($P < 0.05$). N = 8 in each group.

The significantly differential metabolites between the MCE+LPS and LPS groups were enriched in Lipid metabolism, Carbohydrate metabolism, Amino acid metabolism, Metabolism of other amino acids, Metabolism of cofactors and vitamins, Energy metabolism, and others. Compared with the LPS group, MCE supplementation altered the pathways of Sphingolipid metabolism, Neuroactive ligand-receptor interaction, Alanine, aspartate and glutamate metabolism, Apelin signaling pathway, Calcium signaling pathway, Aminoacyl-tRNA biosynthesis, Gap junction, Arginine biosynthesis, Apoptosis, MAPK signaling pathway and others.

Correlation analysis between gut microbiota and metabolites

As shown in Figure 8A, at the phylum level, in the negative mode, the relative abundance of *Firmicutes* was negatively correlated with N-Desthienylethyl-rotigotine. A negative correlation was observed between the relative abundance of *Proteobacteria* and (+/-)-Tryptophan. The relative abundance of *Actinobacteriota* was positively correlated with lysophosphatidylcholine (LysoPC)(17:0) and LysoPC(18:0) and was negatively correlated with Myo-Inositol. The relative abundance of *Bacteroidota* showed a negative correlation with gamma-glutamylphenylalanine. As shown in Figure 8B, in the positive mode, A positive correlation was observed between the relative abundance of *Firmicutes* and 2-Hydroxycinnamic acid. The relative abundance of *Actinobacteriota* was positively correlated with LysoPC(18:1(11Z)) and phosphatidylcholine (PC)(18:0/0:0) and was negatively correlated with Cinnamic acid and 2-Hydroxycinnamic acid. The relative abundance of *Desulfobacterota* exhibited a positive correlation with 3-O-Acetylepisamarcandin and Triethylamine.

As presented in Figure 8C, at the genus level, in the negative mode, the relative abundance of *Peptostreptococcaceae* was positively correlated with PC(18:1(11Z)/18:2(9Z,12Z)) and PC (16:0/18:2(9Z,12Z)). The relative abundance of *Romboutsia* showed a positive correlation with Citric acid. The relative abundance of *Corynebacterium* was positively correlated with inosine, phosphatidylethanolamine (PE)(20:4/0:0), PC(16:0/20:4 (5Z,8Z,11Z,14Z)), PE(18:0/0:0), and LysoPC(15:0) and was negatively correlated with (+/-)-Tryptophan and Myo-Inositol. As shown in Figure 8D, in the positive mode, the relative abundance of *Rhodococcus* was negatively correlated with 2-Hydroxycinnamic acid. A positive correlation was observed between the relative abundance of *Staphylococcus* and PC (18:0/0:0). The relative

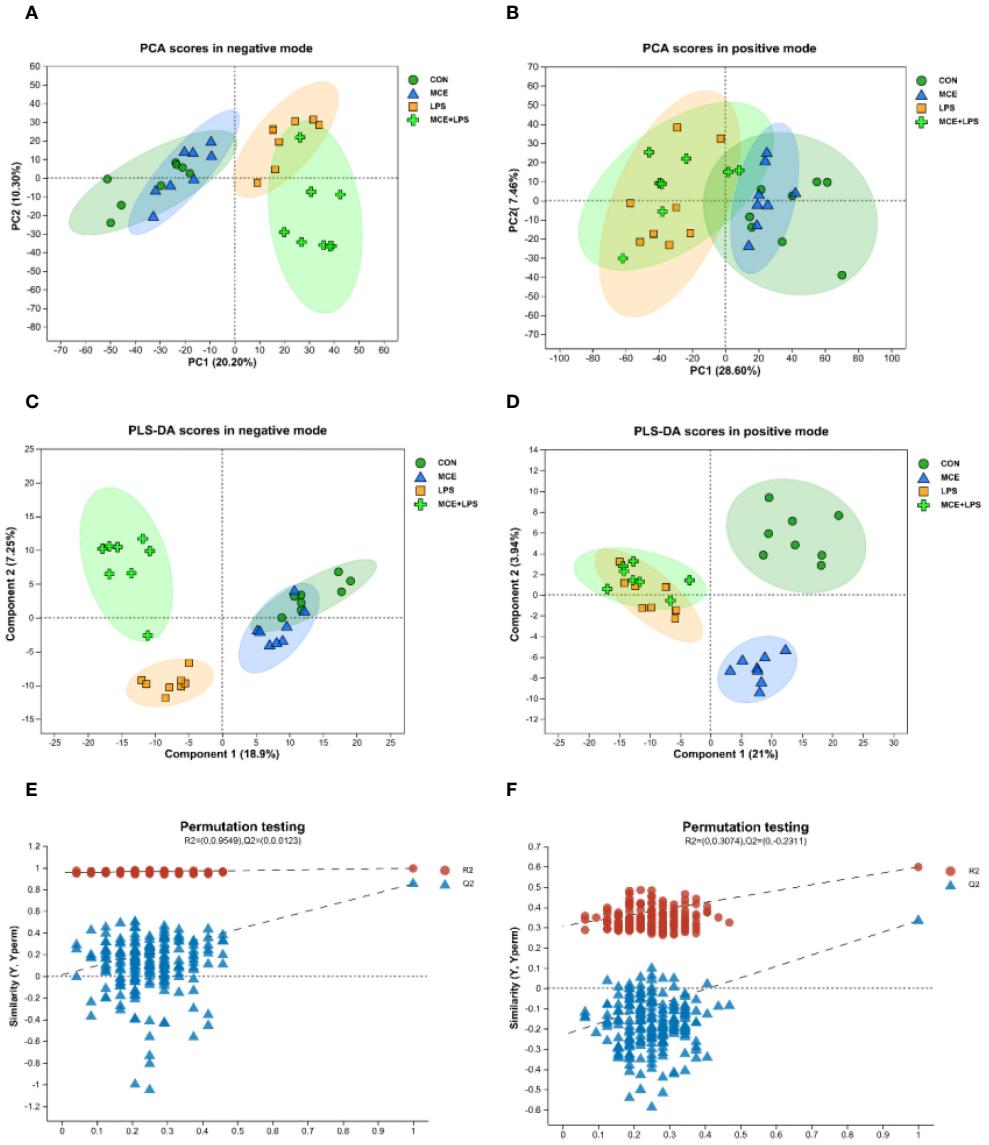

FIGURE 5
PCA and PLS-DA score plots of plasma metabolites in broiler chickens. **(A, B)** PCA scores in negative and positive modes. **(C, D)** PLS-DA scores in negative and positive modes. **(E, F)** Permutation test plots of PLS-DA scores in negative and positive modes. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. N = 8 in each group.

abundance of *Kurthia* diaplayed a negative correlation with PC(14:1(9Z)/22:2(13Z,16Z)). The relative abundance of *Corynebacterium* was positively correlated with PC(20:4/0:0), LysoPC(18:1(11Z)) and PC(18:0/0:0), and was negatively correlated with Betaine.

Discussion

In this study, MCE supplementation significantly increased the ADFI during days 0-14, and, the ADG during days 15-21 was higher in the MCE+LPS group than in the LPS group. The results are in agreement with previous studies. Dietary supplementation of 350 mg/kg MCE effectively alleviated the necrotic enteritis-induced reduction of growth performance in broiler chickens (19). Diet with 0.6 mg/kg of MCE containing protopine and allotypotopine increased the final body weight and ADG and decreased F/G of broiler chickens from d 0 to 42 (28). It was reported that dietary supplementation of 50 mg/kg MCE could enhance the growth performance and intestinal morphology of early-weaned piglets (13). Sanguinarine and chelerythrine have similar structures to aromatic amino acids, which can irreversibly inhibit the activity of intestinal aromatic amino acid decarboxylase (29). Sanguinarine has been proved to promote protein retention by lowering intestinal decarboxylation of aromatic amino acids and increases feed intake by regulating the Trp-serotonin pathway, thus improving animal growth (17, 30).

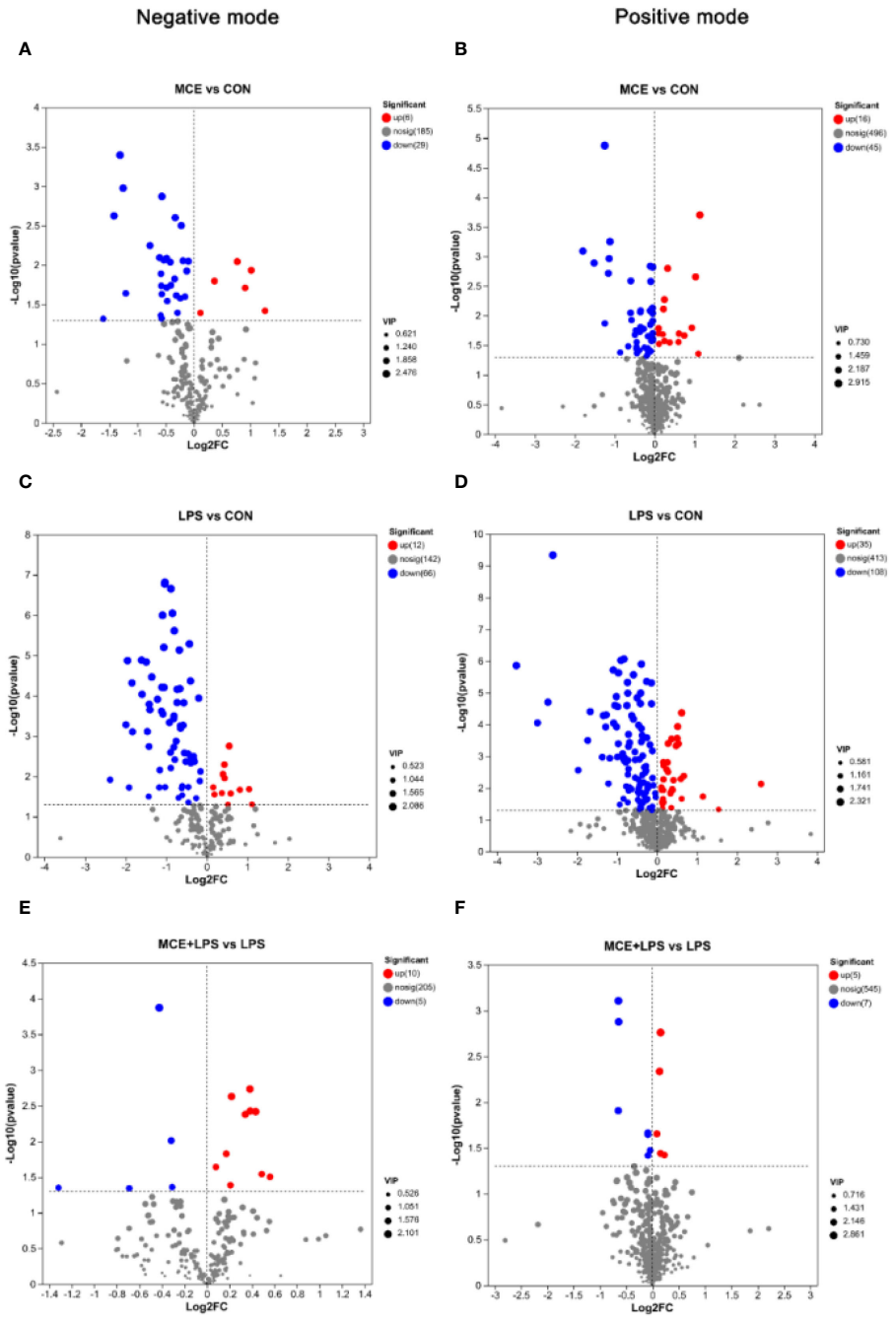

FIGURE 6
Volcano plots showing significantly up-regulated and down-regulated metabolites between different groups of broiler chickens. **(A, B)** significantly differential metabolites between the MCE and CON groups in negative and positive modes. **(C, D)** significantly differential metabolites between the LPS and CON groups in negative and positive modes. **(E, F)** significantly differential metabolites between the MCE+LPS and LPS groups in negative and positive modes. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. N = 8 in each group.

These responses may explain the improved growth performance of broiler chickens by MCE supplementation in this study.

T-AOC represents the capacity of the enzymatic and non-enzymatic antioxidant system in the body (31). CAT, GSH-Px and SOD are important antioxidant enzymes involved in eliminating superoxide anions and hydrogen peroxide during oxidative damage (31, 32). The level of MDA reflects the degree of lipid peroxidation in the body and is an indicator of initial cellular membrane damage (33). Our study revealed that dietary MCE supplementation increased the T-AOC and CAT activity while decreasing MDA levels in the jejunum. Similarly, dietary supplementation of 200 mg/kg MCE observably alleviated oxidative stress induced by enterotoxigenic *Escherichia coli* in mice, as illustrated by lower levels of MDA and increased activities of

TABLE 7 Up- and down-regulated plasma metabolites in broilers between the MCE+LPS and LPS groups in negative and positive modes.

Metabolites identified in the negative mode	Up/ down	M/Z	Retention time	FC	VIP	*P*-value
Gamma-Glutamylthreonine	up	247.09	1.14	1.40	1.38	0.03
D-altro-D-manno-Heptose	up	191.06	1.02	1.27	1.71	<0.001
N-acetylaspartate	up	174.04	1.22	1.16	1.32	0.04
(1R,2S,3S,4R)-p-Menthane-2,3-diol	up	209.09	6.93	1.06	1.46	0.02
Stearic acid	up	321.22	6.91	1.13	1.51	0.01
Threoninyl-Tyrosine	up	281.11	5.13	1.47	1.40	0.03
L-Ribulose	up	149.04	1.06	1.31	1.74	<0.001
L-Aspartic Acid	up	132.03	0.99	1.35	1.72	<0.001
Myo-Inositol	up	179.06	0.90	1.16	1.78	<0.001
L-Glutamate	up	146.04	0.98	1.3	1.81	<0.001
TXB2	down	369.23	6.34	0.40	1.32	0.04
Lamivudine sulfoxide	down	266.02	3.33	0.75	2.05	<0.001
6-(3-ethenylphenoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid	down	333.04	3.27	0.80	1.63	0.01
5-Methoxynoracronycine	down	372.10	2.90	0.62	1.32	0.05
Ribothymidine	down	257.08	2.82	0.81	1.31	0.04
Metabolites identified in the positive mode						
Cucurbic acid	up	245.18	6.93	1.10	2.57	<0.001
Corchorifatty acid A	up	309.20	6.78	1.11	2.74	<0.001
Vinylacetylglycine	up	144.07	2.76	1.11	2.02	0.04
Cyclohex-2-enone	up	97.07	2.14	1.06	2.16	0.02
L-Serine	up	150.01	0.91	1.17	2.00	0.04
Sphingosine-1-phosphate	down	380.26	6.72	0.64	2.78	<0.001
20-Hydroxy-PGF2a	down	371.24	6.52	0.94	2.15	0.02
Oryzalexin E	down	337.27	7.51	0.97	2.03	0.03
Sphinganine-phosphate	down	382.27	6.76	0.64	2.86	<0.001
1-Methyladenine	down	167.10	6.56	0.94	1.99	0.04
Cycloalliin	down	210.08	2.91	0.64	2.32	0.01
(8alpha,10beta,11beta)-3-Hydroxy-4,15-dinor-1(5)-xanthen-12,8-olide	down	242.18	6.09	0.94	2.17	0.02

LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge; M/Z, mass to charge ratio; FC, fold change; VIP, variable important in projection; TXB2, thromboxane B2; 20-Hydroxy-PGF2a: 20-hydroxy prostaglandin F2a. N = 8 in each group.

CAT and GSH-Px (18). It has been reported that sanguinarine not only decreased the ROS level induced by LPS, but also increased the activity of antioxidant enzymes by activating the Nrf2 signaling pathway in mouse mammary epithelial cells (34). Moreover, sanguinarine can inhibit the activities of nicotinamide adenine dinucleotide phosphate oxidase 2, thereby inhibiting the conversion of NADPH to ROS and improving the antioxidant capacity (35).

The intestinal morphology is a crucial indicator for gut health and intestinal absorption capacity. This study demonstrated that diets supplemented with MCE could effectively increase the ileal villus height and VCR in broiler chickens. Similarly, Song et al. (19) has discovered that the diet supplemented with 350 mg/kg MCE significantly improved the growth performance, increased the jejunal villus height and VCR and decreased the intestinal lesion score in broiler chickens with necrotic enteritis. Dietary sanguinarine supplementation at 0.7 mg/kg improved growth performance and increased the villus height and VCR of the duodenum, jejunum and ileum in yellow feathered broiler chickens at 28 days of age (17). Piglets fed a basal diet supplemented with 50 mg/kg MCE exhibited a lower crypt depth of the jejunum, higher villus height of the ileum, and higher VCR of

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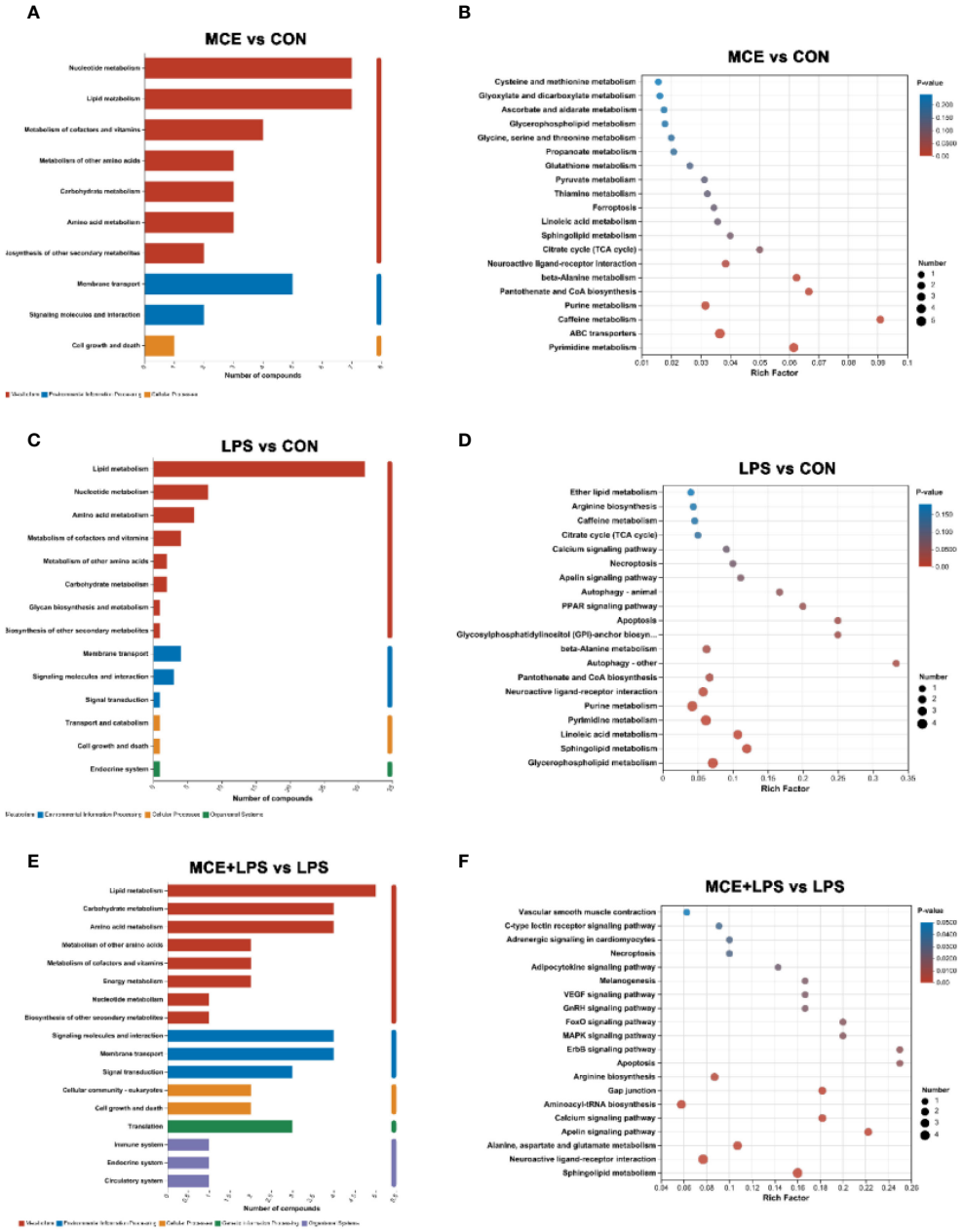

FIGURE 7
Effects of Macleaya cordata extract (MCE) on KEGG pathways enrichment for differential plasma metabolites in broiler chickens. The first and second **(A)** and third **(B)** classification categories of the KEGG pathway between MCE and CON groups. The first and second **(C)** and third **(D)** classification categories of the KEGG pathway between LPS and CON groups.The first and second **(E)** and third **(F)** classification categories of the KEGG pathway between MCE+LPS and LPS groups. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE +LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. N = 8 in each group.

the jejunum and ileum than the animals fed the basal diet (13). According to the findings of this study, MCE may enhance growth performance by improving intestinal morphology, thus facilitating efficient nutrient absorption.

IL-1β is the main pro-inflammatory mediator of systemic inflammatory responses (36). IL-6 is synthesized in the early period of inflammation (37). IL-8 can cause mucosal damage via the release of ROS, metalloproteinases and cytokines (38). IL-17 plays a pro-inflammatory role by promoting the secretion of chemokines (39). This study showed that dietary MCE supplementation decreased the mRNA expression of pro-inflammatory factors *IL-1β*, *IL-8* and *IL-17* in LPS-challenged broiler chickens, which indicated that MCE effectively suppressed the intestinal inflammatory responses induced by LPS challenge. Similar study has reported that pretreatment with MCE dramatically decreased the elevated mRNA expression of pro-inflammatory cytokines, such as *IL-1α*, *IL-1β* and *IL-8* in porcine alveolar macrophages induced by *Glaesserella parasuis* (15).

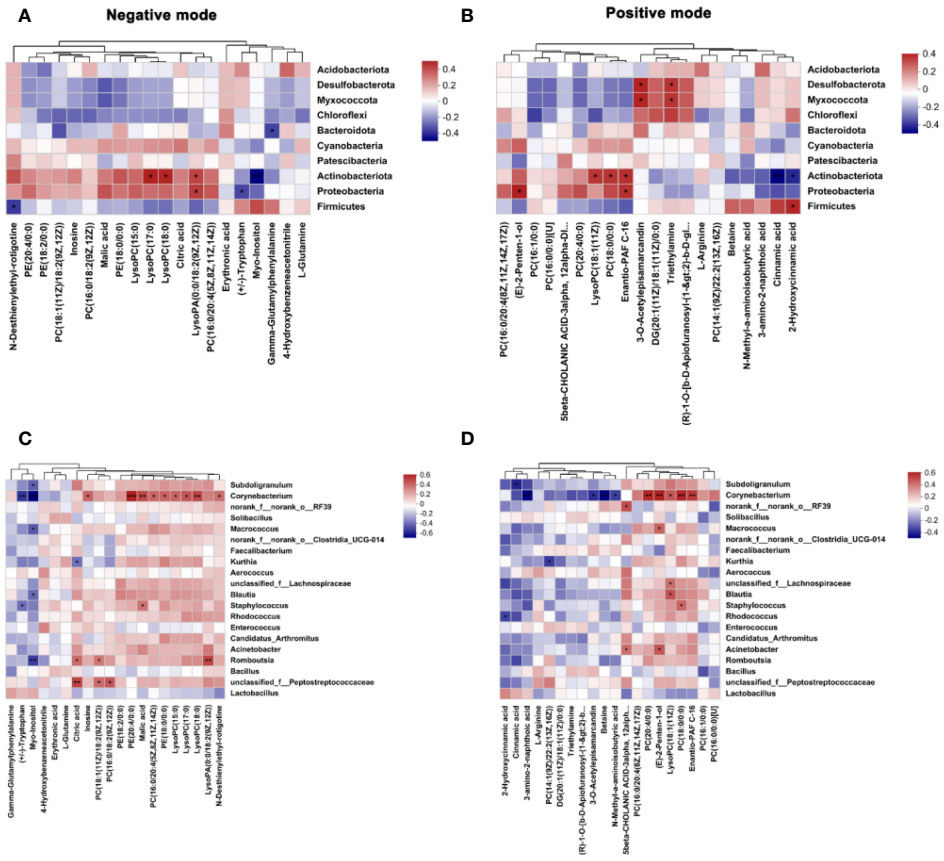

FIGURE 8
Spearman correlation analysis of gut microbiota and plasma metabolites in broiler chickens. **(A, B)** correlation between the top 10 phyla of ileal microbiota and the top 20 differential plasma metabolites in the negative and positive modes. **(C, D)** correlation between the top 20 genera of ileal microbiota and top 20 differential plasma metabolites in the negative and positive modes. CON group, broilers received a basal diet without a LPS challenge; MCE group, broilers received a basal diet supplemented with 400 mg/kg MCE without a LPS challenge; LPS group, broilers received a basal diet and were subjected to a LPS challenge; MCE+LPS group, broilers received a basal diet supplemented with 400 mg/kg MCE and were subjected to a LPS challenge. Significance was set at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N = 8 in each group.

Previous research also found that sanguinarine and chelerythrine are responsible for the anti-microbial, anti-inflammatory and immunomodulatory properties of Macleaya cordata (40). Sanguinarine and chelerythrine displayed anti-inflammatory activity by promoting nuclear translocation of the glucocorticoid receptor and inhibiting activation of NF-κB (14, 15).

Staphylococcus can secrete various toxins and pro-inflammatory factors, alter the junctions between epithelial cells, disrupt the intestinal barrier, and increase intestinal permeability (41, 42). Moreover, *Staphylococcus* has strong drug resistance and can survive inside a variety of immune cells and enter the blood, causing infections of the gut, lung, and other tissues (43–45). *Actinobacteriota* are Gram-positive pathogens with high base pair of G+C, such as *Corynebacterium* and *Nocardia* (46). *Streptococcus* can produce streptolysin O, a pore-forming toxin that can influence the intestinal health (47). The LefSe results demonstrated that *Staphylococcus*, *Actinobacteriota* and *Streptococcus* were the taxonomic biomarkers in broiler chickens challenged with LPS, suggesting that the stimulation of LPS can lead to an increase in the relative abundance of potentially harmful bacteria in the intestine of broiler chickens. *Blautia* can use glucose to produce acetic acid, succinic acid, and lactic acid, and increase the content of volatile fatty acids (48). Besides, it can use carbon dioxide, carbon monoxide, hydrogen, and cellulose that cannot be used by the host as an energy source to regulate intestinal pH and maintain intestinal homeostasis (49). The LefSe results showed that birds in the MCE group has higher relative abundance of *Blautia* compared with other other groups, which may be related to the alleviation of intestinal inflammation and improvement of intestinal morphology.

Firmicutes are the dominant phylum of bacteria in broiler chickens. It has been reported that mice colonized with microbiota abundant in *Firmicutes* from healthy human fecal samples exhibited downregulation of the TH17 pathway and colonic inflammation (50). In this study, MCE supplementation increased the relative abundance of *Firmicutes*, which was similar to the studies of Song et al. (19) in a necrotic enteritis model and Wang et al. (51) in a chronic heat stress model. *Lactobacillus* can inhibit the activity of pathogenic bacteria (such as *Escherichia* coli and *Staphylococcus aureus*) and regulate the activity of immune cells and epithelial cells, thereby improving host immunity and intestinal barrier function (52). The present study showed that MCE supplementation increased the relative abundance of *Lactobacillus*.

This was in agreement with the findings observed in mice, where MCE administration alleviated the decreased *Lactobacillus* population induced by heat stress (53). Meanwhile, dietary MCE supplementation can increase the abundance of *Lactobacillus* and adjust the intestinal pH, which is beneficial to the intestinal luminal environment in early-weaned piglets (13). *Actinobacteriota*, *Peptostretococcaceae* and *Rhodococcus* can promote inflammation by releasing pathogenic toxins such as diphtheria toxin (54). It has been reported that *Actinobacteriota* is highly associated with ulcerative colitis, Crohn's disease, immunodeficiency, and other diseases (55). Our study showed that MCE could alleviate the increase in the relative abundance of *Actinobacteriota*, *Peptostretococcaceae* and *Rhodococcus* caused by LPS, thereby suppressing intestinal inflammation.

The results of differential metabolite analysis and KEGG enrichment analysis between the MCE+LPS and LPS groups showed that the different metabolites were mainly enriched in Lipid metabolism, Nucleotide metabolism, Amino acid metabolism, and Membrane transport. In Amino acid metabolism pathway, the MCE+LPS group significantly increased the levels of L-aspartic acid, L-glutamate, L-serine, β-alanine, and 5-Hydroxy-L-tryptophan compared with the LPS group. L-aspartic acid can inhibit the formation of cell membranes in *Staphylococcus* aureus, and regulate leukocyte phagocytosis and the immune response (56, 57). In addition, it can promote the entry of argininosuccinic acid into the tricarboxylic acid cycle and enhance cell survival (58). L-glutamate is the precursor of glutathione and plays an antioxidant role. It is also the precursor of γ-aminobutyric acid (GABA), which acts as a signaling molecule in brain cells, islet cells and intestinal cells. Moreover, glutamate is the only amino acid that can stimulate afferent gastric vagal nerves (59). L-serine can bind to pyruvate kinase to produce pyruvate and affect glucose metabolism, and it is a necessary amino acid for ceramide synthesis (60). In addition, 5-Hydroxy-L-tryptophan can enhance glucose and lipid metabolism and stimulate the proliferation of T cells and B lymphocytes through the synthesis of 5-hydroxytryptamine (61–64). β-alanine is the rate-limiting precursor for the synthesis of carnosine synthesis, and it can exert antioxidant and neuroprotective effects by regulating carnosine synthesis (65–67). These results suggest that MCE supplementation can regulate the levels of related metabolites and alter amino acid metabolism.

In the present study, hypoxanthine, uracil and deoxyguanosine are enriched in the nucleotide metabolism pathway. Hypoxanthine produces ROS during metabolism, which may cause oxidative stress, trigger caspase-3-induced apoptosis, and increase inflammation (68). Uracil is formed by mismatch due to spontaneous deamination of cytosine (69). Deoxyguanosine and pseudouridine are the biomarkers for oxidative DNA damage and prostate cancer, respectively (70). In this study, we found that dietary MCE supplementation could decrease the levels of these metabolites and regulate nucleotide metabolism. In addition, our study showed that 13-(S)-HODPE, PC and PE were key metabolites in lipid metabolism. It is reported that 13-(S)-HODPE can increase the production of ROS and promote the expression of apoptotic genes such as caspase-3 and p21, thus promoting cell apoptosis (71). The present study showed that MCE decreased the level of 13-(S)-HODPE, suggesting that MCE may inhibit oxidant stress by reducing the 13-(S)-HODPE level. The present study demonstrated that MCE supplementation significantly reduced the level of 13-(S)-HODPE. PC and PE are the most abundant phospholipids in all mammalian cell membranes, and the ratio of PC to PE is a key factor for low-density lipoprotein metabolism and energy metabolism in cellular organelles, and it plays an important role in gut and liver health (72). This study showed that MCE supplementation decreased the levels of both PC and PE, suggesting that MCE supplementation could regulate the lipid metabolic pathway and participate in the regulation of gut inflammation.

The results of Spearman correlation analysis of gut microbiota and plasma metabolites showed that the relative abundance of *Firmicutes* was positively correlated with the level of 2-hydroxycinnamic acid, a well-known antioxidant (73, 74), suggesting that *Firmicutes* could exert antioxidant function by increasing the level of 2-Hydroxycinnamic acid. *Corynebacterium* plays a pathogenic role by secreting phospholipase D (PLD) and diphtheria toxin, and PLD can activate NF-κB in epithelial cells, release inflammatory factors such as IL-6, and increase vascular permeability (75). In addition, *Corynebacterium* is closely associated with inflammatory diseases, such as bacteriemia and endocarditis (76). Our results showed that the relative abundance of *Corynebacterium* was negatively correlated with (+/-)-tryptophan and myo-inositol and positively correlated with inosine, suggesting that *Corynebacterium* could alter the levels of related metabolites, thereby regulating amino acid and nucleotide metabolic pathways.

Conclusion

In conclusion, dietary MCE supplementation at 400 mg/kg benefited the growth performance and alleviated the intestinal injury in broiler chickens challenged with LPS, which might be closely related to the modulation of gut microbiota and plasma metabolites. MCE supplementation could increase the relative abundance of potentially probiotic bacteria (such as *Blautia* and *Lactobacillus*) and decrease the relative abundance of potentially pathogenic bacteria (such as *Actinobacteriota*, *Peptostretococcaceae*, and *Rhodococcus*) in the gut. Moreover, MCE supplementation could alter the composition of metabolites and regulate many metabolic pathways, mainly including Lipid metabolism, Amino acid metabolism, and Nucleotide metabolism in broiler chickens. This study provides a valuable reference for nutritional regulation to prevent the gut damage induced by immune stress in broiler chickens.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: Sequence Read Archive of NCBI, accession number PRJNA1087016.

Ethics statement

The animal study was approved by Animal Care and Use Committee of Qingdao Agricultural University (No. DKY20220513). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XW: Data curation, Investigation, Methodology, Writing – original draft. TZ: Data curation, Investigation, Methodology, Writing – review & editing. WL: Project administration, Supervision, Writing – review & editing. MZ: Project administration, Supervision, Writing – review & editing. LZ: Methodology, Software, Writing – review & editing. NW: Methodology, Software, Writing – review & editing. XZ: Methodology, Software, Writing – review & editing. BZ: Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Is there sufficient evidence to support the health benefits of including donkey milk in the diet?

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Donkey milk has attracted attention due to its distinctive nutritional composition and potential health advantages, particularly because of its whey protein content, which includes lysozyme, α-lactalbumin, lactoferrin, and β-lactoglobulin and vitamin C, among other components. These elements contribute to immunoregulatory, antimicrobial, antioxidant, and anti-inflammatory properties, positioning donkey milk as a possible therapeutic option. In addition, due to the low levels of caseins, the casein-to-whey protein ratio, and the β-lactoglobulin content in donkey milk, it presents an optimal alternative for infant formula for individuals with cow's milk allergies. Moreover, research into donkey milk's potential for cancer prevention, diabetes management, and as a treatment for various diseases is ongoing, thanks to its bioactive peptides and components. Nevertheless, challenges such as its low production yield and the not fully understood mechanisms behind its potential therapeutic role necessitate more thorough investigation. This review consolidates the existing knowledge on the therapeutic possibilities of donkey milk, emphasizing its importance for human health and the need for more detailed studies to confirm its health benefits.

KEYWORDS

donkey milk, lysozyme, lactoferrin, antioxidant, antibacterial, immune regulator, therapeutic potential

1 Introduction

The therapeutic potential, similarities to human milk and high digestibility of donkey milk have captured the attention of researchers in this field (1–4). Donkey milk has low cholesterol, fat and protein level, however, consider a rich source of milk whey proteins (3, 5). In addition, donkey milk has a vitamin C level of 57 mg/L, which is closest to that of human milk (60 mg/L), and higher than cow milk (27 mg/L) (1, 6). The low level of casein and casein to whey protein ratio in donkey milk may contribute to its role in formula milk of infant (3, 7). Today, donkey milk is being marketed as a consumer product and is used by newborns (8, 9), people with cow's milk protein allergies, and the elderly (10).

The growing interest in donkey milk is further justified by its multifaceted bioactivities, encompassing antimicrobial, antiviral, anti-inflammatory, anti-proliferative (2, 11–13) and

antioxidant effects (3, 14). Additionally, its pronounced antibacterial activity against a spectrum of pathogenic bacteria (2) is linked to an extended shelf life of the milk. The immunomodulatory, anti-inflammatory, and antiviral properties of donkey milk can largely be attributed to its constituent bioactive molecules, namely lactoferrin and lysozyme, as corroborated by extensive research (15, 16).

Lysozyme, a pivotal component of the innate immune system, is recognized for its broad-spectrum antimicrobial activities against bacterial (15), fungal, and viral pathogens, thereby serving as a natural defense mechanism against infections. In the realm of pharmaceuticals, the application of milk-derived lysozyme spans the prophylaxis of diseases of bacterial, viral, fungal, and inflammatory etiologies, further supplemented by its immune-stimulatory (17, 18) and antihistaminic potentials (19, 20). The advancements in lysozyme modification open new avenues in clinical medicine (17, 21). The milk whey proteins were suggested to conduce to the destruction of tumors, as it modulates the synthesis of the tumor necrosis factor (TNFα) and also stimulates the production of Type I interferon (INFα, INFβ, INFγ), interleukin-2 (IL-2) and interleu-kin-6 (IL-6) by human lymphocytes (22).

Based on its abundance in the colostrum and milk, LF plays an essential immunomodulatory role that complements immature biological defense functions in neonates (23). Furthermore, LF enhances immune function, which declines with age (24). The previously reported physiological effects of LF include antimicrobial, antiviral, anti-aging, neuroprotective, regulation of iron metabolism, improvement of bone metabolism, and immunomodulatory effects (25).

The use of donkey milk as a therapeutic agent to heal wounds and treat various diseases, such as bronchitis, asthma, joint pain, and gastritis, is being explored (10, 12). Consistently, the anti-cancer (26), antidiabetics (27), atherosclerosis (28) and anti-colitis (29) properties of donkey milk has also been reported. In the current pandemic of the coronavirus, some modified form of lysozyme can be used to stimulate the formation of interferon, an effective substance against coronavirus, and thus reduce the risk of the life-threatening form of COVID-19 up to 79% (30, 31).

This review aims to synthesize existing knowledge on the antimicrobial, anti-inflammatory, immunoregulatory, and antioxidant attributes of donkey milk. In addition, we have highlighted the role of donkey milk as therapeutic agent in prevention of cancer, diabetes, and heart diseases. Furthermore, this review delineated the principal factors that detrimentally affect the milk characteristics of donkey.

2 Materials and methods

This review examines articles that discuss the composition and bioactive components of donkey milk, as well as studies highlighting its potential therapeutic uses. These therapeutic uses include its antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory effects. The data for this review was gathered from reputable platforms such as springerLink, Scopus, Web of Science, PubMed, Google Scholar, and ScienceDirect. Various key terms were utilized in the search, including the composition of donkey milk, its bioactive elements, therapeutic benefits, and its antimicrobial, anti-inflammatory, immunomodulatory, and antioxidant properties. The selection criteria for the articles were rigorous, focusing only on studies published in reputable, peer-reviewed English language journals from 2010 onwards, with the exception of two earlier studies from 2007. Conference summaries, books, and book sections were not considered for this review.

3 Composition and bioactive components of donkey milk

The nutritional composition and beneficial properties of donkey milk have been extensively explored in scientific literature, including in recent studies (32–36). The summary of donkey milk compositions has been provided in Table 1. Donkey milk is particularly valued for its close resemblance to human breast milk, both in terms of its nutritional profile and its health benefits. It is characterized by a balanced mix of essential nutrients, though it differs from cow's milk in several key aspects.

Protein content in donkey milk ranges from 1.5–2.0%, which is less than that found in cow's milk. The proteins in donkey milk include both caseins and whey proteins, such as α-lactalbumin and β-lactoglobulin, but in smaller amounts compared to those in cow's milk. Notably, donkey milk is richer in lactoferrin and lysozyme, two proteins known for their antimicrobial properties, than cow's milk (37). Consistently, the fat content in donkey milk is relatively low, between 0.3–1.0%, and its fat includes a higher proportion of polyunsaturated fatty acids, especially linoleic acid, which contributes to its health benefits (38). Donkey milk is also higher in lactose than cow's milk, with levels between 6.2 and 7.4%, lending it a slightly sweet taste (8).

Moreover, donkey milk is acknowledged for its healthful attributes, such as lower fat and cholesterol levels, and higher amounts of protein, casein, lactose, whey protein, calcium, selenium, and vitamin D3 (36). Consistently another study also reported that donkey milk has a lower cholesterol content, a lower casein to whey protein ratio, a higher calcium to phosphorus ratio, and a higher taurine content compared to bovine milk (39). The donkey milk also contains a variety of vitamins, including both water-soluble (B group and vitamin C) and fat-soluble (vitamins A, D, E, and K) types and minor components such as hormones (6). The vitamin content can fluctuate depending on the donkeys' diet and environment. Donkey milk is a good source of essential minerals like calcium, magnesium, phosphorus, and potassium, though in amounts typically lower than in cow's milk (40). Despite this, the bioavailability of these minerals is high, attributed to the low protein content of the milk.

Donkey milk also features a range of bioactive components that support immunological health, such as immunoglobulins, lactoferrin, lysozyme, and cytokines, which may enhance its immune-boosting capabilities (41, 42). In terms of whey protein content, donkey milk contains about 4.9–9.6 g/L, which constitutes roughly 43–50% of its total protein. The whey protein is primarily composed of β-lactoglobulin, α-lactalbumin, immunoglobulins, lysozyme, serum albumin, and lactoferrin (Figure 1). These proteins not only provide energy but also play roles in antimicrobial, antioxidant, anti-inflammatory, and antitumor activities, contributing to the wide range of health benefits associated with donkey milk consumption (43). The whey protein fraction of donkey milk is predominantly constituted by β-lactoglobulin, with concentrations approximately at 3.75 g/L, and α-lactalbumin, measured around 1.80 g/L. Additionally, the

TABLE 1 Comparative analysis of milk composition: donkey, human and cow.

Major components in milk	Composition	Donkey milk	Human milk	Cow milk
Fatty acids (% of total fatty acid)	PUFA (%)	14.0–30.0	8.00–19.00	2.0–6.0
	Linolenic acid (ALA) (%)	4.5–16.00	0.5–3.00	0.5–1.8
	Linoleic acid (LA) (%)	6–15.2	6.0–17.7	1.2–3.0
	LA:ALA (%)	0.9–6.1	7.4–8.1	2.1–3.7
	DHA (%)	0.04	0.15	0.03
	ARA (%)	0.14	0.37	0.48
Major whey protein (g/L)	α-Lactalbumin	1.8–3.0	1.8–3.5	1.0–1.5
	β-lactoglobulin	3.0–3.2	Absent	3.2–4.0
	lysozyme	1	0.04–0.2	Trace
Major casein (CN) (g/L)	β-Casein	3.9	3.8	8.6–11.0
	αs1-Casein	1.2–2.0	1.0–1.9	3.0–3.9
Water (%)		89	87	87
Lactose (g/L)		60–72	63–70	44–58
Protein (g/L)		14–20	9–19	33–40
DM (g/L)		90–114	103–124	118–130
Fat (g/L)		4–16	20–39	34–53
Energy Value (kJ/kg)		1939.4	2855.6	2983.0
Total whey protein (g/L)		4.9–8.0	6.0–8.4	5.0–7.0
Total casein (g/L)		6.5–10.00	2.0–4.2	24.0–29
Casein: whey protein (g/L)		1.2	0.4–0.5	4.5

PUFAs, Polyunsaturated fatty acids; DHA, Docosahesaenoic; ARA, Arachidonic acid.

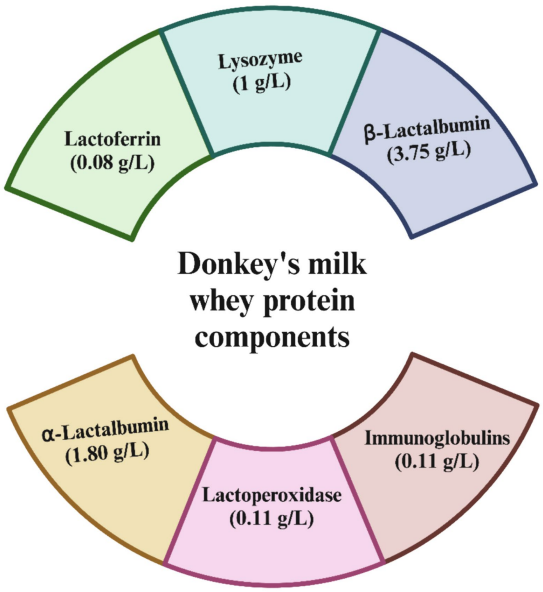

FIGURE 1
Bioactive components of donkey milk.

composition includes immunoglobulins at roughly 1.30 g/L, lysozyme at about 1.00 g/L, serum albumin at nearly 0.40 g/L, and lactoferrin at approximately 0.08 g/L (38). After being consumed, proteins are broken down into smaller peptide fragments through enzymatic hydrolysis in the gastrointestinal tract. This process is essential for the efficient digestion and absorption of proteins and also results in the creation of bioactive peptides. These peptides have specific biological functions, often imitating or boosting the activities of the original proteins, thus enhancing their overall bioactivity. These proteins are not merely sources of energy but also play pivotal roles in various physiological processes. They exhibit a spectrum of bioactive functions, encompassing antimicrobial, antioxidant, anti-inflammatory, and antitumor properties, thereby underscoring the multifaceted health benefits conferred by whey protein constituents in donkey milk.

The contents for Table 1 are adopted from Baloš et al. (1); Zheleva, (2); Vincenzetti et al. (14); Derdak et al. (43); Cimmino et al. (44); Wang F et al. (45); Li M et al. (46); Claeys et al. (47);; Li Y et al. (48); Garhwal et al. (49); Parsad, (50); Altomonte et al. (51); Martemucci and D'Alessandro, (52).

4 Antimicrobial, anti-inflammatory and immunoregulatory effect of donkey milk

4.1 Antimicrobial regulatory properties of donkey milk

Recent *in vitro* investigations have elucidated the antibacterial properties of equid milk, attributing these characteristics to the

elevated concentrations of lysozyme and lactoferrin (53–55). Furthermore, an association has been observed between the antibacterial activities of donkey milk and its high calcium content (56). The spectrum of anti-bacterial efficacy of donkey milk extends to pathogens such as *Staphylococcus aureus* (57), *S. haemolyticus, Listeria monocytogenes* (58), *Escherichia coli,* and *Salmonella enteritidis* (12, 59, 60). The abundance of lysozyme in donkey milk is particularly noted for its capability to degrade the peptidoglycan layer of Gram-positive bacteria, thus facilitating bacterial lysis (61). Recent research by Saju et al. (36) has demonstrated that endosymbiotic bacteria isolated from donkey milk exhibit pronounced antibacterial activities against *Escherichia coli, S. aureus,* and *Salmonella enterica.* The comprehensive antimicrobial efficacy of donkey milk is likely due to the synergistic interactions among its bioactive components, namely lactoferrin, lysozyme, immunoglobulins, and fatty acids, in addition to anatomical factors related to the udder's size and position (8, 62). However, a reduction in lysozyme levels during the later stages of lactation has been linked to the presence of pathogenic bacteria in donkey milk (63, 64).

The antimicrobial efficacy of lactoferrin and lysozyme has been substantiated through a compendium of research efforts (18, 65–71). These studies collectively underscore the critical roles that these molecules play in the innate immune defense, particularly through their bacteriostatic and bactericidal activities. Lactoferrin, a multi-functional protein of the transferrin family, is renowned for its ability to sequester iron, thereby inhibiting the growth of iron-dependent bacteria in inflammatory sites. Con-currently, lysozyme, an enzyme that hydrolyzes the bonds in bacterial cell wall peptidoglycan, contributes to the bactericidal activity, particularly against gram-positive organisms. Further investigations have elucidated the synergistic antimicrobial potential when lysozyme and lactoferrin are combined with immunoglobulins and L-amino acid oxidase, as observed in donkey milk (72, 73). This synergism is attributed to the complementary mechanisms of action of these components, where lysozyme and lactoferrin disrupt bacterial cell walls and sequester essential growth nutrients, respectively, while immunoglobulins provide specific immunity and L-amino acid oxidase generates hydrogen peroxide, contributing to an antimicrobial environment.

The bactericidal mechanisms of lysozyme are multifaceted and can be delineated into three principal categories:

Direct bacteriolysis through peptidoglycan disruption: lysozyme exerts a direct bactericidal effect by cleaving the glycosidic bonds within the peptidoglycan layer of bacterial cell walls, a process that is lethal for certain susceptible bacteria. This disruption leads to osmotic imbalance and cell lysis, particularly in some nonpathogenic Gram-positive bacteria such as *Micrococcus luteus* and various Bacillus species. However, this mechanism is not universally effective across all bacterial species, as some exhibit inherent resistance or acquire resistance through structural modifications of peptidoglycan, such as O-acetylation of N-acetylmuramic acid (NAM) and de-N-acetylation of N-acetylglucosamine (NAG), which impede lysozyme's enzymatic activity.

Indirect antimicrobial effects via enzymatic and non-enzymatic activities: beyond its enzymatic action, lysozyme can exhibit antimicrobial activity through non-enzymatic mechanisms, particularly at elevated concentrations. These activities are attributed to the highly cationic nature of lysozyme, facilitating interactions that can compromise bacterial membrane integrity or induce the release of autolytic enzymes, which are typically involved in bacterial cell wall remodeling. This aspect of lysozyme's function is evidenced by the retention of antimicrobial activity even when the enzyme's active site is altered by site-directed mutagenesis, suggesting a mechanism independent of its enzymatic cleavage of peptidoglycan.

Synergistic enhancement with host defense molecules: the bactericidal efficacy of lysozyme, particularly against pathogenic and gram-negative bacteria, is significantly augmented in the presence of other host defense molecules, such as lactoferrin, antibodies, complement proteins, hydrogen peroxide, and ascorbic acid. These cofactors are believed to facilitate the disruption of the outer bacterial membrane, thereby enhancing lysozyme's access to the peptidoglycan layer. This synergistic interaction underscores the collaborative nature of the innate immune system in combating bacterial infections. Furthermore, recent studies have illuminated lysozyme's role in modulating the host immune response. The enzymatic degradation and subsequent lysis of bacteria by lysozyme result in the release of bacterial components, including peptidoglycan fragments, which can activate pattern recognition receptors on host cells. This process not only contributes to the immediate defense against bacterial invasion but also plays a pivotal role in the activation and regulation of broader immune responses (18, 74–76). Thus, lysozyme's contribution to antimicrobial defense extends beyond direct bactericidal activity, encompassing roles in immune modulation and synergy with other antimicrobial pathways.

Moreover, lactoferrin has demonstrated significant antiviral activity, inhibiting viral attachment to host cells and replication within them. This is achieved through the blockade of key viral receptors [Angiotensin-converting enzyme 2 (ACE), Heparan sulfate proteoglycan (HSPG)] and the induction of antiviral cytokines such as interferon (IFN)-α/β (77, 78). The administration of lactoferrin has also been shown to enhance natural killer (NK) cell activity and Th1 cytokine responses, thereby bolstering defenses against viral pathogens (79). The antimicrobial mechanism of donkey milk has been presented in Figure 2.

4.2 Anti-inflammatory and immunoregulatory role of donkey milk

The anti-inflammatory and immunomodulatory effects of donkey milk have garnered increasing scientific interest, as evidenced by recent studies (Table 2). Consistently, Kocyigit et al. (80) provided a foundational exploration into the anti-inflammatory properties of equid milk, setting a precedent for subsequent investigations in this domain. Our research team further substantiated these findings, demonstrating that the anti-inflammatory and antioxidative attributes of donkey milk contribute significantly to its regulatory effects on health (48). In a detailed study, Taghiloo et al. (81) elucidated the impact of donkey milk on immune cell function, revealing that treatment with donkey milk resulted in elevated levels of interleukin-8 (IL-8) and interleukin-6 (IL-6) in peripheral blood mononuclear cells (PBMCs). IL-8 serves as a chemotactic factor for neutrophils, while IL-6 plays a crucial role as an acute phase protein with protective functions. Furthermore, donkey milk was found to modestly enhance the production of tumor necrosis factor-alpha (TNF-α) from PBMCs, indicating a relatively low pro-inflammatory profile. The stimulation of normal human PBMCs with donkey milk also led to the release of interleukin-10 (IL-10) in supernatants, highlighting its

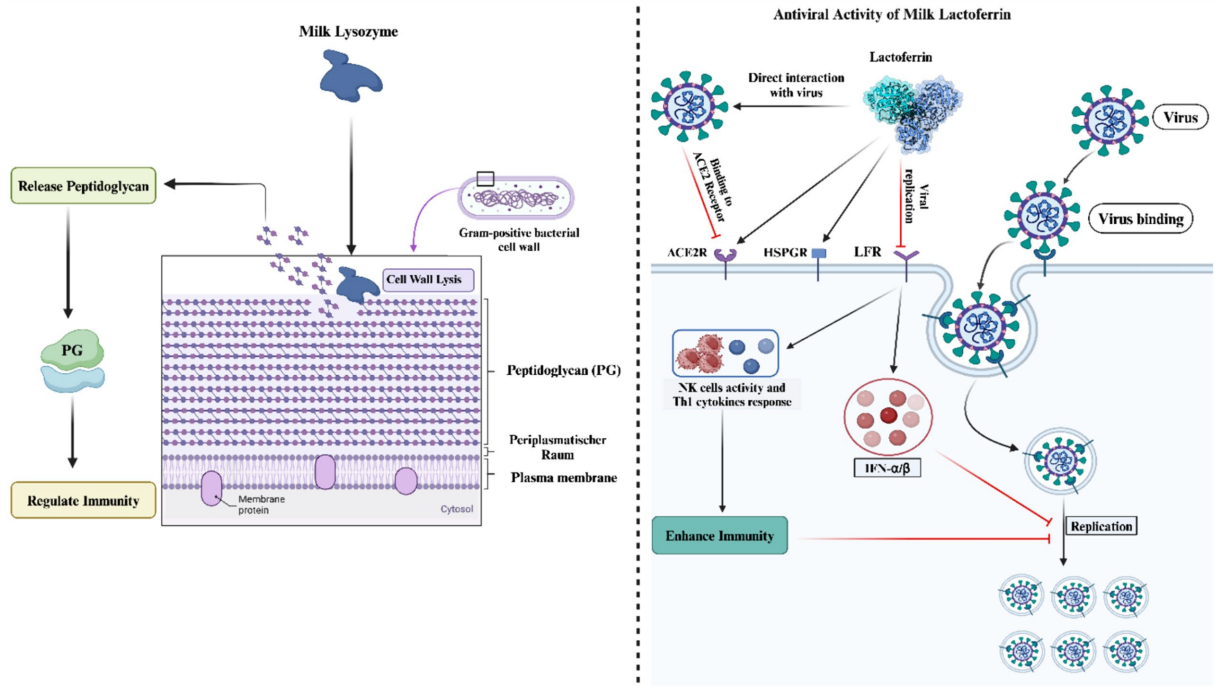

FIGURE 2
The antimicrobial properties of donkey milk.

potential to modulate immune responses and maintain immune homeostasis (81). These cytokines are instrumental in the maturation, differentiation, and regulation of immune cells, thereby facilitating the host's defense mechanisms (82). Previous investigations have shown that donkey milk can induce the production of nitric oxide (NO) from PBMCs and promote adaptive immunity through cytokine production (83). In a mouse model of ileitis, donkey milk exhibited anti-inflammatory properties, which were associated with the normalization of antimicrobial peptides levels (lysozyme and α-defensin) in Paneth cells and a reduction in the dysbiosis typically associated with ileitis (84). Additionally, the impact of donkey milk on gut barrier function was observed in mice subjected to water-avoidance stress, further underscoring its therapeutic potential (85).

The anti-inflammatory capabilities of donkey milk have also been linked to the prevention of allergic asthma, showcasing its broad spectrum of health benefits (86). Moreover, a recent study by Farias et al. (87) demonstrated that asinine milk significantly downregulated salivary cortisol levels and the expression of the IL-1B gene in weaning piglets, thereby mitigating stress-induced inflammatory responses. Collectively, these studies highlight the multifaceted role of donkey milk in modulating inflammation and immune responses, suggesting its potential as a therapeutic agent in various inflammatory and autoimmune conditions.

5 Unlocking the health benefits of donkey milk: *in vitro* insights

5.1 Anticancer activity of donkey milk

Donkey milk is recognized as a reservoir of numerous salutary components, including caseins, Omega-3 fatty acids, lactoferrin, lysozyme, α-lactalbumin, and β-lactoglobulin, which contribute to its potential therapeutic applications. The inclusion of long-chain omega-3 polyunsaturated fatty acids (PUFAs) is of particular interest due to their documented role in the prophylaxis of various carcinomas, including but not limited to breast and colorectal cancers (88–97). Consistently, research delineates the inhibitory effects of α-casein on the activity of breast cancer stem cells and cancer-associated fibroblasts in MDA-MB-231 cells, mediated through the modulation of HIF-1α, STAT3, and STAT19 expression (98). Concurrently, Maurmayr et al. have elucidated the potent anti-tumorigenic properties of lactoferrin, α-lactalbumin, and β-lactoglobulin against a spectrum of human tumor cell lines, including A549, HT29, HepG2, and MDA231-LM2 (99).

Investigations into the bioactivity of donkey milk on A549 human lung cancer cells have revealed its capacity to augment the secretion of a cadre of cytokines, including IL-2, IFN-γ, IL-6, TNF-α, and IL-1β, thereby indicating a dual mechanism of action: direct antiproliferative effects on tumor cells and indirect tumoricidal effects mediated through the activation of lymphocytes and macrophages (100). This assertion is bolstered by Shariatikia et al. (101), who reported dose-dependent cytotoxic effects of donkey milk casein and whey proteins against MCF7 cells. The antiproliferative, antimutagenic, and antibacterial properties of donkey milk kefir have also been documented, with evidence suggesting its role in inducing apoptosis, inhibiting cell proliferation, and reducing the co-expression of iNOS and endothelial-NOS in the context of Ehrlich ascites carcinoma (EAC) in mice (102–105).

Moreover, the comparative analysis of donkey colostrum (DC) and mature milk (DM) on 4T1 triple-negative breast cancer (TNBC) tumors in mice has yielded promising outcomes, with both DC and DM significantly attenuating the primary tumor volume and the relative organ weight of the liver and lungs in the 4T1 mouse model,

TABLE 2 Antimicrobial, anti-inflammatory, antioxidant and immune regulatory role of donkey milk.

References	Donkey milk associated biological effect
Zheleva (2)	• Anti-inflammatory, immunoregulatory, antioxidant and antimicrobial potential
Li et al. (3)	• Antioxidant properties and antiallergic properties
Aspri et al. (13)	• ACE-inhibitory activity
Akan (27)	• Antioxidant and antidiabetic potential
Tafaro et al. (28)	• Immunoregulatory properties
Saju et al. (36)	• Antibacterial potential of donkey milk endosymbiotic bacteria against *Escherichia coli, Staphylococcus aureus* and *Salmonella enterica*
Spada et al. (72)	• Antibacterial properties of Donkey milk
Li et al. (48)	• Antioxidant and anti-inflammatory properties
Taghiloo et al. (81)	• Immunoregulatory effect
Jirillo and Magrone (82)	• Anti-inflammatory and antiallergic properties
Yvon et al. (85)	• Anti-inflammatory and antimicrobial potential
Garhwal et al. (49)	• Antimicrobial, anti-inflammation, antioxidant, and hypo-allergenicity properties
Beghelli et al. (164)	• Antioxidant properties
Bhardwaj et al. (165)	• Antioxidant properties
Simos et al. (116)	• Antioxidant and anti-platelet properties
Trinchese et al. (147)	• Improved antioxidant efficiency and anti-inflammatory status

without adverse effects on body weight. This antitumor efficacy is attributed to the modulation of apoptotic and angiogenic markers, including an increase in cleaved-caspase-3 and Bax expression and a decrease in MMP2 and CD31 levels (26). Additionally, Akca et al. (106) have identified the anti-proliferative and genotoxic effects of donkey milk on lung cancer cells, with a specificity that spares normal lung cells, further underscoring the potential of donkey milk as a functional food with therapeutic implications in oncology.

5.2 Antidiabetics potential of donkey milk

Bioactive peptides, derived from a variety of protein sources with a notable emphasis on milk proteins, are increasingly recognized for their multifaceted biological functionalities. These include, but are not limited to, antioxidant, antimicrobial, anti-diabetic, antihypertensive, anticancer, and antitumor properties (107, 108). Type II diabetes mellitus, also referred to as non-insulin dependent diabetes, emerges as a pre-dominant metabolic disorder characterized by persistent hyperglycemia, with a significant global incidence annually (27, 109). The etiology of Type II diabetes is multi-factorial, with obesity, β-cell dysfunction, and insulin resistance in peripheral tissues being primary contributors (110–112).

A pivotal aspect of Type II diabetes management involves the modulation of di-peptidyl peptidase-IV (DPP-IV) and α-glucosidase activities, enzymes integral to the inactivation of incretins that are crucial for normoglycemic regulation (113, 114). The inhibition of these enzymes constitutes a strategic approach in the therapeutic management of this condition. Empirical evidence from *in vivo* investigations has under-scored the regulatory influence of food proteins on serum glucose concentrations (115). Corroborating these findings, *in vitro* studies have demonstrated that peptides derived from casein significantly attenuate the activity of DPP-IV and α-glucosidase, while concurrently enhancing antioxidant capacities, thereby contributing to the management of Type II diabetes (27).

Furthermore, the antidiabetic potential of donkey milk has been substantiated through various research endeavors (2, 42, 116, 117). Consequently, Li et al. (117) elucidated that donkey milk not only ameliorates the viability of compromised pancreatic β cells but also enhances insulin sensitivity in target organs without directly stimulating insulin secretion from the damaged β cells. This is attributed to the presence of α-lactalbumin in donkey milk. Additionally, donkey milk has been shown to decrease glycosylated hemoglobin levels and exert a therapeutic effect on diabetes by downregulating the expression of key hepatic gluconeogenesis enzymes, namely phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase (117). Collectively, these studies highlight the significant potential of bioactive peptides, particularly those derived from milk proteins, in the prevention and management of type II diabetes through various biochemical mechanisms. The key findings of studies reported the role of donkey milk in health improvement have been summarized in Table 3.

5.3 Use of donkey milk in cow's milk protein allergy children

The research development on donkey milk as alternative for infants with cow milk allergy has been summarized in Figure 3. Cow's Milk Protein Allergy (CMPA) delineates an aberrant immunologic response toward proteins presents in cow's milk, manifesting in a subset of individuals. As a prevalent food allergy, particularly in developed nations, cow's milk proteins often represent the initial exogenous proteins introduced to infants, with an incidence rate of 2–7% in children under 6 months, which notably diminishes to 0.1–0.5% in adults (118, 119). The allergic manifestations to cow's milk can be attributed to diverse immunological pathways, including immediate IgE-mediated hypersensitivity, characterized by symptom onset within 30 min post-ingestion, and delayed non-IgE mediated reactions, where symptoms emerge hours to days subsequent to consumption (120, 121). The main allergens in cow's milk are primarily the caseins (α-s1- and β-caseins). However, β-lactoglobulin and α-lactalbumin are also involved to a lesser extent (118). It is worth noting that the lower allergenic potential of certain types of milk, such as that from non-ruminant animals like donkeys or horses, is not only due to their lower casein content but also to significant sequence differences between the proteins in their milk and those found in ruminants' milk. Additionally, the reduced allergenicity observed in non-ruminant milk may largely be a result of substantial sequence differences between β-lactoglobulin in non-ruminants and its homologous protein in ruminants.

Addressing CMPA necessitates the comprehensive exclusion of cow's milk from the diet. Given the critical role of milk as a nutritional mainstay up to the age of two, outright elimination is inadvisable; instead, alternative milk sources with appropriate nutritional profiles, low allergenic potential, palatability, and economic viability are recommended. While hypoallergenic formulas are preferred for managing CMPA, the incorporation of milk from other species

TABLE 3 Donkey milk role in human health.

References	Health linked bioactive components in donkey milk	Health benefit
Li et al. (26)	• Milk α-casein, lactoferrin, α-lactalbumin, and β-lactoglobulin	• Triple-negative breast cancer
Akan (27)	• milk casein	• α-glucosidase inhibitor and Antidiabetic
Tafaro et al. (28)	• Rich in omega-3 fatty acids	• Donkey colostrum and milk induced nitric oxide (NO) release from PBMCs
Yvon et al. (85)	• lysozyme	• Used in treatment of ileitis
Lu et al. (86)	• Donkey milk powder (15% protein, 64% carbohydrate and 6% fat, including 8% whey protein and 0.5% polyunsaturated fatty acid)	• Alleviated ovalbumin-induced asthma • Relieved airway hyperresponsiveness, injury and fibrosis of airway epithelium • Reduced airway eosinophilia and the increased Th2 cytokines in bronchoalveolar lavage fluid as well as serum immunoglobulin E, and inhibited NF-κB P65 activity.
Mao et al. (100)	• Milk lysozyme	• Prevent lung cancer
Shariatikia et al. (101)	• Casein and whey proteins	• Prevent Breast cancer
Esener et al. (104)	• milk kefir	• Ehrlich ascites carcinoma
Khan et al. (107)	• Milk lactoferrin and lysozyme	• Cytotoxic and genotoxic on human
Trinchese et al. (166)	• –	• Improved heart mitochondrial metabolic flexibility and antioxidant potential • Prevent hypertension and heart failure
Tang et al. (167)	• Donkey milk protein	• Enhanced the gastrointestinal function and reduce the incidence of gastrointestinal motility disorder in patients with severe pneumonia
Kocic et al.; Li et al. (168, 169)	• Donkey milk	• Enhance the skin fibroblast survival and their proliferative and regenerative potential • Restored skin barrier by upregulating the level of filaggrin • Enhance wound healing

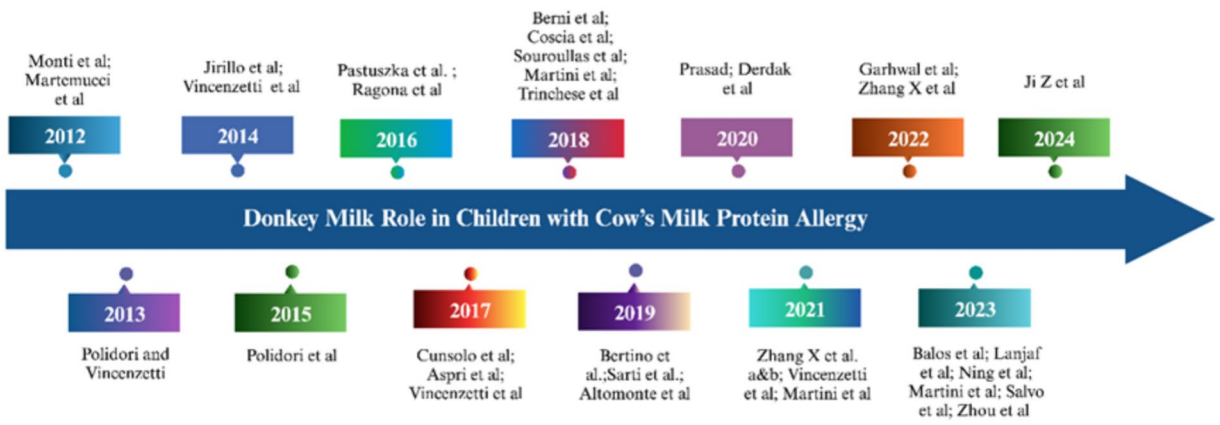

FIGURE 3
Research development of donkey milk as alternative in infants with milk cow allergy (1, 6, 7, 12–14, 38, 43, 50–52, 63, 82, 124, 147–163).

warrants careful pediatric oversight. The diminished allergenicity of donkey milk is chiefly ascribed to its lower casein content (42, 122). Moreover, the high whey protein ratio in donkey milk facilitates easier digestion (123). Despite its lower fat and caloric density, the nutritional value of donkey milk can be enhanced through the addition of medium-chain triglycerides (8). This fat supplementation strategy is equally applicable to lyophilized donkey milk, which according to Vincenzetti et al. (42), retains its nutritional integrity in comparison to its raw counterpart.

6 Factors compromising the compositional properties and quality of donkey milk

The compositional properties and quality of donkey milk are influenced by a multifaceted array of factors, which include but are not limited to the method of preservation, the stage of lactation, and dietary considerations. These elements have been identified as pivotal in determining the nutritional and biochemical profile of donkey

milk, with implications for both its quality and utility in various applications (124–126). The factors affecting quality and quantity of donkey milk are summarized in Table 4.

6.1 Lactation stage and its impact on donkey milk composition and quality

The stage of lactation has been observed to exert a significant influence on the compositional attributes of donkey milk, particularly with respect to its protein, lipids and volatiles contents (127). Research indicates a progressive decline in milk protein concentration as the lactation period extends (128, 129). This trend is corroborated by Malacarne et al. (40), who noted a discernible impact of the lactation phase on the protein and mineral content of donkey milk, albeit with caseins being an exception. Contrarily, Zhou et al. (63, 64) documented a fluctuating pattern in the relative expression levels of various casein proteins, which initially increased before diminishing as lactation progressed. Complementing these findings, dos Santos et al. (130) reported a decline in total solids and fats in correlation with advanced lactation stages, whereas Salgado et al. (131) observed an enhancement in total polyunsaturated and n − 3 fatty acids over time. Intriguingly, aging was linked with an elevation in protein and fat concentrations in milk, alongside a notable impact on the principal whey proteins, whose contents were also modulated by the lactation stage (130). The expression levels of lactoferrin and other whey proteins such as albumin, lysozyme, β-lactoglobulin, and α-lactalbumin exhibit-ed dynamic changes during the lactation period, with a significant portion of donkey milk protein being constituted by whey proteins (40). The lipid profile of donkey milk was also found to be significantly altered by lactation, with hormonal shifts, particularly in prolactin levels, being implicated in the observed reduction in lipid content (132, 133).

6.2 Dietary influences on donkey milk production traits

Nutritional strategies play a crucial role in optimizing the health and productive performance of livestock, including lactating donkeys. There is a substantial body of evidence underscoring the positive effects of nutrition on the overall well-being and output of livestock animals (134–141). Recent investigations have highlighted the potential of nutritional management during lactation to mitigate oxidative stress and enhance the health and lactational efficiency of donkeys (142–144). Specifically, dietary supplementation with selenium yeast (0.3 mg/kg) has been shown to significantly improve the composition and quality of donkey milk, as well as the overall lactational performance (142). Furthermore, the inclusion of dietary roughage has been associated with an upregulation of lipid molecules and volatile organic compounds in donkey milk, indicating the profound impact of diet on milk compositional traits (145).

6.3 Effect of breed on milk production traits in donkeys

The variability in donkey milk production and composition is closely linked to the specific breed of the animals involved. Studies

TABLE 4 Effect of lactation stage, diet and method of preservation/treatment on donkey milk production traits.

Biological factors	Effect on donkey milk production traits	References
Diet effect		
selenium yeast supplementation (0.3 mg/kg)	• Improved milk components and lactational performance	(142)
Dietary roughage supplementation	• Improved milk lipid molecules and volatile organic compounds (VOCs)	(145)
Crude protein (14.2%)for 10 weeks	• Enhanced milk yield and yields of protein, lactose, solid-not-fat, total solid, and contents of protein, total solid and milk urea nitrogen	(170)
Concentrate to forage feed ratio (30:70) 8 weeks	• Increased milk protein and most amino acid (AA) production in milk	(171)
Lactation stage		
	• Altered protein profile	(63)
	• Alteration in milk microbiota	(64)
	• Changes in milk mineral contents (cupper and selenium concentrations decreased; manganese increased)	(172)
Treatment/preservation effect		
HTST plus HPP treatment at 300 MPa HTST plus HPP treatment at 400 MPa HTST plus HPP treatment at 300 MPa	• Maintained the milk lysozyme, α-lactalbumin and β-lactoglobulin contents in donkey milk. • Reduced Donkey milk stability • Promoted shelf life of donkey milk	(32, 173)
Combination of pasteurization and HPP (600 MPa/180 s and 400 MPa/180 s)	• Compromised the quality of donkey milk • Unfit for human consumption	(173)
UHPH at 100 MPa	• Maintained the minimum shelf-life of donkey milk for 28 days. • Did not affect the level of milk lysozyme	(174)
Freeze drying method	• Preserved the bioactive components and overall shelf life of donkey milk • Reduced the transport and the storage costs of donkey milk	(175, 176)

HHP, High hydrostatic pressure; HTST, high-temperature short time pasteurization; HHP, high hydrostatic pressure; UHPH, Ultra-high-pressure homogenization.

have demonstrated that certain breeds exhibit markedly higher milk yields compared to others. For instance, Istrian donkeys have been shown to produce significantly more milk than Littoral Dinaric donkeys (146). Similarly, higher milk yields were observed in the Martina Franca and Ragusana breeds when compared with the Amiata jennies (129). Beyond yield, there are notable differences in the compositional traits of milk across various donkey breeds (40, 58, 144, 146). These findings underscore the critical influence of breed as a determining factor in both the quantity and quality of milk produced by donkeys. The breed-specific differences in milk composition likely reflect variations in genetics, physiology, and lactation dynamics, emphasizing the importance of considering breed as a primary factor in the selection of donkeys for milk production purposes. These findings collectively underscore the intricate interplay between lactation stage, dietary practices, and preservation/treatment methods in shaping the compositional quality of donkey milk. Understanding these dynamics is essential for devising strategies to optimize the nutritional value and functional properties of donkey milk for various applications.

7 Future perspectives and limitations

Future studies could focus on the in-depth analysis of the bioactive compounds present in donkey milk, such as lysozyme, lactoferrin, and immunoglobulins, which contribute to its antimicrobial and immunomodulatory activities. The exploration of these components could lead to the development of functional foods or supplements aimed at enhancing immune function and overall health. Rigorous clinical trials are necessary to substantiate the health claims associated with donkey milk. This includes its purported benefits in managing allergies, improving gut health, and supporting the immune system. Establishing a strong evidence base will be crucial for gaining regulatory approval and consumer trust in donkey milk as a health-promoting food. As interest in donkey milk increases, sustainable and ethical farming practices must be developed to meet demand without compromising animal welfare or environmental integrity. This includes addressing the challenges of low milk yield and seasonal lactation in donkeys through advancements in animal husbandry and dairy technology. Efforts should be made to increase consumer awareness and acceptance of donkey milk as a nutritious and health-promoting food. This involves overcoming cultural and perceptual barriers, as well as establishing a premium market segment for donkey milk products, akin to those for other non-conventional dairy products like goat or sheep milk.

One of the primary limitations in the use of donkey milk is its low production yield. Donkeys produce significantly less milk than cows, goats, or sheep, and the milking process can be labor-intensive, contributing to higher production costs and limited availability. There is currently a lack of standardization in the production and processing of donkey milk, which can lead to variability in product quality and nutritional content. Establishing industry standards and regulatory guidelines will be essential for ensuring consistency and safety in donkey milk products. Despite promising preliminary research, there are still significant gaps in our understanding of the health benefits and nutritional properties of donkey milk. More comprehensive studies are needed to elucidate its mechanisms of action and potential therapeutic applications. The economic viability of donkey milk production on a large scale is questionable due to the aforementioned production challenges and market acceptance issues. Developing cost-effective production methods and creating value-added products could help mitigate these concerns.

8 Conclusion

In conclusion, donkey milk presents an intriguing option for health-promoting foods, with its unique nutritional profile and potential therapeutic properties. However, still need deep studies involving both invitro and *in vivo* experiments to establish the molecular mechanism through which donkey milk showed therapeutic effect against several diseases like diabetes, cardiovascular diseases, asthma, hypoallergy and various types of cancers. In addition, realizing its potential will require overcoming significant challenges related to production, research, and market development. Future studies and innovations in dairy technology will play a critical role in addressing these limitations and unlocking the potential of donkey milk as a valuable addition to the functional food market.

Author contributions

MK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. WC: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. WR: Investigation, Software, Writing – review & editing. BH: Data curation, Investigation, Writing – review & editing. XK: Data curation, Investigation, Methodology, Writing – review & editing. QU: Data curation, Investigation, Writing – review & editing. LW: Data curation, Investigation, Software, Writing – review & editing. TW: Investigation, Validation, Writing – review & editing. AK: Methodology, Software, Writing – review & editing. ZZ: Data curation, Methodology, Validation, Writing – review & editing. LL: Data curation, Investigation, Methodology, Writing – review & editing. CW: Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. ML: Conceptualization, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Chitooligosaccharides improves intestinal mucosal immunity and intestinal microbiota in blue foxes

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Objective: Gut health is critical to the health of the host. This study was conducted to investigate the effects of Chitooligosaccharides (COS) on intestinal morphology, intestinal barrier, intestinal immunity and cecum microbiota of blue foxes.

Methods: Seventy-two 125-day-old blue foxes were randomly divided into basal diet (BD) group, 200 ppm COS1 (1.5 kDa) group and 200 ppm COS2 (3 kDa) group for 8 weeks.

Results: We elucidated that dietary COS1 supplementation promoted the development of intestinal villus morphology in blue foxes. Importantly, COS1 increased the number of goblet cells in duodenum, jejunum and ileum by 27.71%, 23.67%, 14.97% and S-IgA secretion in duodenum, jejunum and ileum by 71.59% and 38.56%, and up-regulate the expression of Occludin and ZO-1 by 50.18% and 148.62%, respectively. Moreover, COS1 promoted the pro-inflammatory and anti-inflammatory balance of small intestinal mucosa, and increased the diversity of cecum microbiota of blue foxes, especially Lactobacillus_agilis and Lactobacillus_murinus, and up-regulated the signaling pathways related to polysaccharide decomposition and utilization.

Conclusion: Here, we present dietary COS1 (1.5 kDa) can promote intestinal villus development, enhance intestinal barrier function, regulate intestinal immune balance and cecum microbiota homeostasis.

KEYWORDS

chitooligosaccharides, microbiota, intestinal barrier, mucosal immunity, blue fox

1 Introduction

The blue fox (*Alopex lagopus*) is a mammal of the Arctic fox genus Canidae of the carnivoran order, with a circumpolar distribution in the northern hemisphere (1). The blue fox fur is soft and luster, with dense plush and straight needle hair (2). The main purpose of breeding blue foxes is to obtain high yield and quality fur for high economic benefits (3). The blue fox is a kind of typical carnivore, which inhabits Arctic islands, mainly feeds on Marine resources such as seabirds, new-born seal pups, and fish and shrimp (4). The intestine of the blue fox is short, about 4.3 times the length of the body (about 235 cm), and the time for food to pass through the gastrointestinal tract is only 20-25 hours (5, 6). Weaning, mother-child separation, and environmental factors can trigger weaning stress in newborn animals, reduce circulating antibody levels, cause microbiota imbalance, change intestinal morphology and function, and weaken digestion and absorption capacity (7). The growing period (125 days of age) is the key period of post-weaning development of blue foxes. The health status of blue fox is a key determinant of fur quality.

Chitosan oligosaccharides (COS) are co-oligomers of N-acetylglucosamine and D-glucosamine linked by β - (1→4) glucoside bonds (8). COS is usually prepared by hydrolysis or deacetylation of chitin/chitosan, the primary source is abundant marine biological resources, such as shrimp shells and crab shells (the second most abundant polysaccharide in nature) (9). The mean molecular weight (MWs) of COS is less than 3.9 kDa and the degree of polymerization is between 2-20 (10). COS has the characteristics of high degree of deacetylation, high water solubility, high biocompatibility and low viscosity (11), which endows it with significant antibacterial activity, immune activity, probiotic activity and lipid lowering activity (12–15). Moreover, it showed to protect the intestinal barrier and regulate the intestinal flora (16). In mouse models of colitis and IPEC-J2 cells, COS was found to directly enhance intestinal barrier function, and COS intervention also promoted the richness and diversity of intestinal flora. It was confirmed that COS-modified fecal microbial transplantation can enhance intestinal barrier function (16). In mice, one *in vivo* study demonstrates that COS (200 mg/kg-1) treatment promotes the population of Bacteroidetes, but inhibits the Proteobacteria phylum. At the genus level, COS treatment reduces the population of probiotic Lactobacillus, Bifidobacterium, and harmful Desulfovibrio bacteria (17). Meanwhile, *in vitro* fermentation assessments conducted in the same study show that COS decreases the number of Escherichia/Shigella pathogens (17). In pigs, dietary supplementation of COS (Mw =1.5 kDa) increases the number of Bifidobacteria and Lactobacilli (18).Therefore, COS is widely used in livestock and poultry production as a natural growth promoter, immunomodulator and potential prebiotic (19).

In this study, we selected COS as a potential prebiotic as a feed additive to explore its effects on intestinal morphology, intestinal barrier, intestinal immunity and cecum microbiota of blue foxes. We hope to provide scientific data for marine-derived oligosaccharides regulating gut microbiota and gut health in carnivores.

2 Materials and methods

2.1 Source of materials

COS1, chitooligosaccharides (molecular weight 1.5 kDa), and COS2, chitooligosaccharides (molecular weight 3 kDa) were purchased from Golden-Shell Pharmaceutical Co., Ltd. (Zhejiang, China). All animal-specific procedures were approved and authorized by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee, and the Institute of Special Animal and Plant Sciences Wild Animal and Plant Subcommittee (ISAPSWAPS2016000302).

2.2 Animals and experimental design

A total of 72 125-day-old blue foxes were randomly assigned into 3 dietary groups supplemented with nothing, COS1 or COS2 at 200 ppm. The composition and nutrient levels of the basal diet are shown in Table 1. The addition concentration of COS was set according to the previous literature (20). Each group contained 3 replicates with 8 foxes per replicate (half male and half female). The adaptation period was 7 days, and the formal experiment lasted for 60 days. Each blue fox was housed in a single cage with a size of 110 cm × 90 cm × 90 cm. Foxes were fed daily at 8:00 and 16:00 and had water *ad libitum*. Feeding experiments were conducted outdoors under natural light. All foxes remained in good health during the whole feeding period. In experiment 1 and 60 days respectively weighing blue fox weight on an empty stomach in the morning, used to measure the average daily gain (ADG) of blue fox.

TABLE 1 Basal diet composition and nutrient level of blue fox during growing period (%, as-fed basis).

Ingredients (%)		Nutrient composition	
Corn	35.50	ME (MJ/kg)	12.97
Soybean meal	16.40	CP (%)	29.08
Fish meal	17.50	EE (%)	12.40
Meat and bone meal	5.00	Lys (%)	2.28
Chellocken meal	5.00	Met (%)	1.23
Corn gluten meal	5.00	Ca (%)	2.40
Corn germ meal	4.00	P (%)	1.18
Fish oil	3.00		
Lard	4.50		
NaCl	0.10		
Premix[1]	4.00		
Total (%)	100.00		

ME, metabolic energy; CP, crude protein; EE, ether extract; Lys, lysine; Met, methionine; Ca, calcium; P, phosphorus.

[1] Premix provided per kilogram of diet: vitamin A, 200,000 IU; vitamin D_3, 40,000 IU; vitamin E, 2,500 IU; vitamin B1, 125 mg; vitamin B2, 200 mg; vitamin B3, 500 mg; vitamin B6, 200 mg, vitamin B12, 2.5 mg; vitamin K3, 40 mg; pantothenic acid, 800 mg; biotin, 7.5 mg; folic acid, 100 mg; vitamin C,7,500 mg;Fe, 2000 mg; Zn, 1500 mg; Cu, 500 mg; Mn, 400 mg; I, 15 mg; Co, 7.5 mg; Se, 5 mg.

2.3 Sample collection

The slaughter test was carried out on day 60 of the feeding experiment, and the test animals were fasted and had free access to water for 12 h before sacrifice. Nine blue foxes (3 in each replicate) were randomly selected, blood samples were collected from the hind limb vein, serum was isolated from blood samples after centrifugation and stored at -20°C for the detection of cytokines content. Blue foxes were killed by injection of 5 mL of succinic acetylcholine, skinned and dissected. The tissues of duodenum, jejunum and ileum were collected for histological examination. The mucosa of the duodenum, jejunum, and ileum and cecum contents were quickly frozen in liquid nitrogen and stored at -80°C for subsequent detection of relative quantification of genes and detection of microbiota diversity.

2.4 Histological examination

Duodenum, jejunum and ileum tissue of 5 blue foxes from each group were fixed with 4% paraformaldehyde and Carnoy's Fluid, embedded in paraffin. Serial sections (5 to 8 μm) for hematoxylin - eosin (H&E) and Alcian staining. Images were acquired through the Lycra microscope. Villi height is measured from the tip of the villi to the junction of the recess (μm). Villus width is defined as the width at the widest part of the villus (μm). Crypt depth was defined as the depth of adjacent villi invagination (μm). Intestinal morphology and goblet cell number were determined by measuring at least 6 different areas in each section using the Image-Pro-Plus Image analysis system.

2.5 RNA isolation and real-time quantitative PCR in jejunum mucosa

The RNA was extracted using the RNAiso kit and assayed for purity and concentration. The cDNA was synthesized using a reverse transcription kit (PrimeScript ^{TM} PT Master Mix). Real-time PCR for measuring gene expression was conducted using TB Green Premix Ex Taq ^{TM} kit. Gene-specific primer sequences for Occludin, zonula occludens-1 (ZO-1), interleukins-10 (IL-10), transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) are list in Table 2. The cycling conditions used to amplify cDNA were as follows: 95°C for 5 min; 40 cycles for 95°C for 10 s, 60°C for 35 s. The relative gene expression levels were calculated using the $2^{-\delta\delta Ct}$ method.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay kits were used to detect the levels of IgG, IgA, IgM, IL-2, IL-6, IL-1β, TNF-α, IL-10, DAO and D-lactate in serum and jejunal mucosa, and the content of S-IgA in duodenum, jejunum and ileum mucosa. The lower limit of detection was: IgG (2.01 mg/g), IgA (408.37 pg/g), IgM (326.77 μg/g), IL-2 (49.81 pg/g), IL-6 (11.71 pg/g), IL-1β (216.04 pg/g), TNF-α (18.48 pg/g), IL-10 (14.30 pg/g), DAO (27.07 pg/g), D-lactate (0.79 mmol/L) and S-IgA (98.10 pg/g). Fox Immunoglobulin G (IgG) kit (No. 01.01.0143.10075.00), Fox Immunoglobulin A (IgA) kit (No. 01.01.0143.10076.00), Fox Immunoglobulin M (IgM) kit (No. 01.01.0143.10077.00) Fox interleukin-2 (IL-2) kit (No. 01.01.0143.10078.00), Fox interleukin-4 (IL-4) kit (No. 01.01.0143.10079.00), Fox interleukin 1β (IL-1β) kit (No. 01.01.0143.10081.00), Fox tumor necrosis factor α (TNF-α) kit (No. 01.01.0143.10082.00), Fox interleukin10 (IL-10) kit (No. 01.01.0143.10080.00), Fox diamine oxidase (DAO) kit (No. 01.01.0143.10083.00), Fox lactic acid (LD (kit) (No. 01.02.0153.10002.00). The above kits were purchased from Shanghai Guchen Biotechnology Co., LTD. Secretory immunoglobulin A(S-IgA) (No. H108-2) was purchased from Nanjing Jiancheng Biotechnology Co., LTD.

2.7 Sequencing of cecum microbiota

DNA extraction and sequencing were performed by Nuohezhiyuan Technology Co., LTD., Beijing, China. 16S rRNA

TABLE 2 Primer sequences for qPCR.

Gene	Forward primers (from 5′ to 3′)	Reward primers (from 5′to 3′)
β-Actin	ACCCACACGGTGCCCATC	CTTGATGTCACGCACGATTTCC
Occludin	ATCCAACTGCCCAGGCTTCT	ATCACCATGAACCCCAGGACA
Zo-1	CATAACAGATACAGACCAGAAGCACAG	AGGAGGGACAACCGCAGCAC
IL-10	CTGGACAACATACTGCTGACCG	CTTGATGTCTGGGTCGTGGTTC
TGF-β1	AGTGCCTGAGCCTGTCTTGC	CCAGTGACATCAAAGGACAGCC
IL-1β	CAAATACCTGGTGCTGTCTAACTCG	GGGCTTCCCATCCTTCATCA
TNF-α	ATGTTGTAGCAAACCCCGAAGC	CAAAGCGGCTGATGGTGTGG

ZO-1, Zonula occludens-1; IL-10, Interleukin-10; TGF-β1, transforming growth factor-β; IL-1β, Interleukin-1β; TNF-α, Tumor necrosis factor-α.

genes of distinct regions (16SV4) were amplified used specific primer (515F-806R) with the barcode. Sequencing libraries were generated using NEB Next® Ultra™ II FS DNA PCR- free Library Prep Kit. Quality filtering on the raw tags were performed using the fastq software to obtain high-quality Clean Tags. Species annotation was performed using QIIME2 software. The row reads were deposited into NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1066055). The Nuohezhiyuan cloud platform was used for bioinformatics analysis (https://magic.novogene.com).

2.8 Statistical analysis

The experimental data were analyzed using one-way ANOVA and differences were compared using Ducan's Multiple Range Test in SPSS 26.0 software, and expressed as mean ± standard deviation (SD). The statistical significance is defined as $P < 0.05$. Graph pad Prism 8 was used to plot bar chart.

3 Results

3.1 COS had no adverse effect on the body weight of blue foxes

The effect of dietary supplement COS on body weight of blue foxes are showed in Table 3. During the 8-week feeding trial, there were no significant differences in initial body weight, final body weight and average daily gain (ADG) in COS supplement group compared with the BD group ($P > 0.05$). The results showed that supplementation of 200 mg/kg COS1 and COS2 had no adverse effects on body weight of blue foxes during growing period.

3.2 COS1 increased the development of intestinal villus morphology in blue foxes

In the duodenum, compared with the BD group, the villus height, villus width and muscular thickness in COS1 group increased the most (42.06%, 31.19% and 28.83%, respectively) ($P < 0.05$) (Figures 1A–C, F), while the VH/CD ratio increased by 30.3% in the COS2 supplement group ($P < 0.05$), suggesting a risk of hindering villi growth (Figures 1A, E). In the jejunum, compared with the BD group, the villus height was increased by 10.83% in the COS1 group, and the villus width and muscular thickness were increased in the COS1 and COS2 groups ($P < 0.05$) (Figures 1A, G, H, K). COS1 supplementation showed the highest increase incrypt depth of jejunum, and villus height, villus width, crypt depth, and muscular thickness in the ileum ($P < 0.05$) (Figures 1A, I, L–N, P). COS had no significant effects on duodenal crypt depth and VH/CD values of jejunum and ileum ($P > 0.05$) (Figures 1D, J, O). These results indicated that supplementation of 200 mg/kg COS1 was beneficial to stimulate the villus morphology and development of ileum in growing blue foxes.

3.3 COS improved intestinal barrier function parameters of blue foxes

Compared with BD group, the number of goblet cells in duodenum, jejunum and ileum in COS1 group increased by 27.71%, 23.67% and 14.97%, respectively ($P < 0.05$) (Figures 2A–D). Meanwhile, the S-IgA content in jejunum and ileum mucosa of COS1 group was increased by 71.59% and 38.56% compared with BD group, respectively ($P < 0.05$) (Figure 3A). Moreover, the jejunal mucosa of blue foxes in COS1 group showed up-regulated *Occludin* and *ZO-1* mRNA level. ($P < 0.05$) (Figures 3B, C). In the jejunal mucosa and serum, the levels of DAO and D-Lactate in COS1 group all decreased, compared with those in BD group ($P < 0.05$) (Figures 3D, E). These results suggested that COS1 supplementation has the potential to enhance intestinal barrier function in growing blue foxes.

3.4 COS promoted intestinal immune balance in blue foxes

In the jejunal mucosa, compared with the BD group, the mRNA levels of pro-inflammatory cytokines *TNF-α* and *IL-1β* in the COS1 and COS2 supplementation groups were decreased, and *TNF-α* in the COS1 group decreased the most by 79.80% ($P < 0.05$) (Figures 4A, B). Compared with the BD group, the mRNA levels of anti-inflammatory factors *IL-10* and *TGF-β1* in COS1 supplementation group increased the most, which were 72.66% and 106.32%, respectively ($P < 0.05$) (Figures 4C, D). In the jejunal mucosa, the contents of pro-inflammatory cytokines IL-2, IL-6 and TNF-α were the lowest in COS1 supplementation group, especially the content of IL-6 was decreased by 81.03% compared with BD group ($P < 0.05$) (Figure 4E). In serum, the concentrations of proinflammatory cytokines IL-6 and IL-1β were lower in the COS2 group than in the BD group ($P < 0.05$) (Figure 4F). Antibodies IgG, IgA and IgM play an important bridging role in the immune response (21). Compared with the BD group, the jejunum mucosa of IgG and IgM concentration in COS1 and COS2 supplementary groups in are increased, the serum IgA and IgM in COS1 supplementary group content increased ($P < 0.05$) (Figures 4G, H). These results proved that COS1 supplementation could promote the dynamic balance of intestinal mucosal immunity in blue foxes.

3.5 COS modulated cecum microbiota of blue foxes

Compared with the BD group, the Ace, Chao and Shannon indexes of the COS1 supplementation group were significantly increased by 266.82%, 275.06% and 20.17% ($P < 0.05$) (Figures 5A–D), indicating that COS1 supplementation increased the diversity of intestinal groups of blue foxes. The weighted_unifrac distance considered the effects of microbial

TABLE 3 Effects of chitosan oligosaccharide on growth performance of blue fox[1].

Items	BD	COS1	COS2	*P*-Value
Initial body weight/kg	6.39 ± 0.56	6.35 ± 0.60	6.35 ± 0.29	0.969
Final body weight/kg	12.11 ± 0.50	12.26 ± 0.69	12.04 ± 0.33	0.692
Average daily gain/(g/d)	95.42 ± 2.14[ab]	98,54 ± 3.50[a]	96.39 ± 2.94[b]	0.031

BD, basal diet group; COS1, basal diet supplemented with 200 ppm chitooligosaccharides (molecular weight 1.5 kDa) group. COS2, basal diet supplemented with 200 ppm chitooligosaccharides (molecular weight 3 kDa) group.
[1] Performance data represent the mean values ± standard deviation of 24 blue fox.
[a,b] Datas shows as mean values ± sd in the same row with different letters differ at $P < 0.05$.

species abundance and species on community structure composition, while the unweighted_unifrac distance only considered the effects of species. In both distance algorithms, the sample points in the COS1 group and the COS2 group are clustered separately (Figures 5E, F), indicating that there are differences in the species and abundance of microorganisms between the two groups.

At the phylum level, the gut microbiota of blue fox is mainly composed of Firmicutes and Bacteroidetes (Figure 6A). Fusobacteria and Phascolarctobacterium were enriched in BD group, while reduced in COS1 and COS2 group (Figures 6A, B). Compared with BD and COS2 group, Acidobacteriota, Chloroflexi, and Verrucomicrobiota in COS1 group were more abundant (Supplementary Figures S1, S2). At the genus level, COS1 supplementation resulted in enrichment of *Alloprevotella* classified in the phylum Bacteroides, *Bifidobacterium* classified in the phylum Actinobacteria and *Lactobacillus* classified in the phylum Firmicutes (Figure 6B). COS2 supplementation resulted in enrichment of *Prevotella* classified in the phylum Bacteroides, and *Catenibacterium*, *Megamonas*, and *Megasphaera* classified in the phylum Firmicutes (Figure 6B). In order to reveal which lactobacillus is responsive to COS1, the enrichment of *Lactobacillus* at species level in each group was further explored. The datas showed that compared with BD group, only the relative abundance of *Lactobacillus_agilis* and *Lactobacillus_murinus* in COS1 group increased by 130.84% and 134.45% ($P < 0.05$) (Figures 6C, D).

In the prediction of microbial gene function, the signaling pathway of L-ornithine biosynthesis (GLUTORN-PWY) was up-regulated in both COS1 and COS2 compared with BD group ($P < 0.05$) (Figures 6E, F). Interestingly, compared to the BD group, In COS1 group, signaling pathways such as glycolysis and Entner-

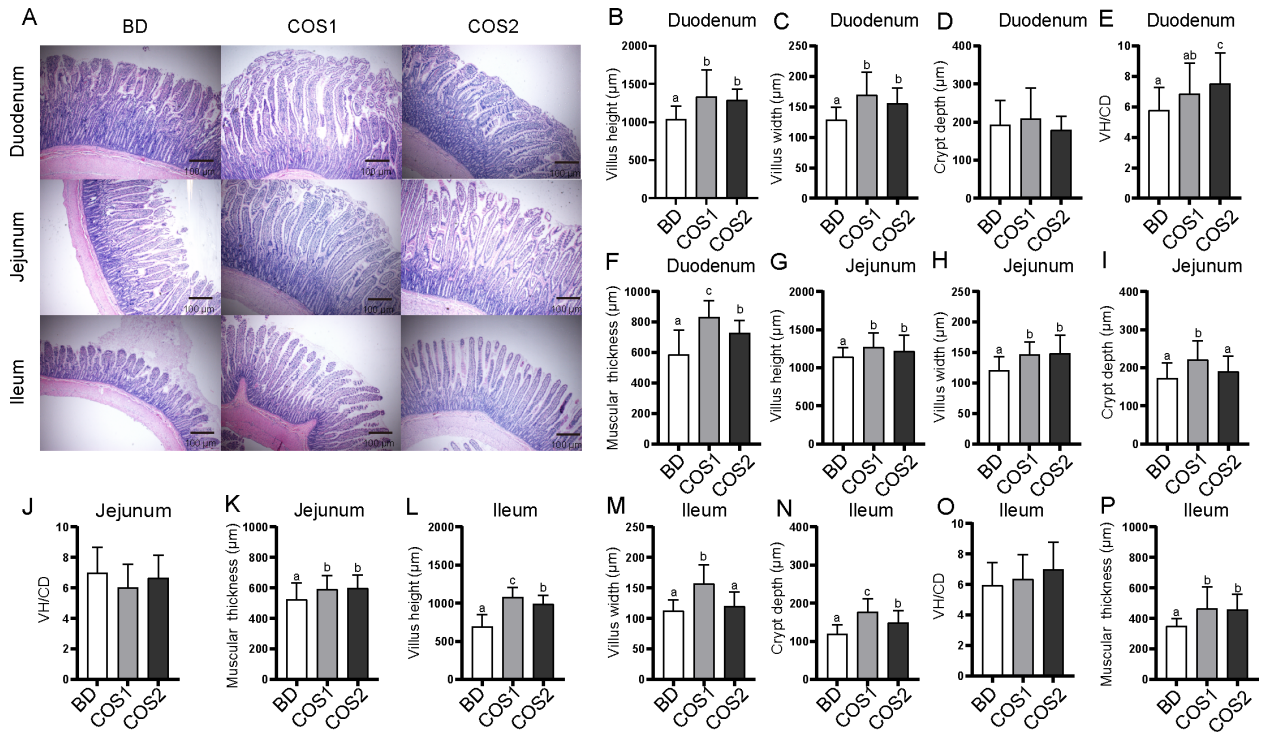

FIGURE 1
Effects of dietary COS supplementation on morphology and development of intestinal villi of blue fox during growing period. Histological examination of duodenum, jejunum, and ileum stained with H&E in BD group, COS1 group (1.5 kDa), COS2 group (3 kDa) (Light microscopy 400×, n = 5) **(A)**. Villus height (μm), villus width (μm), crypt depth (μm), villus height/crypt depth, muscular thickness (μm) in duodenum **(B–F)**, jejunum **(G–K)**, and ileum **(L–P)**. The letters a, b, and c in the bar chart represent significant differences between groups at $P < 0.05$.

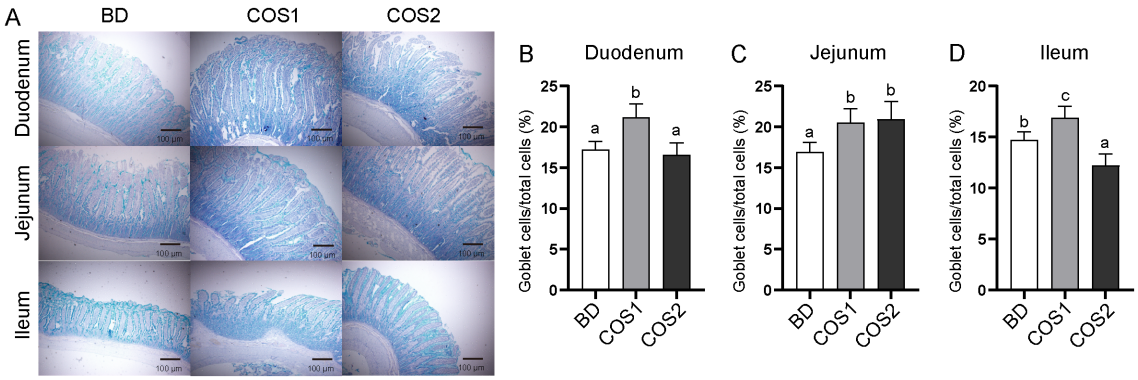

FIGURE 2
Effect of dietary COS supplementation on the number of small intestinal goblet cells in blue foxes during growing period. Representative images of Alcian staining in duodenum, jejunum, and ileum tissue to observe goblet cells (Light microscopy 400×, n = 5) **(A)**. Proportion of goblet cells in duodenum **(B)**, jejunum **(C)**, ileum **(D)**. The letters a, b, and c in the bar chart represent significant difference between groups at $P < 0.05$.

Doudoroff pathway (GLYCOLYSIS-E-D), D-galacturonate degradation I pathway (GALACTUROCAT-PWY), pentose phosphate pathway (PENTOSE P-PWY), and heterolactic fermentation pathway (P122-PWY) were upregulated ($P < 0.05$) (Figure 6E). These up-regulated pathways are related to the decomposition and utilization of polysaccharides.

3.6 Correlation between intestinal barrier factors, intestinal immune parameters, and differential bacteria

Figure 7 shows the Spearman correlation analysis between intestinal barrier factors, intestinal immune parameters and differentially enriched

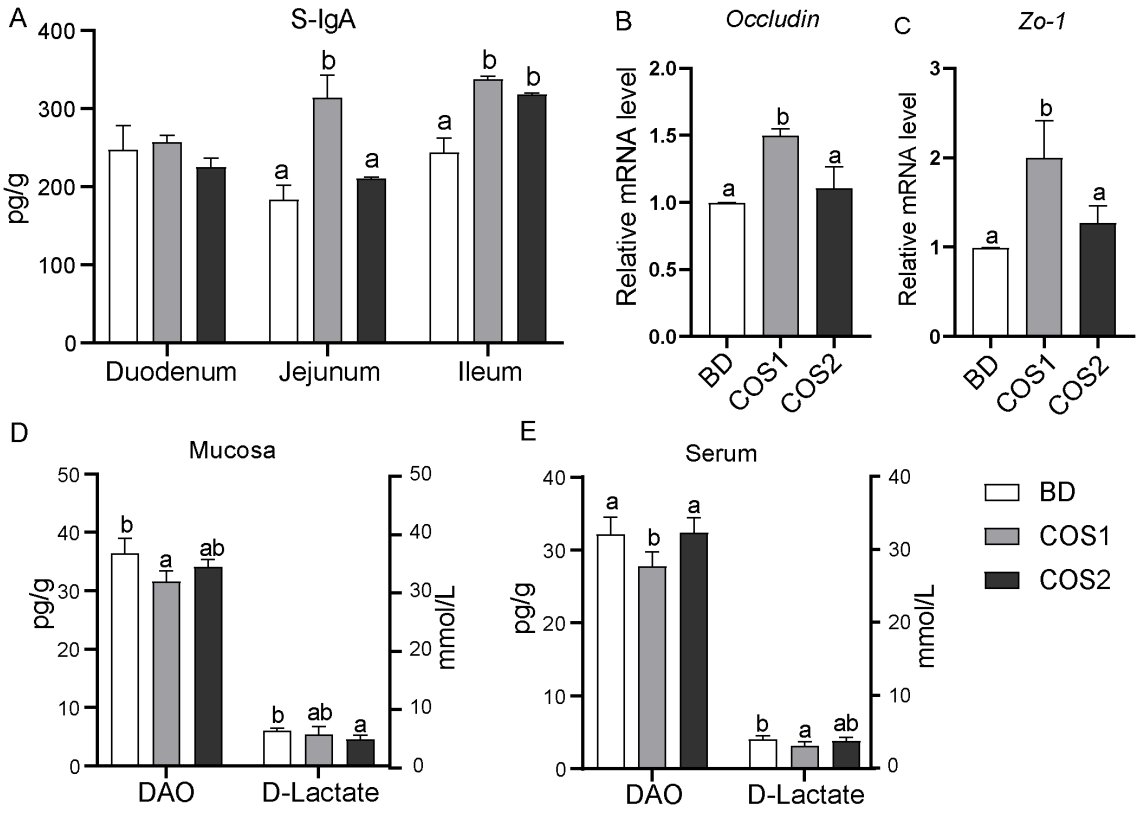

FIGURE 3
Effects of dietary COS supplementation on intestinal barrier indexes of blue foxes during growing period. Content of secretory immunoglobulin A in duodenum, jejunum, and ileum **(A)**. The relative mRNA expression levels of *Occludin* and zonula occludens 1 (*ZO-1*) in small intestinal mucosa were detected by RT-qPCR **(B, C)**. Levels of diamine oxidase and D-lactic acid in intestinal mucosa **(D)** and serum **(E)**. The letters a, b, and c in the bar chart represent significant differences between groups at $P < 0.05$.

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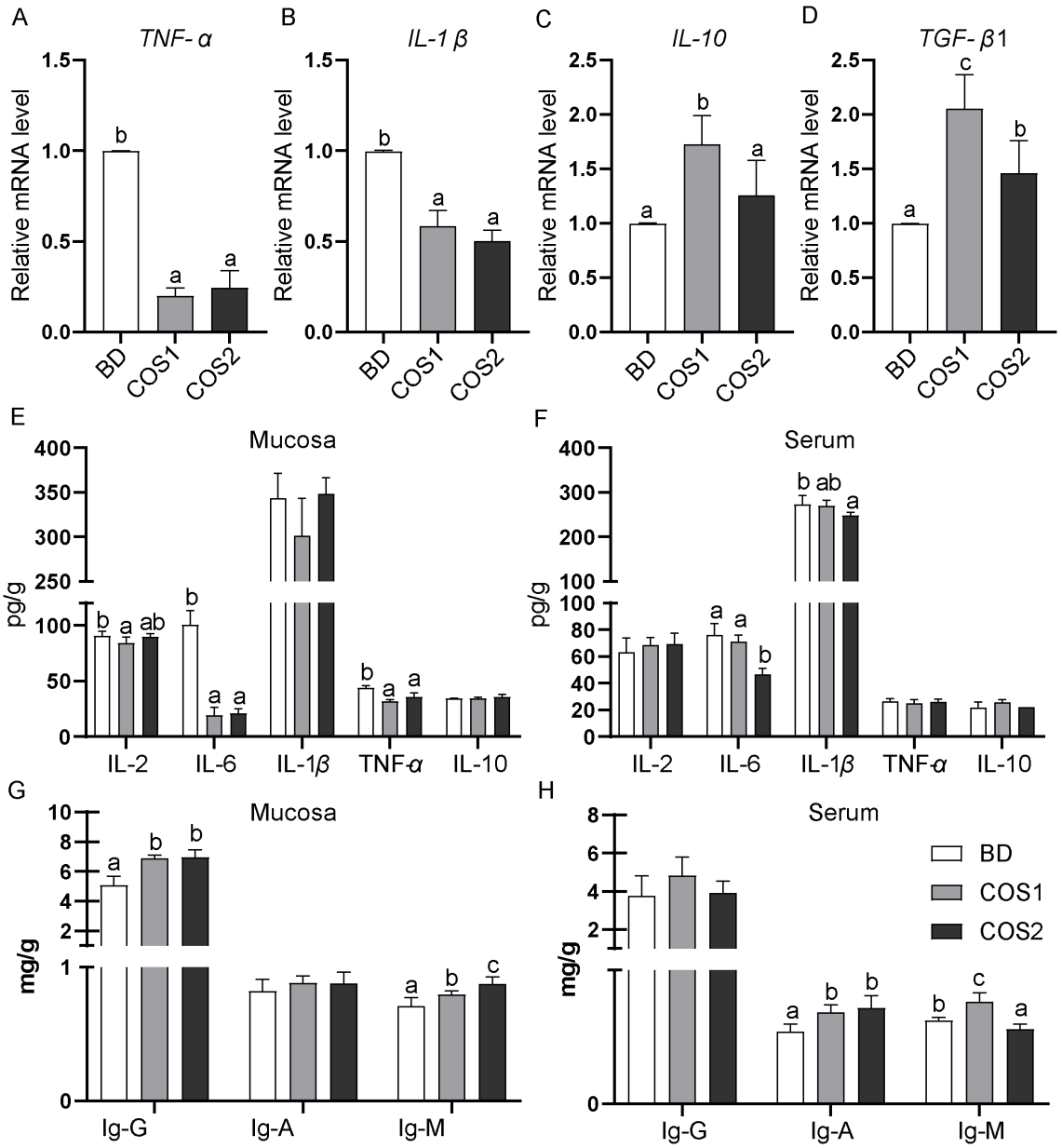

FIGURE 4
Effects of dietary COS supplementation on intestinal immunity of blue foxes during growing period. The relative mRNA expression levels of genes for inflammatory cytokines in small intestinal mucosa were detected by RT-qPCR **(A–D)**. Levels of inflammatory cytokines in intestinal mucosa and serum **(E, F)**. Contents of immunoglobulin-G, immunoglobulin-A, and immunoglobulin-M in intestinal mucosa and serum **(G, H)**. The letters a, b, and c in the bar chart represent significant differences between groups at $P < 0.05$.

bacteria in cecum. Intestinal barrier factors S-IgA, *Occludin*, and *ZO-1* showed strong positive correlation with enriched bacteria in COS1 group (especially *Lactobacillus_agilis* and *Lactobacillus_murinus*) ($R > 0.4$, $P < 0.05$), and weak positive correlation with enriched bacteria in COS2 group ($R > 0.1$, $P < 0.05$). The DAO was strongly positively correlated with the enriched *Mycoplasma*, *Parabacteroides* and *Fusobacterium* in group BD ($R > 0.4$, $P < 0.05$), and was negatively correlated with enriched bacteria in COS1 group and enriched Succivibrio and Megasphaera in COS2 group ($R < -0.3$, $P < 0.05$). The correlation between pro-inflammatory factors *TNF-α* and *IL-1β* and intestinal bacteria was similar to DAO ($P < 0.05$). The correlation between anti-inflammatory factors *IL-10* and *TGF-β1* and intestinal bacteria was similar to intestinal barrier factors ($P < 0.05$). Intestinal immunoglobulin Ig-G was strongly positively correlated with *Lactobacillus*, *Lactobacillus_murinus* and *Megasphaera* ($R > 0.4$, $P < 0.05$). To sum up, the correlation evaluation of COS1 is better than that of COS2. Therefore, we speculate that COS1 may specifically enrich beneficial bacteria, especially *Lactobacillus_agilis* and *Lactobacillus_murinus*, to protect the intestinal barrier and regulate the intestinal immune function.

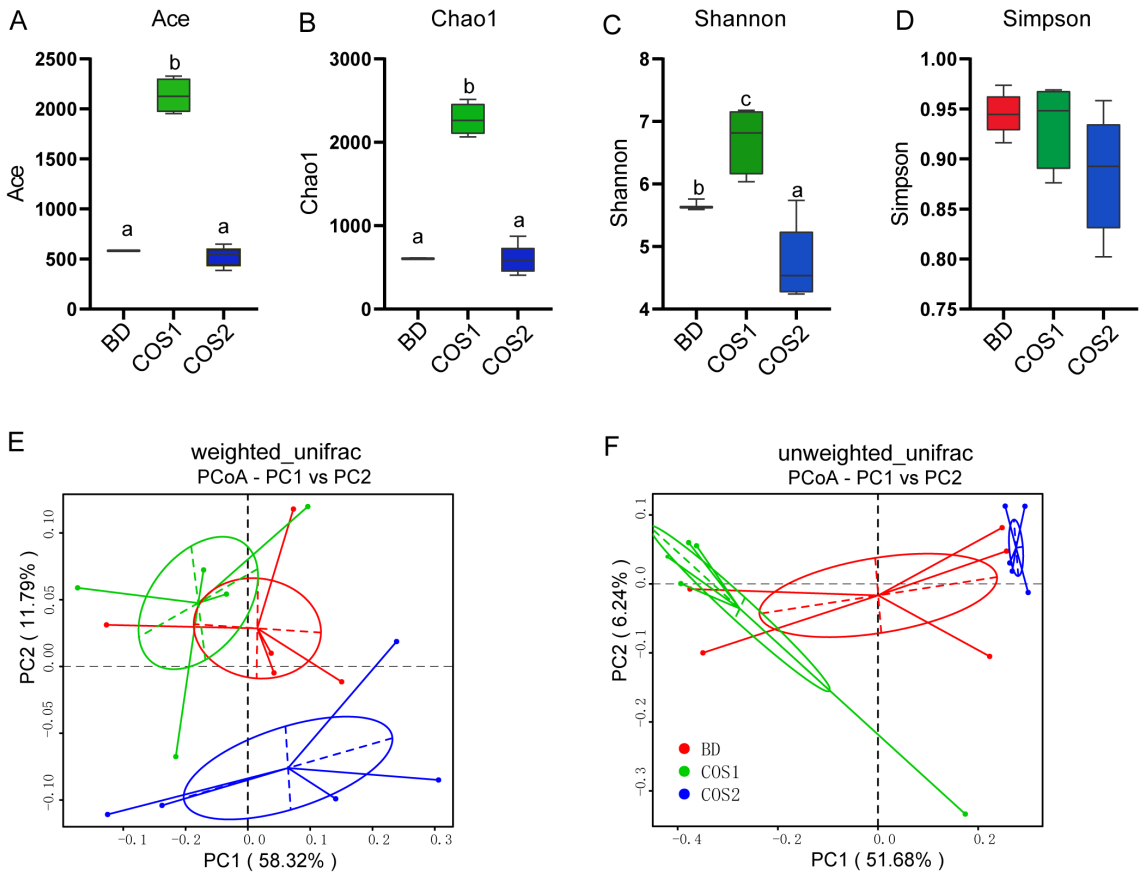

FIGURE 5
Effects of dietary COS supplementation on cecal microflora diversity in growing blue foxes. Abundance index: Ace Index **(A)** and Chaos Index **(B)**. Diversity index: Shannon index **(C)** and Simpson index **(D)**. Principal Coordinate Analysis (PCoA) analyzed community structure diversity: weighted_unifrac **(E)** and unweighted_unifrac **(F)**. The letters a, b, and c in the bar chart represent significant differences between groups at $P < 0.05$.

4 Discussions

Host intestinal health is closely related to intestinal mucosal immunity and intestinal microecology. Chitooligosaccharides (COS) as potential prebiotics can improve the balance of intestinal flora and mediate intestinal immunity (22). However, studies on COS in intestinal immunity and microbial regulation of blue foxes are still limited. Hence, in the present study, diets containing two molecular weights of COS1 (1.5 kDa) and COS2 (3 kDa) were fed to 125-day-old blue foxes for 8 weeks. This study found that short-term feeding COS had no adverse effect on the body weight of blue foxes. Compared with COS2, COS1 has a better effect on promoting intestinal villi development, intestinal mucosal immunity, maintaining inflammatory homeostasis and regulating intestinal microecology. These results demonstrate that COS1 can regulates intestinal mucosal immunity and intestinal microbiota of blue foxes to promoted intestinal health.

The blue fox is an important fur economic animal, and the healthy blue fox shows bright fur plate and dense texture (23). In this study, COS had no effect on the body weight of blue foxes. However, during our experiment, we observed that COS-supplemented blue foxes had smoother and lustrous hair. Whether COS supplementation promotes growth performance in pigs at different growth stages varies depending on the dose, molecular weight of COS, and duration of supplementation (24–26). These results indicated that COS may promote the absorption of nutrients in the fur development of blue foxes, rather than reflected in the weight gain.

COS increased the morphological characteristics of small intestinal villus. The morphological integrity of villi greatly influences the digestion and absorption capacity of the small intestine (27). The morphological characteristics include villus height, villus width, crypt depth and muscular thickness, which mapped nutrient absorption, intestinal barrier function and immunity (28–30). In this study, dietary COS supplementation had the greatest effect on intestinal villi height, villi width and muscular thickness. Consist with our results, Ruixia Lan and Youssef, Islam M. reported that COS increased the ileal villus height and decreased the crypt depth in broilers and laying hens, respectively (31, 32). Similar results were also reported by Dingfu Xiao, who indicated that COS enhanced the villus height/crypt depth in weaned piglets (33). Our results further explore a role of COS in stimulating the morphology of the ileum of blue foxes during growing period.

COS1 had positive effects on mucosal and epithelial barrier function of blue foxes. Goblet cells continuously secrete mucus into the intestinal lumen to remove and block contact with harmful

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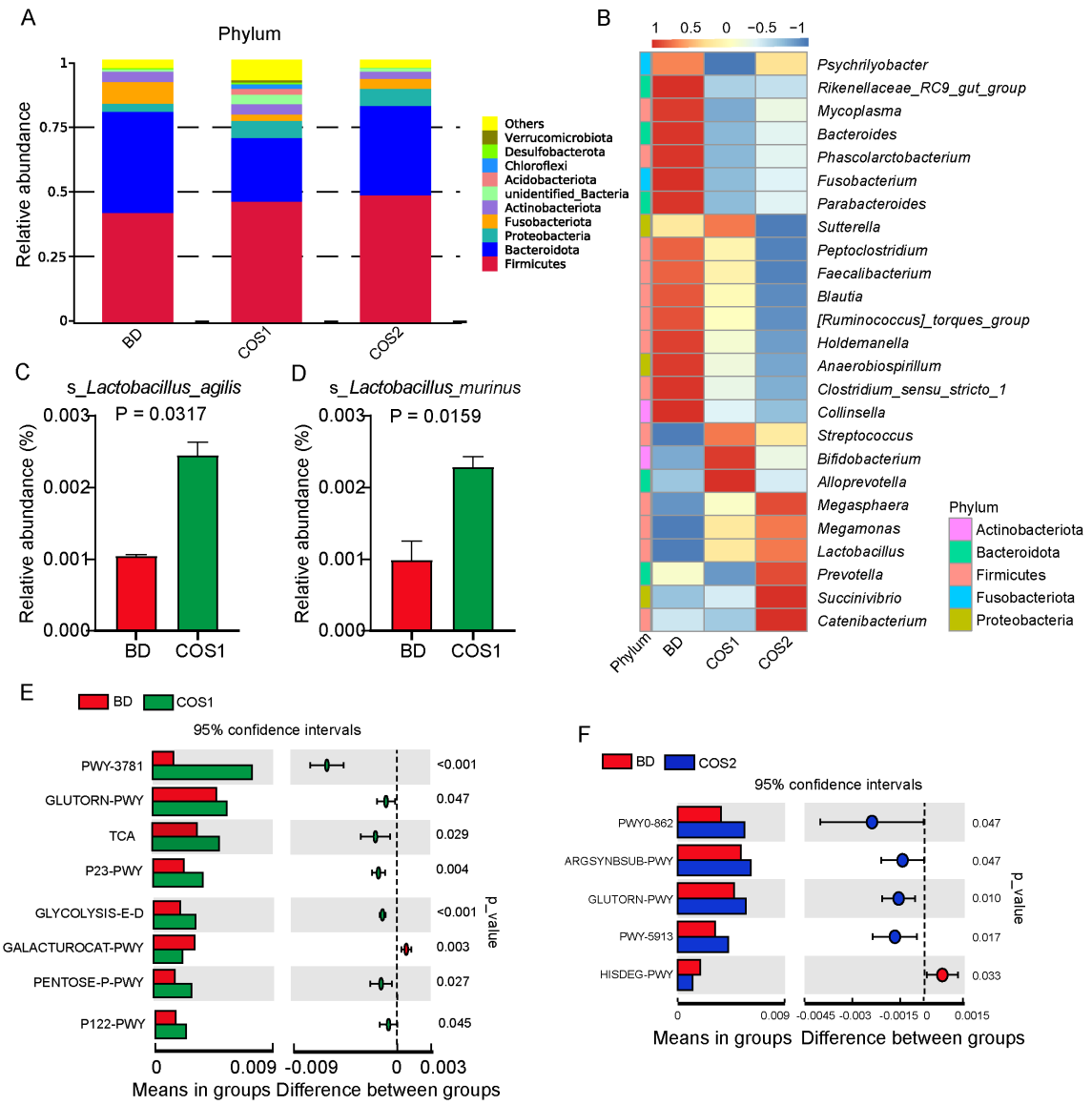

FIGURE 6
Effects of dietary COS supplementation on cecal microflora composition of blue foxes during growing period. Relative abundance histogram at the Phylum level **(A)**. Relative abundance chart at the Genus level **(B)**. The column of *Lactobacillus* with significant difference at Species level ($P < 0.05$) **(C, D)**. Differential enrichment analysis of microbial gene function pathway prediction: BD vs COS1 **(E)**, and BD vs COS2 **(F)**.

bacteria (34). S-IgA distributed in the mucous layer interferes with pathogen adherence or penetration of the mucosal barrier through antibody-dependent specific immunity and glycan-dependent innate immunity (35). In this study, COS1 supplementation increased the number of small intestinal goblet cells and S-IgA content in jejunum and ileum mucosa. These results are consistent with findings for other species, which supplementation COS (1-2.2 kDa) can increase the goblet cells number of piglets and activate the secretory pathway of S-IgA and enhance the intestinal mucosal immunity of mice (33, 36). Tight junctions are important factors for the integrity of the epithelial barrier, and Occludin and ZO-1 are key proteins in intestinal tight junctions (37). Our results was consistent with a previous finding that dietary COS1 supplementation increased the expression of tight junction in the jejunum of LPS-challenged mice (38).D-lactic acid is a byproduct of bacterial metabolism and occurs only in human micromolar concentrations under normal conditions (39). Diamine oxidase (DAO) is an intracellular enzyme at the tip of the intestinal villi in mammals (40). When the intestinal mucosal barrier is damaged, the release of both is increased and enters the bloodstream. In this study, we demonstrated that COS1 supplementation reduced DAO and D-LA levels, suggesting that COS1 can reduce intestinal permeability in blue foxes. Taken together, these results indicated that COS1 improved the intestinal barrier function in growing blue foxes.

COS regulated the homeostasis of inflammation in blue foxes. Lipid overload associated with obesity induces low-grade tissue inflammation in the body (41). The blue fox belongs to the carnivorous canid family, and the fat in its diet is its main source of energy, and the fat supply in the growing period is about 25-40% (42). Previous studies have shown that COS supplementation

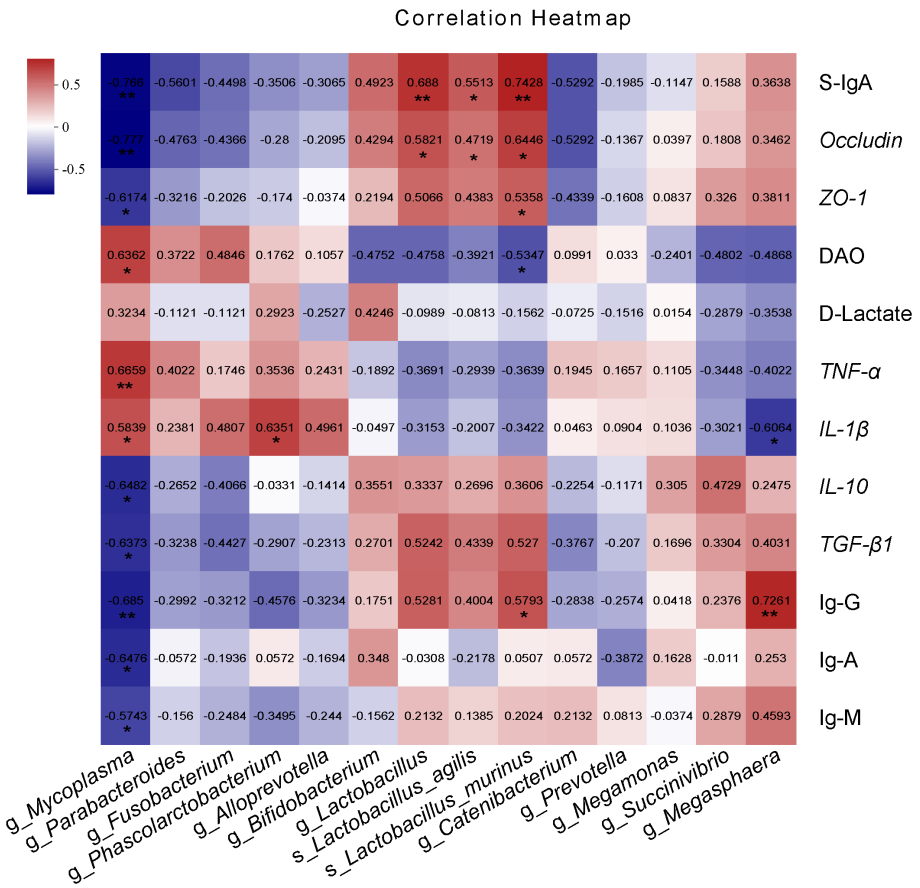

FIGURE 7
Spearman correlation analysis of intestinal barrier factors and intestinal immune parameters with differentially enriched cecum microbiota. The red grid indicates a positive correlation; The blue grid shows the negative correlation. The numbers in the grid represent the correlation coefficient. The "*" "**"represent significant differences between groups at $P < 0.05$, $P < 0.01$.

alleviated chronic inflammation induced by LPS (43). Moreover, dietary COS supplementation significantly reduced the mRNA expression levels and cytokine contents of inflammatory factors TNF-α, IL-β and IL-6 in host tissues (44, 45). Consistent with the results of previous studies, in this study, we also found that COS can reduce the level of pro-inflammatory factors in the intestinal mucosa of blue foxes (46), and increased the level of anti-inflammatory factors (such as IL-10), so as to maintain the dynamic balance of intestinal immunity of blue foxes (47). IgG, IgA and IgM can cooperate with each other to mediate intestinal immune adaptation and immune defense against pathogens, and contribute to intestinal homeostasis (48). In this study, COS1 and COS2 supplementations both increased the immunoglobulin levels. These results indicate that COS may regulate intestinal immunity in growing blue foxes by regulating inflammatory balance and immunoglobulin levels.

The rich diversity of intestinal flora lead to functional redundancy, which could maintain the normal operation of intestinal microbiota when the external environment is disturbed (49). In this study, COS1 supplementation increased the richness index and diversity index of intestinal bacteria in growing blue foxes, which was consistent with Huang et al. 's study in mice (50). In addition, COS1 and COS2 supplementation both reduced the abundance of Fusobacteria and Phascolarctobacterium. Fusobacteria was intestinal commensal bacteria and opportunistic pathogens, and their redundancy was associated with intestinal infections (51). Further studies have found that *Fusobacterium nucleatum* secretes antagonistic substances to repel probiotics (such as *Bifidobacterium*), resulting in microbiota dysbiosis (52). Phascolarctobacterium was accompanied by the development of obesity in the dog (53), moreover, in obese mice models, it was strongly positively correlated with TC and LDL-C in serum and TG and MDA in liver (54). In this study, the supplementation of COS1 enriched the abundance of Acidobacteriota, Chloroflexi, and Verrucomicrobiota. Acidobacteriota can effectively degrade cellulose, hemicellulose and xylan in nature (55). Verrucomicrobia is an abundant and highly specialized degrader of fucosan and other complex polysaccharides (56). In this study, COS1 enriched *Alloprevotella*, *Bifidobacterium* and *Lactobacillus*. Alloprevotella classified to Bacteroides, are typical dietary fiber degrading bacteria (57). This is consistent with the enrichment of glycolysis and Entner-Doudoroff pathway (GLYCOLYSIS-E-D), D-galacturonate degradation I pathway (GALACTUROCAT-PWY), and pentose phosphate pathway (PENTOSE P-PWY) in microbial function prediction after COS1 supplementation in this study. The enrichment of *Bifidobacterium* could regulate the expression of mucin proteins and immune homeostasis (58, 59). Moreover, *Bifidobacterium* can adhere to dietary glycans such as chitin (60), and dehydrolyze oligosaccharides by expressing a variety of extracellular and cytosolic glycosyl hydrolases

(GHs) (61). COS can stimulate the cell density of Lactobacillus more than 4 times, and the mode of promoting the growth of lactobacillus was the same as that of cellobiose (62, 63). These data indicate that COS1 supplementation in the blue fox diet during the growing period may play a role in regulating the intestinal microecological balance through the enrichment of dietary fiber-degrading bacteria. In addition, in this study, both COS1 and COS2 supplementation enriched the pathway of L-ornithine biosynthesis (GLUTORN-PWY). L-ornithine is an amino acid in the liver responsible for the urea cycle, which can convert toxic ammonia into urea, thereby protecting the health of the viscera (64). Therefore, we hypothesized that COS1 supplementation can increase the degradation efficiency of dietary fiber in feed and improve the intestinal immune of blue foxes.

In this study, the concentration of Mycoplasma in the basal diet was negatively correlated with the intestinal barrier index and positively correlated with the imbalance of inflammation. The enrichment of Mycoplasma is a manifestation of intestinal flora disorder (65). Importantly, *L.agilis* and *L.murinus* enriched in COS1 group and showed a strong positive relationship with intestinal barrier indexes and the balance of inflammatory factors. Intestinal endogenous Lactobacillus is generally distributed on the surface of epithelial cells (66). Studies have shown that 84 and 53 carbohydrate-related enzymes, including helper activity (AAs), glycosyltransferase (GTs), carbohydrate esterase (CEs), and glycoside hydrolase (GH), have been annotated by *L. agilis* and *L. murinus* as potential probiotics. They all showed positive effects on intestinal protection in colitis mice (67, 68), which responded to the enrichment of carbohydrate catabolic pathways in COS1 group as predicted by microbial function. Within 2 weeks of caloric restriction diet, lactic acid bacteria microbial community dominated by *L. murinus_147* rapidly enriched in mice, and this strain reduced intestinal permeability and the level of systemic inflammatory markers, such as TNF-α (69), similar to the phenomenon in this study. Further studies have shown that *L. murinus* plays an important role in promoting Treg cell development, inhibiting the development of colitis in mice, and maintaining intestinal immune homeostasis (70). In addition, extracellular vesicles secreted by L. murinus contribute to the polarization of M2 macrophages and the secretion of anti-inflammatory cytokine IL-10 (71). In this study, Succivibrio and Megasphaera enriched in COS2 group also had a certain positive relationship with intestinal barrier and inflammation balance of blue foxes. Succivibrio specializes in the digestion of complex carbohydrates and is a major propionate producer, which is related to feed efficiency (72). Megasphaera, as a probiotic, can ferment lactic acid and mainly produce propionate and valerate, which is related to the improvement of production performance of ruminants (73, 74). The above data explained why the overall supplementation effect of COS1 was better than that of COS2, which may be due to the cascade reaction of intestinal bacteria in response to oligosaccharides with different molecular weights.

In conclusion, dietary COS can improve intestinal morphology and barrier function of growing blue foxes by improving inflammation and immune levels. Our study confirms the strong prebiotic properties of COS in blue fox breeding. The change of COS on the enrichment of different flora in cecum may be the main reason for promoting intestinal health of blue foxes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1066055.

Ethics statement

The animal study was approved by Chinese Academy of Agricultural Sciences Animal Care and Use Committee, and the Institute of Special Animal and Plant Sciences Wild Animal and Plant Subcommittee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JW: Data curation, Software, Writing – original draft, Writing – review & editing. JS: Formal analysis, Writing – review & editing. GW: Writing – review & editing. WL: Formal analysis, Writing – review & editing. ZW: Writing – review & editing. HL: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

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SUPPLEMENTARY FIGURE 1
Differential enrichment analysis of dietary COS supplementation on phyla level of cecal microflora in growing blue foxes: BD vs COS1.

SUPPLEMENTARY FIGURE 2
Differential enrichment analysis of dietary COS supplementation on phyla level of cecal microflora in growing blue foxes: COS1 vs COS2.

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Vitamin, antioxidant and micronutrient supplementation and the risk of developing incident autoimmune diseases: a systematic review and meta-analysis

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Background: Autoimmune diseases pose significant health challenges worldwide and affect millions. In recent years, there has been growing interest in exploring preventive strategies through nutritional interventions using vitamins, antioxidants, and micronutrients to reduce the risk of developing autoimmune diseases. However, excessive supplementation has also been associated with toxicity.

Objective: We aim to assess how the intake of vitamins, antioxidants and micronutrients affect the risk of developing autoimmune diseases.

Methods: This PRISMA-adherent systematic review involved a systematic search of PubMed, Embase and Cochrane for controlled studies that evaluated the risk of incident autoimmune diseases after supplementation. Random effects meta-analyses were used for primary analysis.

Results: 18 studies were included. Overall meta-analyses observed that vitamin D did not influence the risk of autoimmune diseases (RR=0.99, 95%CI: 0.81-1.20). However, among the different vitamin D dosages, subgroup analysis demonstrated that those who were supplemented with 600-800IU/day may have a statistically significant reduction in risk (RR=0.55, 95%CI: 0.38; 0.82). Systematic review suggested that consumption of most vitamins, micronutrients and antioxidants may not have any effect on the risk of autoimmune diseases. Smoking, age, physical or outdoor activity and diet were significant confounding factors that affected the efficacy of such interventions.

Conclusion: We studied the effect of various vitamins, micronutrients and antioxidants on the risk of developing autoimmune diseases. Our study

contributes to the evolving landscape of nutritional immunology, providing a foundation for future research to unravel more definite relationships with supplementation and the development of incident autoimmune diseases.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42024504796.

KEYWORDS

autoimmune diseases, immunology, multiple sclerosis, nutrition, rheumatoid arthritis, systemic lupus erythematosus

1 Introduction

Autoimmune diseases represent a diverse group of conditions characterised by the immune system's aberrant response against the body's own antigens (1) due to loss of immune tolerance (2). These diseases pose significant health challenges worldwide, impacting millions (2, 3). In 2019, the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) estimated the global incidence of autoimmune diseases to be 67 million (4). Women are especially susceptible to autoimmune diseases, making up approximately 80% of the disease population (5, 6).

Traditional methods used to treat autoimmune diseases mainly consist of immunosuppressive medications that dampen the body's immune responses against self (7). Despite these treatments, the prevalence of autoimmune and inflammatory diseases continues to rise. Studies estimate that the annual increases in the overall global incidence and prevalence of autoimmune diseases stand at 19.1% and 12.5%, respectively (8). Currently, no therapy can modulate or reprogram the development of any autoimmune diseases. It can be assumed that the current state of therapies is symptomatic, blocking inflammatory cytokines but leaving the prevention of incident autoimmune diseases largely untouched. Nonetheless, risk factors for autoimmune diseases have further been identified, including genetic factors (9) and lifestyle choices like smoking, stress (10) and physical activity (11).

This rise in incidence and prevalence of autoimmunity necessitates a deeper understanding of modifiable factors that may influence autoimmune disease risk. In recent years, there has been a growing interest in exploring preventive strategies beyond conventional treatments, through improved understanding of the immunological underpinnings of autoimmunity (12, 13). Emerging research suggests that nutritional interventions, specifically through supplementation, could have a pivotal role in altering the risk of developing autoimmune conditions (14, 15). Vitamins, in particular vitamin D (16), and antioxidants such as omega-3 fatty acids, have garnered attention for their potential anti-inflammatory effects (17). They are also postulated to have modulatory effects on the immune system (18). Research has shown that the vitamin D receptor is expressed on multiple cells in the immune system, such as monocytes, dendritic cells and activated T-cells (19). Studies have shown that this binding of vitamin D to its receptor on immune cells can inhibit pro-inflammatory activity by inhibiting T-lymphocyte proliferation and is hence associated with a decrease in pro-inflammatory cytokines interleukin 2 (IL-2) (20) and interferon gamma (IFN-gamma) (21). Vitamin D deficiency has been observed in patients with autoimmune diseases such as rheumatoid arthritis, Sjogren's syndrome, systemic lupus erythematous and systemic sclerosis (22). Furthermore, low levels of vitamin D in these patients have been associated with poorer disease outcome and course (23, 24). On the other hand, excessive intake of vitamin supplements has also been associated with potential toxicity (25).

Dietary patterns rich in antioxidants such as vitamins are also believed to improve overall health and decrease oxidative stress, preventing disease (26). The Mediterranean Diet is one example, focusing on a healthy diet consisting of whole grains, fruits, vegetables, seafood and nuts. This diet has been postulated to have anti-inflammatory effects on the human body, potentially reducing the risk of autoimmune diseases (27). Significantly, a study conducted by Skoldstam et al. found that in a population of patients diagnosed with rheumatoid arthritis, intervention with the Mediterranean Diet obtained a substantive reduction in inflammatory activity, resulting in increased mobility and hence better quality-of-life overall (28). Obtaining a more comprehensive understanding of the components in such diets that can alter immune system activity will be crucial to our knowledge of the dietary factors that can modify the risk of developing autoimmune diseases.

As individuals increasingly turn to complementary approaches for health maintenance (29), using vitamins, antioxidants or micronutrients supplementation, it becomes imperative to rigorously assess the collective evidence supporting the potential risk of certain dietary supplements on the development of incident autoimmune diseases. We aim to consolidate existing literature to assess how the intake of vitamins, antioxidants and micronutrients affect the risk of developing autoimmune diseases.

2 Methods

We report our systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Our protocol was registed on PROSPERO (CRD42024504796).

2.1 Search strategy

Literature search was performed in PubMed, Embase, and Cochrane. Our search strategy combined terms for vitamins, antioxidants, micronutrients, and autoimmune diseases. The database-controlled vocabulary was used to search subject headings. A spectrum of synonyms with appropriate truncations was used to search title, abstract, and author keywords. The search strategy was translated between the databases. Examples of the search strategies for PubMed and EMBASE are available in Supplementary Table S1.

2.2 Inclusion and exclusion criteria

Two reviewers independently screened titles and abstracts of all studies for eligibility. The full text of studies assessed as 'relevant' or 'unclear' was then independently assessed by a third reviewer. All peer-reviewed English-language studies published since 2000 that evaluated the risk of autoimmune diseases following the supplementation of vitamins, antioxidants or micronutrients were included. Non-empirical studies, grey literature, studies without a control-arm and abstracts were excluded. The selection process is shown in Figure 1.

2.3 Data extraction

Two reviewers independently performed the extraction with quality checking performed at the end. Subject matter information included the aim of the study, demographics, and characteristics of control group, and main findings of the study.

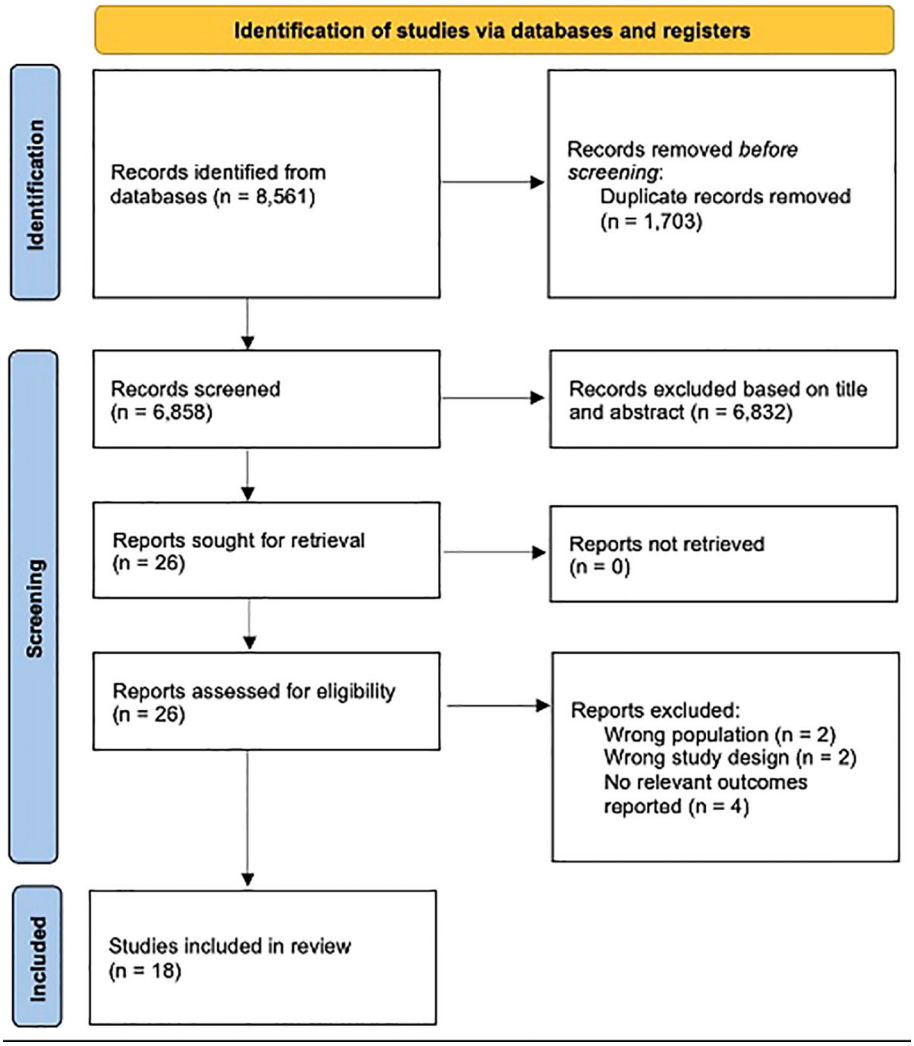

FIGURE 1
PRISMA flowchart.

2.4 Statistical analysis

We conducted all analyses on R (version 4.1.0) using the *meta* and *metafor* packages. A two-sided P value of <0.05 was considered as statistically significant unless specified. Studies were pooled for meta-analyses using the relative risk of the autoimmune disease [measured using risk ratios (RR) compared to controls]. Sensitivity analysis was conducted using identification and exclusion of potential outliers and the leave-one-out analysis. Between-study heterogeneity was represented using I2 and τ2 statistics. I2 of <30% demonstrated low heterogeneity between studies, 30% to 60% revealed moderate heterogeneity, and >60% showed substantial heterogeneity (30). We performed subgroup analyses and meta-regression to determine if any key categorical and hierarchical variables influenced the results. We assessed for publication bias quantitatively using Egger's test. Visual inspection for funnel plot asymmetry was used for qualitative publication bias. If we suspected publication bias, sensitivity analysis was conducted using the trim-and-fill method (R0 estimator, fixed-random effects models) to estimate the pooled effect size after imputing potential studies (31). If publication bias was absent, this assumes a normal distribution of effect sizes around the center of the funnel plot (32).

2.5 Risk of bias

Two independent reviewers assessed for the methodological quality and risk of bias of the included studies using the Joanna Brigg's Institute (JBI) Critical Appraisal tool (33). Any discrepancies were resolved by a third reviewer.

3 Results

3.1 Characteristics of included studies

A total of 18 studies (17, 34–50) were included from 8,561 records (Figure 1). The remaining 8,543 studies were excluded after removing irrelevant studies with the wrong study design, population, outcomes, and the duplicates. Eight studies reported on multiple sclerosis (34–36, 38, 40, 43, 46, 47), eight studies reported on rheumatic arthritis (39, 41, 42, 44, 45, 48–50), two studies reported on systemic lupus erythematosus (48, 49) and two studies did not report the specific autoimmune disease (17, 37). The exposures included vitamin A (38, 41, 48), B (38, 41, 44, 46), C (38, 39, 41, 46, 48), D (17, 34–36, 38, 40–42, 44, 45, 49), E (37, 39, 41, 44, 48), multivitamins (41, 46), iron (38, 40, 41, 44, 46), calcium (38, 41, 45, 47), omega-3 (17, 41, 46), zinc (38, 39, 41, 44, 47), dietary antioxidants (38, 50). Among 945,471 participants, 4,591 patients developed autoimmune diseases. Main characteristics of the included studies can be found in Table 1.

Six studies (17, 34, 35, 40, 41, 45) were included to evaluate the RR of autoimmune diseases amongst those with vitamin D supplementation. Meta-analysis of the six studies (Figure 2) indicated that vitamin D supplementation does not seem to affect the risk of autoimmune diseases (RR=0.99, 95%CI: 0.81-1.20). Subgroup analyses showed that those who consumed both dietary sources and supplementary vitamin D may have significant risks of developing autoimmune diseases (RR=1.26, 95%CI: 1.00-1.58) as compared to those who consumed supplementary vitamin D alone (RR=0.85, 95%CI: 0.73-0.99) (p<0.01) (Table 2). Subgroup analyses showed that the type of autoimmune disease did not significantly affect the risk of developing autoimmune diseases after consumption of supplementary vitamin D (RR=0.99, 95%CI: 0.81-1.20) (Figure 3). Other categorical variables such as country of study, control type, gender, age, study design, and smoking were also not found to significantly reduce the risk of autoimmune diseases (Table 2). Meta-regression was also insignificant (Supplementary Table S2).

Four studies (17, 35, 40, 45) evaluated the RR of autoimmune diseases based on the dosage of vitamin D supplementation. After stratification by dosage, the individual cohorts were reported separately by the studies. Meta-analysis of the 11 cohorts (Figure 4) indicated that the dosage of vitamin D supplementation may reduce the risk of autoimmune diseases, although borderline statistically insignificant (RR=0.88, 95%CI: 0.77-1.00). Subgroup analysis (Supplementary Table S3) of the dosage of vitamin D supplementation suggested a statistically significant reduction in risk of autoimmune diseases among those who were supplemented with vitamin D dosages of 600-800IU/day (RR=0.55, 95%CI: 0.38; 0.82). Subgroup analyses of other categorical variables were not performed due to the possibility of type 1 error.

3.2 Other vitamins, antioxidants or micronutrients

Meta-analyses of supplementation with other vitamins, antioxidants or micronutrients can be found in Supplementary Table S4 and revealed that supplementation with vitamin C, B, multivitamin, iron, omega-3 demonstrated a statistically insignificant decrease in risk of autoimmune diseases. Only supplementation with vitamin E demonstrated a statistically insignificant increase in risk of autoimmune disease (RR=1.17, 95%CI: 0.65; 2.10).

Due to the limited number of studies of each vitamin, antioxidant and micronutrient, systematic review was additionally performed and reported (Supplementary Tables S5, S6).

3.3 Multivitamins and vitamin A, B, C, E

Three studies reported that vitamin A had none (41, 48) to significant reduction (38) in autoimmune diseases. Vitamin A supplementation was shown to reduce risk of multiple sclerosis (38) but not in rheumatoid arthritis (41, 48).

Four studies reported that vitamin B had none (41, 44) to significant reduction (38, 46) in autoimmune diseases. Both Rezaeimanesh et al. (46) and Abdollahpour et al. (38) demonstrated reduction in risk of developing multiple sclerosis as an adult after consuming vitamin B during adolescence while the other two studies investigated the incidence in older adults.

TABLE 1 Overall characteristics.

Author	Publication year	Region of study	Gender male (%)	Mean age (SD)	Characteristics of the controls	Proportion of smoking (%)	Autoimmune disease	Exposures	Number at risk (exposed)	Number of events (exposed)	Number at risk (control)	Number of events (control)
Dehghan (34)	2018	Iran	23.5	30.9 (3.51)	Matched healthy	14.2	Multiple Sclerosis	Vit D	120	46	360	98
Butzkueven (35)	2023	Australia	28.6	37.0 (10.3)	Placebo	14.6	Multiple Sclerosis	Vit D	49	25	50	27
Cavalla (36)	2022	Italy	29.0	35.4 (9.36)	Matched healthy	NR	Multiple Sclerosis	Vit D	83	NA	83	NA
Hahn (17)	2021	USA	49.4	67.1 (7.1)	Placebo	7.2	All	Vit D, Omega-3	12927	123	12944	155
Karlson (37)	2008	USA	0.0	54.6 (7.0)	Placebo	49.0	All	Vit E	19576	50	19568	56
Abdollahpour (38)	2022	Iran	41.1	30.8 (8.2)	Matched healthy	NR	Multiple Sclerosis	Vit D, Vit A, Vit B6, Vit C, Calcium, Iron, Zinc, Dietary Antioxidant Index	547	NA	1057	NA
Cerhan (39)	2003	USA	0.0	61.4 (NR)	Nil	33.0	Rheumatoid Arthritis	Vit C, Vit E, Zinc	29368	152	NA	NA
Cortese (40)	2015	Norway	27.9	45.6 (10.7	Matched healthy	52.0	Multiple Sclerosis	Vit D, Iron	953	79	1717	160
Kronzer (41)	2022	USA	30.0	64.0 (NR)	Matched healthy	44.0	Rheumatoid Arthritis	Vit D, Vit A, Vit B, Vit C, Vit E, multivitamin, Calcium, Omega-3, Iron, Zinc	212	159	636	482
Merlino (42)	2004	USA	0.0	61.5 (4.2)	Nil	33.0	Rheumatoid Arthritis	Vit D	29368	152	NA	NA
Munger (43)	2011	USA	0.0	39.9 (NR)	Matched healthy	46.0	Multiple Sclerosis	Vit D	121024	33	121024	318
Pedersen (44)	2005	Denmark	48.0	57.0 (7.0)	Nil	NR	Rheumatoid Arthritis	Vit D, Vit E, Vit B, Iron, Zinc	56691	69	NA	NA
Racovan (45)	2011	USA	0.0	62.3 (6.92)	Placebo	7.5	Rheumatoid Arthritis	Vit D, Calcium	16238	83	16197	80
Rezaeimanesh (46)	2021	Iran	22.0	37.2 (10.5)	Matched healthy	NR	Multiple Sclerosis	Vit B12, Vit C, Omega-3, Multivitamin, Iron	143	2	400	51
Cortese (47)	2019	Iran	0.0	NR	Nil	NR	Multiple Sclerosis	Zinc, Calcium	175431	479	NA	NA
Costenbader (48)	2010	USA	0.0	NR	Nil	NR	Rheumatoid Arthritis and Systemic Lupus Erythematosus	Vit A, Vit C, Vit E	184643	737	NA	NA
Hiraki (49)	2010	USA	0.0	NR	Nil	NR	Rheumatoid Arthritis and Systemic Lupus Erythematosus	Vit D	119173	976	NA	NA
Moradi (50)	2022	Iran	21.9	43.7 (11.46)	Matched Healthy	7.1	Rheumatoid Arthritis	Dietary Antioxidant index	100	NA	197	NA

NR, Not reported; NA, Not available.

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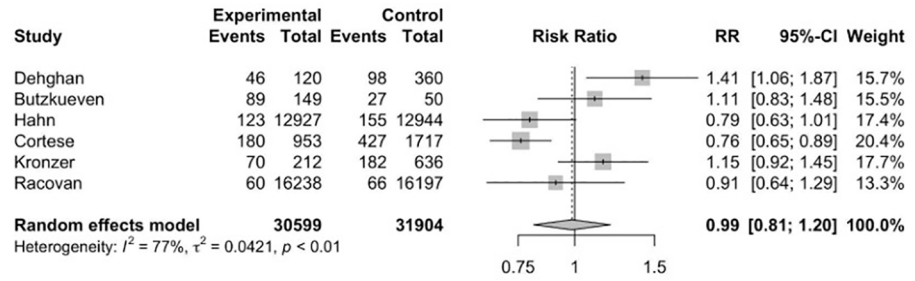

FIGURE 2
Relative risk ratio of Vitamin D supplementation and autoimmune diseases. RR, risk ratio; CI, confidence interval.

TABLE 2 Subgroup meta-analyses of vitamin D supplementation and relative risk ratio of autoimmune disorders using the random effect model.

Variable	Cohorts	Number at risk (Exposed)	Number at risk (Controls)	Risk Ratio	95% CI	I2	Test of interaction (p-value)
Overall	6	30599	31904	0.99	0.81; 1.20	77%	NA
Control = Matched	3	1285	2713	1.05	0.79; 1.39	89%	0.55
Control = Placebo	3	29314	29191	0.92	0.68; 1.25	34%	
Country = Iran	1	120	360	1.41	1.06;1.87	NA	0.12
Country = Australia	1	149	50	1.11	0.83;1.48	NA	
Country = USA	3	29377	29777	0.95	0.75;1.19	61.0%	
Country = Norway	1	953	1717	0.76	0.65;0.89	NA	
Gender = <25%	2	16358	16557	1.15	0.80;1.65	73.0%	0.32
Gender = ≥25%	4	14241	15347	0.93	0.73;1.17	75.0%	
Design = Case control	3	1285	2713	1.05	0.79;1.39	89.0%	0.55
Design = RCT	3	29314	29191	0.92	0.68;1.25	34.0%	
Age = <50	3	1222	2127	1.03	0.77;1.39	88.0%	0.68
Age = >50	3	29377	29777	0.94	0.70;1.28	61.0%	
Intake = Both	2	332	996	1.26	1.00;1.58	13.0%	**<0.01**
Intake = Supplement	4	30267	30908	0.85	0.73;0.99	45.0%	
Type = MS	3	1222	2127	1.03	0.76;1.41	88.0%	0.68
Type = RA	2	16450	16833	1.04	0.70;1.54	22.0%	
Type = Various	1	12927	12944	0.79	0.63;1.01	NA	
Smoking = <30%	4	29434	29551	1.03	0.79;1.34	70.0%	0.64
Smoking = >30%	2	1165	2353	0.92	0.65;1.31	89.0%	

NA, Not Available; NR, Not Reported; MS, Multiple Sclerosis; RA, Rheumatoid Arthritis.

Five studies reported that vitamin C had none (41, 48) to significant reduction (38, 39, 46). Vitamin C supplementation was shown to reduce risk of multiple sclerosis (38, 46) but not in rheumatoid arthritis and systemic lupus erythematosus (41, 48).

All five studies on vitamin E (37, 39, 41, 44, 48) and two studies on multivitamins (41, 46) did not find any significant effect on the risk of autoimmune diseases.

3.4 Micronutrients and antioxidants

Three studies reported that omega-3 had none (41) to significant reduction (36, 40). Cortese et al. (40) showed reduction in risk of developing multiple sclerosis in adulthood with intake of cod liver oil containing omega-3 during adolescence. Cavalla et al. (36) found that the dietary omega

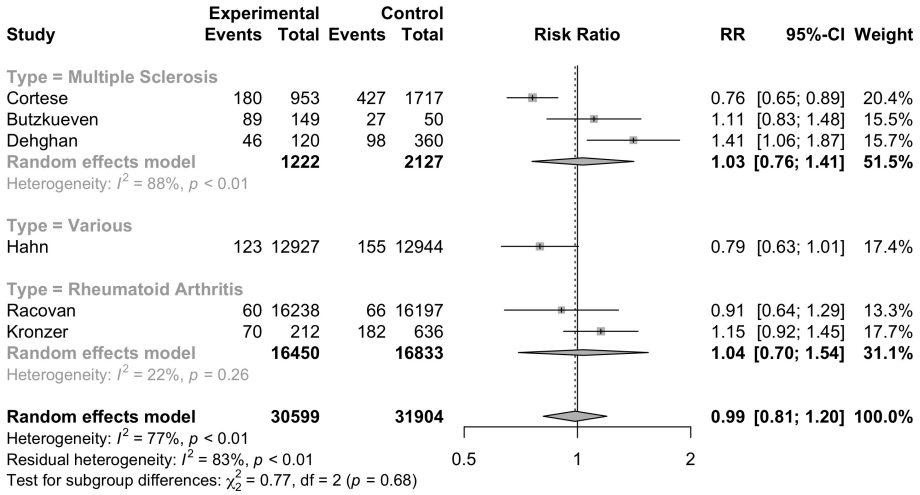

FIGURE 3
Relative risk ratio of Vitamin D supplementation and autoimmune diseases stratified based on disease type. RR, risk ratio; CI, confidence interval.

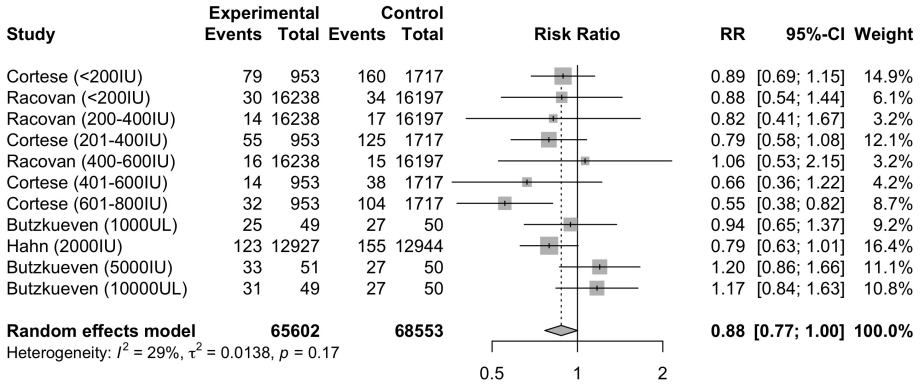

FIGURE 4
Relative risk ratio of Vitamin D supplementation and autoimmune diseases stratified based on studies with reported dosage. RR, risk ratio; CI, confidence interval.

6/omega 3 polyunsaturated fatty acids ratio was higher in patients than in controls, causing reduction in risk.

Four studies on calcium reported none (41, 45, 47) to significant reduction (38). This is possibly related to age, with the younger population having reduced risk (38) while this correlation was not observed in the older populations (41, 45, 47). Interestingly, Kronzer et al. (41) reported that while there was no correlation between intake of supplemental calcium and the risk of rheumatoid arthritis, dietary calcium intake of two to three times a day was associated with a reduced risk of rheumatoid arthritis.

Five studies on zinc reported none (41, 44, 47) to significant reduction (38, 39). Cortese et al. (47) reported that women with the highest total zinc intake and highest intake of supplemental zinc-including multivitamins showed a reduced risk of multiple sclerosis, however no correlation was found between intake of zinc-only supplements. Cerhan et al. (39) reported that intake of supplemental zinc was associated with a reduction in risk of developing rheumatoid arthritis, while intake of dietary zinc was associated with an increased risk of developing rheumatoid arthritis.

Five studies on iron (38, 40, 41, 44, 46) and two on dietary antioxidants index (38, 50) did not observe any significant effect in risk of developing autoimmune diseases.

3.5 Diet

Six (34, 36, 38, 42, 44, 46) out of seven studies (34, 36, 38, 41, 42, 44, 46) reported a significant association between diet and risk of autoimmune diseases (Supplementary Table S7). Three (38, 42, 46) out of the six studies saw a significant reduction in risk of autoimmune diseases while the other three (34, 36, 44) saw a significant increase in risk. Abdollahpour et al. (38) showed a reduced risk of multiple sclerosis with egg, red meat, poultry and dietary supplement intake. Rezaeimanesh et al. (46) demonstrated

that more intake of dairy, seafood, red meat, vegetable, fruit and nut during adolescence resulted in reduction in risk of multiple sclerosis in adulthood. Merlino et al. (42) revealed that an intake of more than 68 servings of milk products in a month reduced risk of developing rheumatoid arthritis. Deghan et al. (34) suggested that a carnivorous diet increased risk of multiple sclerosis when compared to a vegetarian diet. Pedersen et al. (44) found that consuming medium fat fish containing 3-7grams of fat per 100 grams was associated with increased risk of rheumatoid arthritis. Cavalla et al. (36) saw that more intake of rapid absorption carbohydrates, lesser vegetal proteins, and more animal proteins were observed in patients with a first demyelinating event.

3.6 Demographical characteristics

Four studies (17, 34, 35, 45) reported a significant association between personal characteristics variables and risk of autoimmune diseases (Supplementary Table S8). Two studies (35, 45) reported that the risk significantly increases with age. One study (34) evaluated that those with lower income had a higher risk. Hahn et al. (17)showed a significantly increased efficacy of omega-3 fatty acids in reducing risks among those with a family history of autoimmune diseases.

3.7 Smoking status

Three studies (36, 38, 44) investigated the association between smoking and risk of autoimmune diseases (Supplementary Table S9). Abdollahpour et al. (38) found a significantly increased risk of developing multiple sclerosis with lifetime second-hand smoking. Pedersen et al. (44) reported a significantly increased risk of developing rheumatoid arthritis for current smokers as compared to former smokers. Cavalla et al. (36) showed an increased risk of developing a first myelinating event in someone who had ever smoked.

3.8 Physical or outdoor activity

Three studies (34, 38, 41) revealed a significant association between physical or outdoor activity and reduced risk of autoimmune diseases (Supplementary Table S10). Interestingly, Abdollahpour et al. (38) demonstrated that the risks were reduced the most in those with more than five hours of sunlight exposure daily.

3.9 Risk of bias and publication bias

We scored for the quality of the methodology of the 18 studies using the JBI checklist and presented the results in Supplementary Table S11. Overall, there was no significant risk of bias identified. Sensitivity analyses using funnel plots, trim-and-fill, Egger's test showed some publication bias (Supplementary Figures S1–S3, S5). However, leave-one-out analyses showed no singular studies that would affect the overall results (Supplementary Figure S4).

4 Discussion

To the best of the authors' knowledge, this is the first systematic review and meta-analyses to examine the relationship between supplemental vitamins, micronutrients, and antioxidants on the risk of autoimmune diseases. This systematic review and meta-analysis sought to investigate any modifiable risk of developing autoimmune diseases with dietary consumption of various vitamins, micronutrients, and antioxidants. Our results demonstrated that vitamin D supplementation may not have any effect on modifying the risk of developing autoimmune disease. However, subgroup analysis by dosage of vitamin D showed that the dosage consumed at 600-800IU may reduce the risk of autoimmune diseases. Those who consumed both dietary and supplementary vitamin D had a higher risk of autoimmune disease as compared to those on supplementation alone. Systematic review suggests that consumption of most vitamins, micronutrients and antioxidants may not have any effect on the risk of autoimmune diseases. Smoking, age, physical or outdoor activity, and diet were significant factors that affected the efficacy of such interventions.

Current literature suggests that vitamin D levels had been most closely associated with modifying the risk of developing multiple sclerosis compared to other autoimmune diseases (51). A landmark investigation by Munger et al. (52) determined that higher concentrations of serum 25-hydroxyvitamin D (25(OH)D) levels lead to a decreased risk of developing multiple sclerosis. The VITAL randomised controlled trial (17) further validated the potential role of vitamin D supplementation in significantly reducing the risk of autoimmune diseases. Despite this, it is pertinent to recognise that many lifestyle and physiological factors are involved in altering normal serum 25(OH)D levels (53), and supplementation with oral cholecalciferol does not linearly increase serum 25(OH)D levels (53). Hence, a fixed dose of vitamin D supplementation may not apply the same risk reduction in a cohort of subjects with varying characteristics. Furthermore, our study investigated different autoimmune diseases which exert vastly different systemic physiological changes (54) and possibly altered individuals' response to vitamin D supplementation across different cohorts (55).

Vitamin D plays a key role as a hormone in regulating the immune system and influencing immune responses by functioning as a pro-survival molecule, protecting cells from harmful signals by suppressing inflammatory reactions (56). It does so by modulating pathways that affect the differentiation of T-helper-2 cells, M2 macrophages, and regulatory T cells, thereby helping to maintain immune homeostasis through the promotion of a tolerogenic state (56). In our review, most of the autoimmune diseases reported by the different studies were on multiple sclerosis and rheumatoid arthritis. Multiple sclerosis is characterised by an autoimmune response that targets the myelin sheath that insulates nerve fibres, leading to demyelination and impairing the ability of neurons to transmit electrical signals efficiently (57). Over time this repeated

demyelination causes the scar tissue formation, leading to axonal damage further impairing neurological function (57). Although the exact pathophysiology of how vitamin D supplementation reduces risk of developing multiple sclerosis has yet to be elucidated, certain mechanisms have been proposed (58). For example, proteomic analysis revealed that after high dose vitamin D supplementation, 125 proteins were differentially regulated in the brains of 1,25(OH) 2D-treated mice during remyelination, compared to placebo (59). Those upregulated proteins were primarily associated with calcium binding and mitochondrial function (59). Rheumatoid arthritis is a systemic inflammatory autoimmune disorder in which both T and B lymphocytes play a key role in pathogenesis (60). Vitamin D supplementation can influence both T and B lymphocyte populations, helping to regulate the immune response needed to prevent or manage the disease (61, 62).

While there is evidence that vitamin D supplementation can reduce the risk of autoimmune diseases, the high degree of variability between participants' response to supplementation may explain the insignificant results. Genetic variants have been linked to the high variability in the efficacy of vitamin D supplementation (63). For instance, Ammar et al. reported a 18.8% in response variability among three single nucleotide polymorphisms post supplementation (63). Higher body mass index has also been shown to reduce supplementation efficacy in a subset of 16515 patients in the VITAL trial (64). Considering these variations in response can help optimise vitamin D supplementation to achieve a better response. Another explanation could be the studies having a lack of proper control group for vitamin D interventional studies as it is not ethically possible to keep individuals on long-term vitamin D deficiency. However, subgroup analysis suggested that vitamin D dosages of 600-800 IU/day significantly reduced the risk of autoimmune diseases. Cortese et al. described a strong protective effective of vitamin D 600-800 IU/day using cod liver during winter but not in other seasons for Norwegian adolescents developing incident multiple sclerosis during adulthood (40). This finding potentially underscores the importance of considering not just dosage variability but also seasonality when assessing the impact of vitamin D supplementation on autoimmune disease risk. We also noted a bimodal pattern of vitamin D supplementation when <200 IU/day or ≥5000 IU/day (Supplementary Table S3), with extremes of doses leading to paradoxically higher incident autoimmune diseases. This observation mirrors the findings of Lim et al., where a bimodal influence of vitamin D inducing inflammatory responses and fungal burden was observed in a mouse model of candidiasis (65). Lately, Carlberg et al. (66) has put forth this concept of a vitamin D response index that clusters the population to high, mid and low responders based on their transcriptome changes after supplementation. Further research should explore optimal dosages and interventional trials (e.g., N = 1 approach) tailored to specific populations, seasons and disease profiles, considering the multifaceted nature of both vitamin D metabolism and autoimmune pathophysiology (66).

Diet has been well alluded to as a possible modifiable risk factor of autoimmune diseases (67). Specifically, diets characterised by an abundance of fat and sodium, typified by the Western dietary pattern, have been observed to amplify systemic inflammation and exert negative effects on immune responses (68). Conversely, the Mediterranean diet has exhibited its potential in mitigating chronic inflammation (69). This dietary approach has also demonstrated its potential protective role against multiple sclerosis (67). Interestingly, Barrea et al. (70) demonstrated that the Mediterranean diet has the potential to significantly increase serum 25(OH)D levels in a cohort of 617 Caucasians. Vitamin D intake can be derived from specific dietary sources, notably oily fishes, and certain nuts (71), which are often found within a Mediterranean diet (72). While these factors suggest a potential scenario wherein dietary vitamin D may mitigate the risk of autoimmune diseases, our subgroup analyses unveiled a contrasting observation; individuals concurrently utilising both supplemental and dietary sources of vitamin D may have a higher risk of developing autoimmune diseases. Notably, one study included in our analysis (41) reported a prevalent consumption of fatty foods, poultry, and dairy among participants, while another study (34) reported a varied dietary pattern among its participants. We hypothesise that this observation could be attributed to the broader impact of an individual's overall dietary patterns leading to high doses of vitamin D from supplementation and food intake, which may exert a more substantial influence than singular vitamin D supplementation in shaping the risk of autoimmune disease development. This is also in keeping with the bimodal response of vitamin D in preventing incident autoimmunity described above (Supplementary Table S3). More thorough interventional studies should be conducted to investigate the comparative effects of specific dietary patterns and micronutrients on the development of autoimmune disease.

While vitamin D stands as the most extensively investigated vitamin in relation to its role in modulating autoimmunity and the development of autoimmune diseases, our findings and systematic review also highlight the potential contributions of other vitamins, micronutrients, and antioxidants in reducing the risk of autoimmune diseases. Vitamin C has been well established as an effective antioxidant with immunoregulatory effects (73). Similarly, vitamin B, particularly vitamin B6, has established anti-inflammatory roles (74). Beyond vitamins, supplements like omega-3 are accruing evidence for their capacity to decrease morbidity and mortality associated with various autoimmune diseases (75), owing to pro-anti-inflammatory and immunoregulatory effects (76). The VITAL trial, while not reaching statistical significance, reported a 15% reduction in the risk of autoimmune diseases with omega-3 supplementation (17). Intriguingly, the direct impact of serum iron or ferritin supplementation on immune regulation or protection against immune diseases was not well studied. However, existing literature suggests that disturbances in physiological iron metabolism may potentially influence individual immunity (77). Like other vitamins, vitamin E has been demonstrated to exhibit immunoregulatory effects, especially for infectious disease (78). However, our findings did not demonstrate any significant reduction in risk of developing autoimmune diseases with vitamin E supplementation. While our meta-analysis primarily focused on the correlation between vitamin D supplementation and autoimmune disease risk, the inclusion of other vitamins, micronutrients and antioxidants adds complexity to our findings. Limited data for these factors underscores the need for caution in drawing definitive conclusions. Our study opens avenues for future research to build upon by expanding sample sizes and

employing longitudinal designs to better understand temporal relationships. Stratified analyses and exploration of interactions among micronutrients may reveal nuances not captured in our study. Rigorous randomised controlled trials with defined dosages and consideration of confounding factors would further enhance causal inference.

This study also revealed a noteworthy correlation between specific demographic factors and modifying the risk of developing autoimmune diseases. Older age, smoking status and lack of physical or outdoor activity were found to be significant risk factors for autoimmune diseases. While most of the autoimmune diseases we investigated have a peak age of onset of less than 60 years old (79), older age consistently remains a significant risk factor for developing autoimmune diseases (80) and other pathologies (81–83). Similarly, smoking has been a well-established risk factor for autoimmune diseases (84). On the contrary, we noted a significantly reduced risk of developing autoimmune disease with increased physical outdoor activity. On top of the direct benefits of physical activity on immunoregulation (85) and reducing systemic inflammation (86), outdoor activities in areas with sufficient sunlight exposure has been shown to be a protective factor against multiple sclerosis, vis-à-vis via endogenous vitamin D production (87).

Our study should be interpreted in due consideration of the limitations. Firstly, we anticipated high heterogeneity in the reporting of outcomes. Data on the development of autoimmune diseases with vitamin D supplementation was only available for six studies. We were also unable to account for the different subgroups of vitamin D responders. The type of autoimmune disease was also different for each study, further contributing to this heterogeneity. However, we were able to perform subgroup analyses based on the type of autoimmune diseases. Thirdly, not all studies had consistent doses of the supplementation within the intervention group, hence we are unable to quantify the true dose-dependent extent of each supplementation's role in risk modification of developing autoimmune disease. However, we were still able to analyse specific dose ranges in our subgroup and mixed-effect meta regression analysis.

5 Conclusion

In conclusion, we demonstrated the role of various vitamins, micronutrients, and antioxidants in modifying the risk of developing autoimmune diseases. We highlight the importance of dosage variability when considering prophylactic usage of such supplements, especially for vitamin D. Our study contributes to the evolving landscape of nutritional immunology, providing a foundation for future research to unravel more definite relationships between micronutrients and autoimmune diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CL: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft, Writing – review & editing. SL: Data curation, Writing – original draft, Writing – review & editing, Formal analysis. NC: Data curation, Writing – original draft, Writing – review & editing, Formal analysis. AL: Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Methodology, Project administration. ST: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1453703/full#supplementary-material

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