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Microbiota, nutrition and stress: Modulators of immunity

Edited by
Sergey Ponomarev, Isabelle Mack and Alexander Chouker

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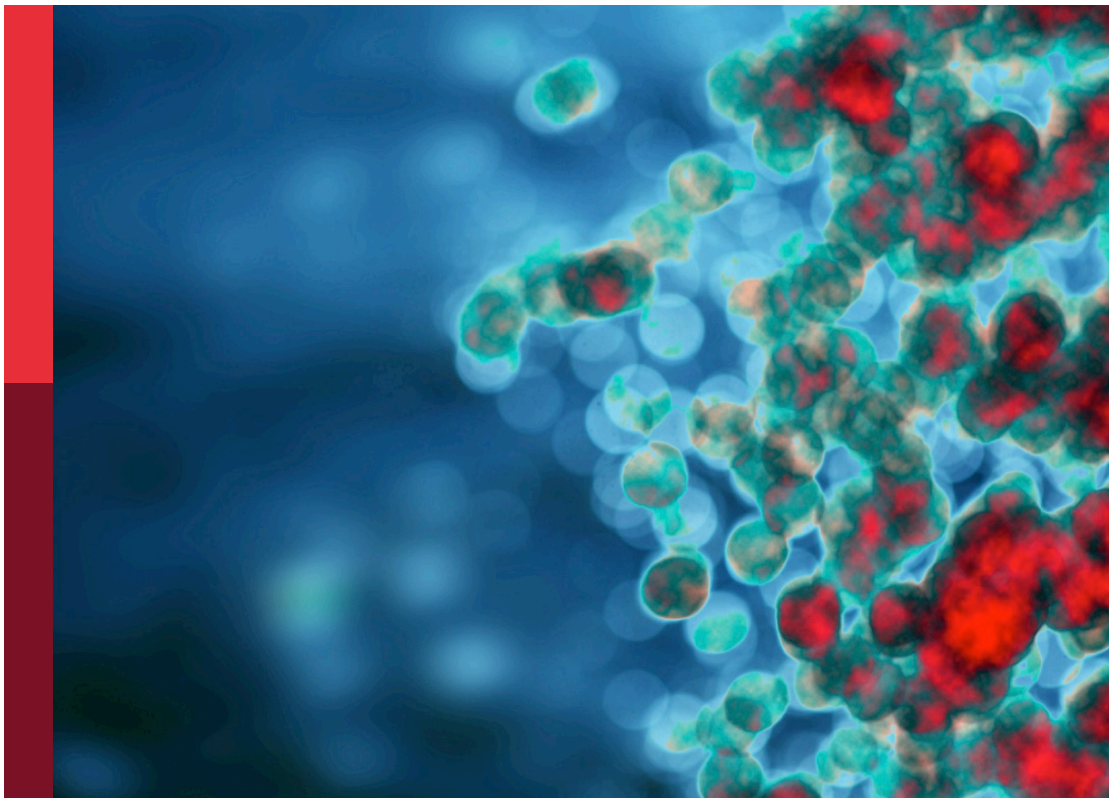

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Microbiota, nutrition and stress: Modulators of immunity

Topic editors

Sergey Ponomarev — Institute of Biomedical Problems, Russian Academy of Sciences (RAS), Russia

Isabelle Mack — University Hospital and Faculty of Medicine, University of Tübingen, Germany

Alexander Chouker — LMU Munich University Hospital, Germany

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Willem Van Eden,
Utrecht University, Netherlands

*CORRESPONDENCE
Sergey Ponomarev
✉ dr.grey@bk.ru

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Editorial: Microbiota, nutrition and stress: modulators of immunity

Sergey Ponomarev[1]*, Isabelle Mack[2] and Alexander Choukér[3]

[1]Laboratory of Immune System Physiology, State Scientific Center of Russian Federation, Institute of the Biomedical Problems, Russian Academy of Sciences, Russian Federation, Moscow, Russia, [2]Department of Psychosomatic Medicine and Psychotherapy, University Hospital Tübingen, Tübingen, Germany, [3]Laboratory of Translational Research 'Stress and Immunity', Department of Anesthesiology, LMU University Hospital, Ludwig-Maximilians-Universitó Munich (LMU), Munich, Germany

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Editorial on the Research Topic
Microbiota, nutrition and stress: modulators of immunity

Vertebrate immunity is a complex system that involves a large number of cells of different organs and intracellular pathways in charge of the protection and maintenance of the body's integrity and homeostasis. Inefficient coordinated action and balancing functions of the complex interplay of the human immune system increase the risk for acute and deadly infectious diseases, while excessive immune responses may lead to autoimmune disorders and subsequent morbidity. Unlike other body systems, immunity functioning patterns are highly individual and genetics only partially defines them. Along with the host immune system, the microbiota of the host is thought to be pivotal in the maintenance of human health by affecting energy metabolism, intestinal function, and immune response among others. In addition, environmental factors such as diet and stress exposures on the one hand, and intrinsic factors such as age and sex on the other hand, modulate immunity by altering gene expression patterns within immunity-related cells, shifting their number in subpopulations and affecting their phenotype and key properties. In the 10 articles within the current Research Topic, these aspects are considered, creating an opportunity to have an idea of the factors that determine the functioning of immunity in animals and humans, respectively.

"It is the start of a new era when people are finally ready to embrace the microbial world." This anticipated direction has been truly voiced by the science journalist and Pulitzer prize winner Yong (1), and the microbial role in the concept of the intestine-immunity axis is widely accepted today (2). Both animal and human studies confirm the potential role of gut microbiota in immunomodulation (3). Interestingly, Hou et al. revealed the causal relationships between gut bacteria and gout, a widespread metabolic disorder, which has a strong autoimmune potential (4). On the other hand, gut microbiota largely depends on a diet. Ali et al. report that food rich in dietary fibers positively affects the microbiome composition in meat geese, which, in turn, induces antimicrobial, antioxidant, and anti-inflammatory properties of gut barriers. At the same time, according to Mardi et al., a proper diet in humans enhances patient survival and improves the overall effect of COVID-19 therapy. Besides dietary fibers and pre- and probiotics, a healthy diet should include nutrients, namely, essential amino acids, choline, non-saturated fatty acids, carbohydrates, and vitamins. A diet rich in fat, as well as prolonged fasting, may result in ketoacidosis, which negatively affects immune system functioning. The mechanism of this impairment

was recently revealed in the work of He et al., demonstrating that one of the ketone bodies, namely, β-hydroxybutyrate, enhances neutrophil adhesion by inhibiting autophagy. In the review of Khan et al., the anti-inflammatory and anti-oxidative role of methionine, lysine, and choline is discussed in relation to periparturient ruminants, while Zhang et al. established the antioxidant capacity of tryptophan in fish. Surprisingly, in an experimental setting in mice, the regular consumption of an acidic polysaccharide extracted from green parts of *Chuanminshen violaceum*, a Chinese endemic plant, significantly improved their state of health. Zou et al. managed to show lowered inflammatory and oxidation indices in the murine blood. Apparently, like dietary fibers, carbohydrates primarily benefit healthy gut bacteria, which mediates the positive effects of CVP-AP-I.

Enzymes' catalytic properties rely on cofactors, many of which are either vitamins or metal ions. Munteanu and Schwartz systemically review the role of vitamins and micronutrients for proper immunity functioning, discussing in detail the to-date data on the role of vitamins A, C, and D, as well as cholesterol in the development of cancer and their therapeutic usage. In addition to their role as transcription regulators and coenzymes, the fat-soluble vitamins (A and E), as well as vitamin C, are known to be a part of the non-enzymatic module of the antioxidant system in the human body, reducing inflammation processes as induced by ROS. Hung et al. propose intravenous vitamin C as a monotherapy to reduce mortality in critically ill patients although the underlying mechanism and optimal dose, inclusion, and exclusion criteria remain to be thoroughly elucidated, especially while other antioxidant therapy mono-approaches, e.g., using essential trace elements [e.g., selenium (Se)], have not shown the expected clinical effectiveness in critical care patients (5).

Stress factors do affect and hereby modulate the immune response effectiveness as a function of the intensity of the stressors. In the current Research Topic, there is only one paper by Klos et al. that links the effects of isolation or confinement as an environmental stress factor and human immunity. The authors systematically review and summarize the data on microbiota composition shifts in different human trials in isolated and confined environments. The results of the study indicate that internal factors that shaped the microbiota over time appeared to be the primary drivers of its composition and function in response to isolation in antigen-deprived conditions. However, a preliminary trend indicates a decreased diversity of gut microorganisms in people inhabiting extreme conditions, which raises concerns about the risk of dysbiosis and associated diseases, including immunity dysregulation (3). Additionally, besides microbiota, there is evidence that emotional stress experienced by people in confinement directly affects their immune system's effectiveness. Numerous experiments in which they model space flight factors, including short-term and prolonged isolation, demonstrate impaired immunity functioning (6). In this regard, potential immunologic countermeasures are being developed to minimize the risks of immune system dysfunction during space exploration full of different stress factors (7).

In summary, the results of the abovementioned studies and the data from the reviews give insight into complex factors affecting immunity functioning. The studies illustrate well the interdependence of nutrition, microbiota, and immunity. On the one hand, the immune homeostasis seems too prone to alterations due to its susceptibility to various environmental factors. On the other hand, one may consider the power of little efforts in the maintenance of a healthy lifestyle to keep immunity in balance.

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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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EDITED BY
Sergey Ponomarev,
Institute of Biomedical Problems (RAS), Russia

REVIEWED BY
José Luis Fachi,
Washington University in St. Louis, United States
Daniel Roeth,
Beckman Research Institute, United States
Evanthia Tourkochristou,
University of Patras, Greece

*CORRESPONDENCE
Betty Schwartz
betty.schwartz@mail.huji.ac.il
Camelia Munteanu
camelia.munteanu@usamvcluj.ro

†These authors have contributed equally to this work

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The relationship between nutrition and the immune system

Camelia Munteanu[1*†] and Betty Schwartz[2*†]

[1]Department of Plant Culture, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Cluj-Napoca, Romania, [2]Robert H. Smith Faculty of Agriculture, Food and Environment, The School of Nutritional Sciences, The Institute of Biochemistry, Food Science and Nutrition, The Hebrew University of Jerusalem, Rehovot, Israel

Nutrition plays an essential role in the regulation of optimal immunological response, by providing adequate nutrients in sufficient concentrations to immune cells. There are a large number of micronutrients, such as minerals, and vitamins, as well as some macronutrients such as some amino acids, cholesterol and fatty acids demonstrated to exert a very important and specific impact on appropriate immune activity. This review aims to summarize at some extent the large amount of data accrued to date related to the modulation of immune function by certain micro and macronutrients and to emphasize their importance in maintaining human health. Thus, among many, some relevant case in point examples are brought and discussed: (1) The role of vitamin A/all-trans-retinoic-acids (ATRA) in acute promyelocytic leukemia, being this vitamin utilized as a very efficient therapeutic agent via effective modulation of the immune function (2) The involvement of vitamin C in the fight against tumor cells via the increase of the number of active NK cells. (3) The stimulation of apoptosis, the suppression of cancer cell proliferation, and delayed tumor development mediated by calcitriol/vitamin D by means of immunity regulation (4) The use of selenium as a cofactor to reach more effective immune response to COVID vaccination (5). The crucial role of cholesterol to regulate the immune function, which is demonstrated to be very sensitive to the variations of this macronutrient concentration. Other important examples are reviewed as well.

KEYWORDS

nutrition, health, immunity, nutrients, vitamins, minerals, amino acids, cholesterol

Introduction

Food, nutrition and health are highly interrelated and consumption of specific nutrients have a profound impact on human health. The amount and type of nutrients consumed are tightly linked to the metabolic stage and the immune health and thus, inappropriate nutrient consumption is associated with development of major human diseases due to an immune system not properly functioning (1).

The inflammatory mechanisms that compose the innate immunity are strongly influenced by nutrition, and this interaction, when perturbed, can profoundly affect disease development. The immune system is able to destroy antigens through both innate and adaptive immune cells and finally through antibodies that are specific for each pathogen (2).

The number of studies related to the impact of nutrition on immune system is continuously increasing. The initial studies published were related to nutritional-modulation of the immune function were mostly based on the effects of micro and macronutrients (3). Lately, a wide variety of phytochemicals and other chemical biocomponents found in nutrients has been added to the list of nutritional-immuno-modulators. These biocomponents affect the immune function but are not crucial for maintaining normal cell metabolism and function (3). Cases in point are several phytochemicals demonstrated to exert impressive positive immune effects (3).

In light of the strong effects, that we will list in the following paragraphs, nutrients have on the immune system it can be concluded that a rich-nutrient diet is rigorously required in order to maintain an adequate health status. This is in addition to the fact that nutrients are the main factors for survival, including cell proliferation, specialization, development of tissue and organs growth, energy supply, and the immune defense function (4).

To clarify all these aspects, it is essential to understand the meaning of an adequate diet and concomitantly to recognize the harmful effect of processed foods that impact the immune system (**Figure 1**).

Moreover, nutritional deficiencies are closely associated with impaired immune response and loss of the host resistance to infection (3). On the one hand, in less developed regions malnutrition continues to be a major health problem (5–7) since it is associated with a higher incidence of morbidity and mortality usually linked with the higher prevalence of bacterial and parasitic infection diseases in these regions (3). In contrast, developed countries confront with inadequate diet consumption, with no real nutritional value, accompanied by excess calories (8). Therefore, malnutrition due to undernutrition or to consumption of poor diets, deficient in macro- and micronutrients, reduce the effectiveness of the immune system, not only by causing a deterioration of the immune protection but also reducing its efficacy in appropriate elimination of the pathogens, thus making people unprotected to a vast variety of diseases.

In addition to food consumption, an important question that arises is regarding the bioavailability of nutrients. Could we treat certain deficiencies using supplements if we are aware of them? Or should we try to build an adequate and complex menu that ensures the proper and desired bioavailability? Another important question refers to where does the absorption of the nutrients take place? It is important to know that the pathogenic agents such as bacterial products, bacteria and some toxic alimentary particles from food and the intestinal microbiota are often responsible for triggering an immune response (9). This is very important because prevention against pathogens is mainly maintained through the intestinal epithelial barrier. The gastrointestinal tract has an essential role due to its lymphoid tissue and for this reason, represents an essential part of the immune system. It has been demonstrated that the epithelial barrier contains cells that present antigens to dendritic cells (DCs) in the lamina propria. The most important are CD103 + CX3CR1- DCs. These imprint the intestinal lymphocytes to stimulate the development of regulatory T cells, the production of IgA, and dendritic cells responsible for the development of Th17 cells and the production of TNFα (10). Additionally, nutrients can control the expression of pro and anti-inflammatory cytokines via interaction with Toll-like receptors (TLRs), which are proteins known to play a key role in the control of the innate immune system. They are located in cells such as macrophages and dendritic cells and as such they control immune cell activity via appropriate crosstalk and signaling. As a result, immune cell's enzymatic activity is affected and therefore molecular and chemical changes linked to oxidative stress and inflammation take place finally affecting the immune function. Most of the activities are associated with oxidative reactions, affecting neutralized cytotoxicity (11).

The normal standard diets of human beings (except for vegetarians and vegans), include vegetables, eggs, milk, dairy products, and meat. From a biochemical perspective, the foods can be converted into micronutrients and macronutrients that ensure the organism's well-functioning (12).

This review aims to summarize at some extent the large amount of data accrued to date related to the modulation of immune function by certain micro and macronutrients and to emphasize their importance in maintaining human health.

In order to achieve this goal, research articles and reviews, found in several international databases, have been researched using phrases and keywords. We used the following keywords: nutrition, health, immunity, nutrients, vitamins, minerals, amino acids, and cholesterol.

The review material covers an extended period of time from 1973 to 2022. To achieve this aim some of the issues addressed were: the correlation of nutrition with the immune system in order to obtain good health, certain nutrients involved in the modulation of immune function through mediating pro- and anti-inflammatory responses, and cholesterols' role in the immune response.

The effect of nutrition on optimal immune response

As alluded to earlier, nutrition plays an essential role in the regulation of optimal immunological response, by providing adequate nutrients in sufficient concentrations to the immune

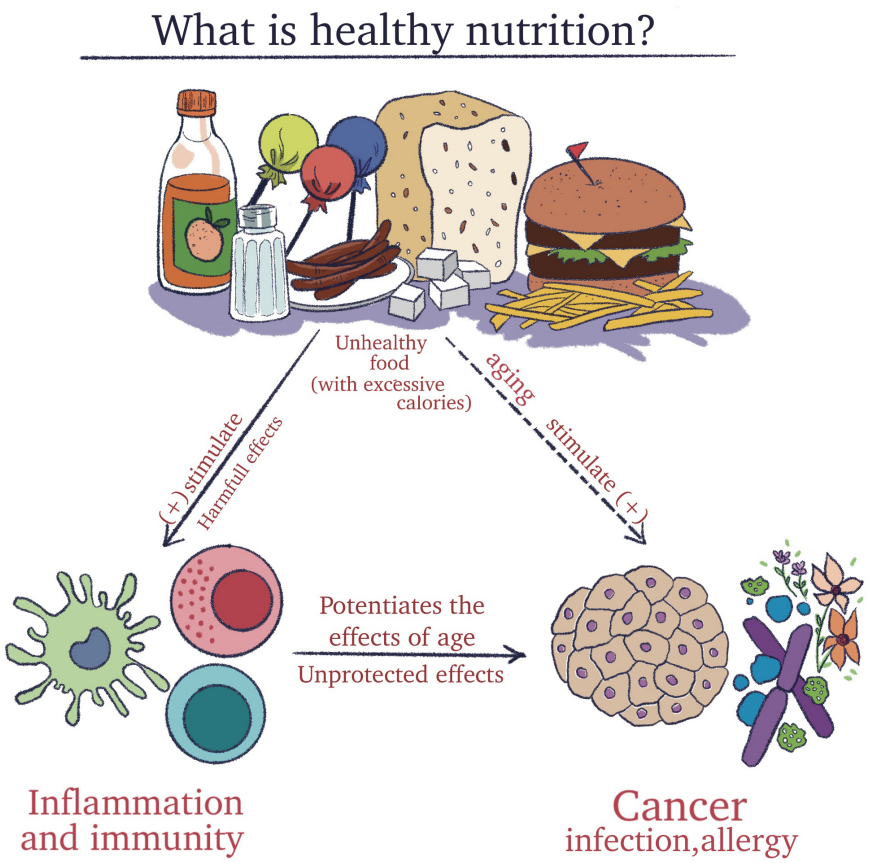

FIGURE 1
The importance of understanding the meaning of a healthy diet and concomitantly to recognize the harmful effect of pro-inflammatory foods that impact the immune system.

cells. In such a manner, the immune system can initiate effective responses against pathogens. In order to avoid chronic inflammation, nutrients stemmed from the diet exert significant effects in initiating this quick response (13). When the dietary nutrients are insufficient or inefficient, the supply of these elements to the immune system cells is significantly spared and immunity is compromised.

There are certain micronutrients such as vitamins and minerals as well as some macronutrients such as specific amino acids demonstrated to exert a very important and particular impact on immune modulation. Amino acids such as L-arginine and L-tryptophan are responsible and critical for macrophages' appropriate immune activity. Macrophages are characterized by variations in their plasticity and polarization in response to changes in the intracellular environment. They are capable to transform into different subtypes depending on the intracellular microenvironment and to the different signaling molecules (**Figure 2**). L-arginine is associated with a well-known immunoregulatory mechanism exploited by M2 macrophages. The mechanism involves arginase 1, which consumes L-arginine and the genes responsible for M1inhibition, concomitantly with M2 promotion (14). Furthermore, arginine and methionine together, are in charge of the synthesis of polyamines. Due to their ability to maintain cell membrane stability and keep DNA homeostasis, they stimulate cell proliferation (**Figure 2**). In addition, many studies show the involvement of these kinds of amino acids in tumor cell growth metabolic pathways as well as in immune antitumor response (15). Through their degradation, these kinds of amino acids supply chemical precursors for a number of biological reactions (16). Regarding insulin-like growth factor -I and insulin growth hormone these can use as strong secretagogue arginine. Metabolic syndrome and type 2 diabetes as well are major worldwide public health problems which are strongly related to nutrition. There are some amino acids implicated in the synergistic stimulation of insulin release from pancreatic β-cells. One of the most well-known mechanisms is related to the fact that arginine in the presence of glucose can depolarize the plasma membrane at a neutral pH. This gating mechanism is called cationic (17).

Indoleamine 2,3-dioxygenase 1 (IDO1) is a powerful immunosuppressive enzyme involved in the catalysis of the first and rate limiting step of L-tryptophan catabolism. IDO1

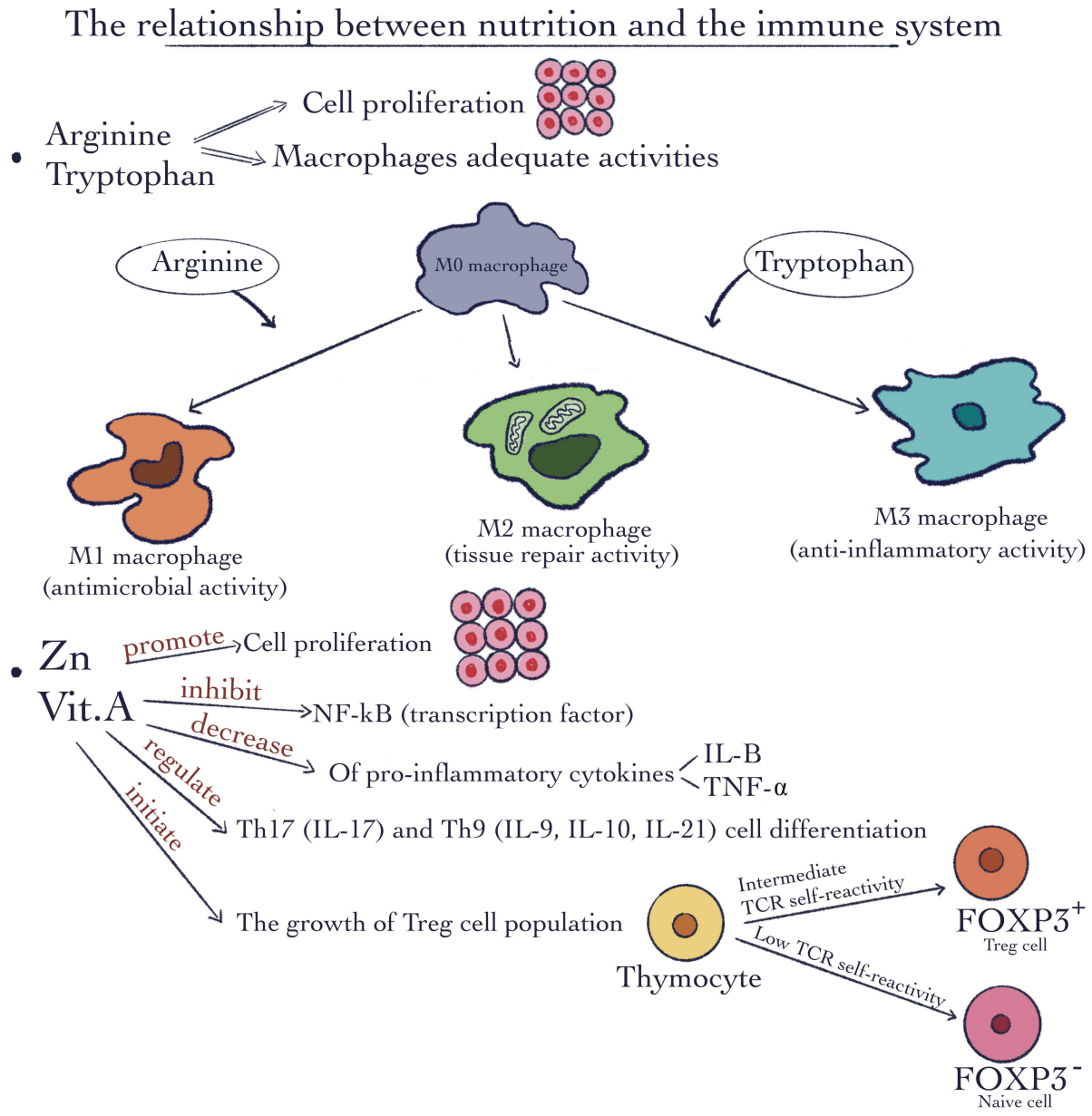

FIGURE 2
The relationship between nutrition and the immune system. Macronutrients such as arginine and tryptophan are involved in cell proliferation and macrophages' adequate activities. Micronutrients like Vitamin A and Zinc can promote cell proliferation; inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway; decrease the pro-inflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α); regulate the Th17 and Th9 cell differentiation; initiate the growth Treg cell population.

depletes L-tryptophan storage and induces the production of immunoregulatory molecules interferon-γ (IFN-γ), tumor-necrosis factor (TNF) and IL-1 (18). High IDO1 expression and catalytic activity occur in dendritic cells (DCs)— in response to IFN-γ (18). Tryptophan metabolism leads to the synthesis of NAD +, which is known as a cofactor capable of redox reactions. In immune tolerance, arginine catabolism may determine the initiation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Arginine may also provide a substrate for the growth and survival of the cells and concomitantly exerts a key role in differentiation and appropriate gene expression (16).

The decline of protein metabolism that is related to the diminishing concentration of certain amino acids, leads to the endoplasmic reticulum (ER) stress. As a result, the T cells which produce pro-inflammatory cytokines are activated (19). The deficiency of Arg is correlated with reduced T cell ability to trigger tumor immunity (15).

Vitamins and minerals such as vitamin A and Zn in addition to their involvement in cell division and proliferation (**Figure 2**) are involved in immune-modulation. For example, the rate of antibody synthesis can be modified by these micronutrients (20).

Vitamin A (**Figure 2**), which is involved in biosynthesis of carotenoids and retinyl esters, molecules well known to affect appropriate immune function (21). It can also exert a role as transcription factor if it is bound to retinoic acid receptors (RARs). As a result, it can be responsible for lipid homeostasis, cell division, growth, and specialization by regulating the expression of certain specific genes (21). Vitamin A deficiency has repercussions on immune functions, such as impaired neutrophil function, suppressing the activity of natural killer (NK) cells, as well as a decline in their number, and damaged capacity of phagocytosing of macrophages. In addition, it may affect the growth and differentiation of B cells (2). In this way, the predisposition for infection disease can increase (22).

Zinc represents another example of the micronutrient group. The transcription factor NF-κB (23) can be inhibited by Zinc (**Figure 2**). Also, the pro-inflammatory cytokines IL-1β and tumor necrosis factor (TNF-α) production (24) may be repressed, as a result of modulation of the Toll-Like Receptor 4 (TLR4) signaling pathway. Moreover, the pro-inflammatory specific Th17 and Th9 cell differentiation pathway (25, 26) can be moderated by Zn. Treg cell population can be increased after Zinc administration (27, 28) thus Zn is considered an important factor for immune cell development. Specific effects include impaired lymphocyte proliferation, Delayed-Type Hypersensitivity (DTH) response, and natural killer (NK) cell activity (29–31). As we mentioned before, there are strong and dynamic relationships between nutrition and the immune system, which are important for maintaining good health. We will discuss further in more details the role of specific nutrients in the mediation of pro-and anti-inflammatory responses.

Nutrients involved in mediation of pro-and anti-inflammatory responses

The immune system consists of cells belonging to the two types of immune responses, i.e., the innate and adaptive mechanisms. Once the pathogens enter the body, the first reaction is mediated by the cells belonging to the innate immunity system. This system consists phagocytes, dendritic cells, eosinophils, neutrophils, mast cells, and some additional cells (32). In this case, the immune response acts quickly. The difference between the innate immunity system and the adaptive response is that the former is unspecialized and less efficient (13). In contrast, the adaptive response is capable to recognize each pathogen, and furthermore, remember if it has been encountered before, therefore T cells being the most important in antigen identification. They are also involved in immune response regulation. Furthermore, there are two kinds: cytotoxic T cells/T8 (CD8 inducer), which are implied directly in killing infected cells (33) and tumor cells, and the T4 helper cells (CD4 inducer), which are useful in modulating other cell's responses. Furthermore, in the function of type cytokines produce by them, there are some subtypes of T helper cells: Th1, Th2, Th17 (13, 34). Th1 cells are responsible for fighting against bacteria and viruses. The main role of these cells is to produce Interferon γ (IFNγ) and IL-2. IFNγ, like IL2, is a cytokine created by both immune adaptive and innate cells like Th1, T8 lymphocytes as well as innate lymphoid cells and NK (35). At the same time, the immune function is activated by Th2 cells. They are capable to produce other interleukins (ILs) (36). Induced cells apoptosis can be caused by activated macrophages and cytotoxic CD8 + T. Interestingly, the other immunity regulatory T cells are involved in the suppression or the blockage of cytokine secretion by the immune response (37). In light of this information, they have a crucial role in peripheral tolerance through the initiation and continuance of this stage (38).

B lymphocytes, which also belong to the adaptive immune system are involved in the synthesis of antibodies. Similar to T cells, they have the ability to specifically respond to each antigen (39). Antigens can actually produce damage to the tissue that they attack. It makes sense that the pathogens in the tissues and around this region promote an inflammatory response. Its main role is to repair the damage tissue in certain ways that can eliminate the antigens and their effects, and decrease their extension (40). After that this process takes place, some physiological changes occur which are responsible for increasing the phagocytes number in the place where this process is happening. As a result, pro-and anti-inflammatory cytokines, prostaglandins, and complements are delivered, especially through the activation of phagocytes. All these changes cause the growth of the inflammatory response (1). Based on the knowledge of which cells are involved in each inflammatory response pathway, it is now feasible to shed light on the effects of specific nutrients on each of these processes.

To this end, we selected to discuss the influence of certain vitamins such as: A, B1, B2, B3, B12, C, and D, minerals like: Zinc, and Selenium as well as certain amino acids such as arginine and tryptophan and some fatty acids.

Vitamin A

Vitamin A plays an essential role in the regulation of innate and cell-mediated immunity, and antibody responsiveness through the activity of either all-trans retinoic acid, 9-cis retinoic acid or other metabolites and nuclear retinoic acid receptors (41). Vitamin A and associated retinoid metabolites exert an important regulatory function of the immune system. This essential role is evidenced when Vitamin A is deficient and an augmented susceptibility to infections is evident (42).

Vitamin A deficiency affects processes related to appropriate cytokines release and antibody production. Additionally, vitamin A deficiency is associated with a reduced production of natural killer cells, monocytes or macrophages, and impaired maturation and proliferation of T- and β-lymphocytes. Vitamin A deficiency impairs innate immunity by impeding normal regeneration of mucosal barriers damaged by infection, and by diminishing the function of neutrophils, macrophages, and natural kill cells. Vitamin A supplementation cuts down morbidity and mortality in various infectious diseases (43). In the case of vitamin A deficiency, the integrity of the mucosal epithelium is altered, resulting in enhanced accessibility to various pathogens to the gastrointestinal tracts and other organs, being children the most affected population (44). In children, severe vitamin A deficiency causes almost the disappearance of goblet cells present in the upper layer of the epithelial line, therefore the production of mucus by these cells is compromised, and bacterial adherence to the epithelial lining is reinforced thus becoming the major factor for the development of the bacterial disease (45). Additionally, vitamin A deficiency is associated with diminished phagocytic activity and macrophage oxidative breakdown that takes place during the process of inflammation along with a reduction in the number of natural killer (NK) cells (42).

It has been demonstrated that vitamin A (Tab.1) stimulates the expansion and differentiation of Th1 and Th2. Thus, vitamin A is capable of promoting the Th2 anti-inflammatory response by repression of IL-12 and IFNγ which are synthesized by Th1 lymphocytes (46). In addition, some studies suggest a positive relationship between vitamin A and mitogen-induced pro-inflammatory cytokine (IFN-γ) and anti-inflammatory cytokine (IL-10) (47). It is important to know that retinol, retinoic acid (RA), and retinal are the three forms of vitamin A. It has been shown that RA is involved in a lot of biological activities (48). According to Rampal et al. (49) under inflammatory conditions, RA might sustain or cause stimulation of intestinal inflammation (50). Moreover, through the release of certain cytokines, such as: IL-1, IL-6, IL-12, and nitric oxide, RA can affect the macrophages' activity (51). When it comes to hypovitaminosis in children, vitamin A administration reduces mortality caused by diarrheal diseases (52). Vitamin A might be responsible for antitumor effects on human pancreatic cell lines (53). In metastasis of renal carcinoma, it seems that all-trans-RA (ATRA) have a similar effect (54). ATRA represents a nutrient that is required in small quantities and it is synthesized in the human body from the A vitamin (55). In acute promyelocytic leukemia (APL), ATRA is utilized as a very efficient therapeutic agent. Furthermore, together with arsenic trioxide (ATO), they are able to increase life expectancy. Due to this combination, the recovery of this disease is approximately 95% of cases (56). It was observed that, in breast cancer, after the administration of vitamin A, cytotoxic effects have been seen, but the healthy cells weren't influenced. As a result, vitamin A is capable of reducing some negative chemotherapy effects (57). The next question worth asking would be whether there are more nutrients involved in mediating pro- and anti-inflammatory responses.

Vitamins B1, B2, B3, and B12

The group of B-vitamins comprise eight water soluble vitamins in charged to carry out essential, inter-related roles for appropriate cellular functioning. These vitamins act as efficient co-enzymes in a vast array of catabolic and anabolic enzymatic reactions and they are essential cofactors for many important cellular metabolic pathways. We therefore cannot refer to life or to cellular life without referring to the B vitamins. In this respect, the crucial enzymes responsible for the regulation of vital functions in cells use specific cofactors such as: Nicotinamide Adenine Dinucleotide/B3, Flavin Mono Nucleotide/Flavin Adenine Dinucleotide/B2, and Thiamine Pyro Phosphate/B1 (58). However, an important aspect to be considered in terms of B vitamins, is that when it comes to the human body, an important source of vitamins B is determined by the activity of the gut microbiota except for some that may be ingested by the diet. The absorption of B vitamins takes place in two different intestinal locations, the large and small intestines. The large intestine represents the main absorption place for most bacterial-produced B vitamins. At the same time, the small intestine represents the place where dietary B vitamins are absorbed. It is tentatively to surmise whether two specific immune responses result from the two different absorption places (59). We surmise that the immune activities at the two specific locations are different since the population of gut immune cells are different (59).

Vitamin B1 or Thiamine (**Table 1**), exerts an anti-oxidative role due to its protective action on sulfhydryl groups from the surface of neutrophils. As a result, the synthesis of cytokines from macrophages is blocked furthermore. Regarding the stimulation of antimicrobial oxidative reactions myeloperoxidase (MPO), H_2O_2, and a halide (HRP/H_2O_2/Nal) determined by the activation of polymorphonuclear leukocytes, thiamine together with other compounds can prevent and inhibit this oxidative system of PMNL (60). The NF-κB pathway involved in the control of the oxidative stress, is prevented by Thiamine. This role is highlighted by suppressing the phosphorylation and catabolism of inhibitory kappa B (IκB), which subsequently inhibits the nuclear translocation of the transcription factor-sensitive redox NF-κB (58, 61). From a biochemical and immunological point of view, we can conclude that thiamine derivates are involved in the control of immune metabolism through the regulation of cells' immune activities. These properties are a result of its function in maintaining an equilibrium between glycolysis and the TCA cycle (62). As we mentioned before, they are cofactors for enzymes participating in these pathway's. The TCA energy cycle represents the main

source of naïve T cells, rest macrophages, and T-regulatory cells. Interestingly, activated T helper cells need energy from aerobic glycolysis because the amount of energy from TCA is not sufficient (63). Due to the significant effects of thiamine on these pathways, B1 deficiencies have so significant side effects. One of the side effects is linked to the stimulation of IL-1, IL-6, and TNF-α (pro-inflammatory cytokines) expression and neuro-inflammation. Finally, neuronal death may occur due to the inhibition of CD 40 and CD 40L regulation (64). It was observed that B1 could be used in the treatment of neurodegenerative diseases through its involvement in the suppression of the pro-oxidative activity of microglial cells (65). Additionally, in regards to B vitamins, we should pay attention to their role in oncogenesis and more over is extremely important to clearly make a distinction between healthy and sick individuals. Some speculations exist regarding the role of B1 in cancer due to its involvement as a cofactor in proliferation and energy pathways that are essential in the development of tumor cells. Further research is needed in order to clearly distinguish Thiamine's possible oncogenic effects (58, 66).

Riboflavin, or vitamin B2 is crucial for energy metabolism through its function as a cofactor (67). It also plays an important role as an anti-inflammatory and anti-oxidant modulator, especially in lungs (68, 69). Some specific aspects regarding the link between major histocompatibility complex (MHC) and B2 bacterial compounds are worth mentioning. This function on the innate mucosal results in the stimulation of invariant T cells. Riboflavin and its precursors selectively activate mucosa-associated invariant T cells (MAIT) that represent the largest population of innate-like T cells in humans. Their synthesis as well as the link with the major histocompatibility complex through the major histocompatibility complex-protein (MR1) are not fully understood. It was observed that the activation of MAIT cells is dependent on genes that encode enzymes responsible for the formation of intermediate compounds in the synthesis of bacterial riboflavin. (70). These types of cells are known for their function in the inflammation and defense activity in gut mucosal due to their production of IL-17 and IFN-γ (71). The proliferation of neutrophils and monocytes as well as the stimulation of macrophages and neutrophils activities might be boosted by the activity of riboflavin (72, 73). The catabolism of inhibitory kappa B (IκB) is responsible for the activation of the pro-inflammatory factor Kappa B (NF-κB). Following this catabolic pathway, the inflammatory signaling pathway becomes activated. At the end of this signaling pathway, the activation of pro-inflammatory cytokines, such as TNF-α and ILs, takes place. In this signaling process vitamin B2, act as an anti-inflammatory suppressor and it may block the activation of the NF-κB (74). Furthermore, through the overexpression of catalase and nitric oxide synthase vitamin B2 could reduce oxidative stress (75).

Vitamin B3, niacin (**Table 1**) is known as NADP and NAD precursor. Similarly, to all B vitamins, it is a cofactor for a wide variety of enzymes involved in several metabolic pathways. In contrast to other B vitamin groups, human cells can synthesize NADP and NAD cofactors through independent pathways. From a biochemical point of view, niacin and the resulting cofactors are involved in redox reactions. NAD is responsible for genomic equilibrium and epigenetic regulation may represent its mechanism of action (76). Additionally, there is a positive correlation between high concentrations of NAD and the blockage of ROS synthesis (77). Furthermore, NAD can be considered an anti-inflammatory micronutrient due to its inhibitory and deacetylation actions, which were observed in the NF-κB pathway (78). Also, it has an inhibitory effect on inflammatory cytokines as well as on animal tumor cells (79). NAD is also considered an efficient anti-inflammatory component since it induces the reduction of certain cytokines released from alveolar macrophages (80).

B12, cobalamin (**Table 1**) affects pro- and anti-inflammatory responses. A negative correlation has been observed between vitamin B12 and TNF-α (81). It has been demonstrated that an increase of TNF-α induce the exhaustion of antioxidants involved in the defense against free radicals (82). As a result, pro-inflammatory cytokines and some other pro-inflammatory compounds are activated (83). Interestingly, in human anemia with cobalamin deficiency, the number of CD8 + T cells decreases compared to the levels in healthy individuals. In contrast, an increase in the number of CD4 + T cells has been observed in patients with cobalamin deficiencies, which differed compared to healthy people. In these cases, the CD4 + /CD8 + ratio is pathological higher. Additionally, in these patients, the activity of NK cells is decreased (84). Interestingly, hyperhomocysteinemia is the result of vitamin B12 deficiency (85), leading to chronic diseases such as insulin resistance (86) and coronary heart disease (87) through the expansion of inflammatory processes. Since vitamin B12 deficiency is associated with abnormal TNF-α activity, it can also lead to insulin resistance (88, 89). Regarding cancer activity, a study by Cheng et al. (90) from a genetic perspective found no correlation between B12 and certain types of cancers such as squamous cell carcinoma, prostate, breast, and colorectal cancer. In the case of lung cancer, B12 administration was not considered a risk factor (91). On the contrary, a higher intake of B12 was considered dangerous for many types of cancer as indicated in a big meta-analysis of cancer patients (92).

Vitamin C

Vitamin C (**Table 1**), is considered an essential micronutrient (93) in humans since they cannot synthesize it. Human absorption of vitamin C is higher compared to other species that are capable to synthesized it (94, 95). Vitamin C is involved in the modulation of a wide variety of immune functions and play a role as a regulator of cell-signaling. Vitamin C is also, involved in gene transcription as well as in

TABLE 1 Vitamins with pro-and anti-inflammatory effects as well as pro-tumor and anti-tumor effects.

Nutrients	Anti-inflammatory effects	Pro-inflammatory effects	Antitumor effects	Tumor effects
Vitamin A	It is capable of promoting the Th2 anti-inflammatory response through repression of IL-12 and IFNγ which are synthesized by Th1 lymphocytes (46). Stimulates production of anti-inflammatory cytokine (IL-10) (47).	It is a positive relationship between vitamin A and mitogen-induced pro-inflammatory cytokine (IFN-γ) (47). Under inflammatory conditions RA may sustain or cause stimulation of intestinal inflammation (50). Through the liberation of certain cytokines such as IL-1, IL-6, IL-12, and nitric oxide is shown that RA may affect macrophages' activity (51).	It has antitumor effects on human pancreatic cell lines (53). It has antitumor effects in metastasis renal carcinoma by ATRA. It seems that all-trans-RA (ATRA) has an antitumor effect (54). In acute promyelocytic leukemia (APL), ATRA is utilized as a very efficient therapeutic agent (56).	
Vitamin B1(Thiamin)	Anti-inflammatory effects are observed due to the fact that B1 deficiencies side effects is linked to stimulation of IL-1, IL-6, and TNF-α (pro-inflammatory cytokines) expression and neuro-inflammation (64). B1 may be used in the treatment of neurodegenerative diseases through its involvement in the suppression of the pro-oxidative activity of microglial cells (65).			There are some speculations regarding its role in cancer due to its involvement as a cofactor in proliferation and energy pathways that are essential in the development of tumor cells (60, 66).
Vitamin B2 (Riboflavin)	Anti-inflammatory and anti-oxidant modulator, especially in lungs (68, 69). Vitamin B2, act as an anti-inflammatory suppressor. It may block the activation of the NF-κB (74).	B2 bacterial compounds stimulate innate mucosal through invariant T cells which are recognized by their inflammation and defense function in gut mucosal by their products IL-17 and IFN-γ (71).		
Vitamin B3 (Niacin)	Through deacetylation and suppression of NF-κB, NAD may be an anti-inflammatory nutrient (78). It has inhibition effects on inflammatory cytokines (79). It is responsible for diminution of certain alveolar macrophages cytokines such as IL-6, IL-1α, and tumor necrosis factor -α after niacin administration (80). Niacin was considered an inhibitory factor for pro-inflammatory cytokines (80).		It inhibits proliferation of animal tumor cells (79).	
Vitamin B12 (Cobalamin)	It has been found a negative relationship between vitamin B12 and TNF-α (81). Vitamin B12 deficiency is recognized to increase in chronic diseases like insulin resistance (86) and coronary heart disease (87) the inflammatory processes.		No correlation was found between B12 and certain types of cancer like squamous cell carcinoma, prostate, breast, and colorectal (90).	Highintake of B12 was considered hazardous for all types of cancer in a big meta-analysis of cancer patients (92).
Vitamin C	Vitamin C is responsible for preventing activity of pro-inflammatory cytokines and launching the NF-κB reaction (98). In peripheral blood cultures that are stimulated with LPS (lipopolysaccharide) after vitamin C administration was observed an enhancement of IL-10 and a reduction of TNF-α and IFN-γ (99). Vitamin C may be an antioxidant protector for the skin in the fight against ROS as a result of external factors' synergistic work, particularly pollutants (102).			

(Continued)

TABLE 1 (Continued)

Nutrients	Anti-inflammatory effects	Pro-inflammatory effects	Antitumor effects	Tumor effects
	(104) et al., 2015 confirmed the decreased inflammation effects in hypertensive and/or diabetic adults through a moderate decline of inflammatory markers such as hs-CRP and IL-6. It is involved in the regulation of HIF-1α activity, which is capable to make possible neutrophil viability under hypoxic conditions (105).		It is supposed that vitamin C is involved in the fight against tumor culture cells through the number enhancement of the NK cells (107).	
Vitamin D	Vitamin D may be an anti-inflammatory nutrient, through suppression of NF-κB (116). It is responsible for the inhibition of specific pro-inflammatory Th1 cells cytokines like TNF-α, IFN-γ, IL-6, IL-2, and IL-17 (117, 118). It is capable to increase the number of cytokines such as IL-10, IL-4, and IL-5 as a result of an increase in the activity of Th2 cells (119). At a molecular level, through the suppression of pro-inflammatory cytokines and prostaglandins (PG) action as well as stopping the NF-κB signaling pathway calcitriol is considered an anti-inflammatory nutrient (128).		Anti-cancer action of vitamin D is extrapolated in tumor cells by calcitriol which is the active biologically and hormonally compound of vitamin D (127). The stimulation of apoptosis, the suppression of cancer cell proliferation, and delayed tumor development in cancer are certain effects of calcitriol (126, 127). Calcitriol may be used as a preventive and therapeutic agent in cancer (128).	

ATRA, all-trans-RA; HIF-1α, hypoxia-inducible factor 1-alpha; IL, interleukin; IFNγ, Interferon γ; NK, natural killer; NF-κB, pro-inflammatory factor Kappa B; hs-CRP high-sensitivity C-reactive protein; RA, retinoic acid; ROS, reactive oxygen species; Th, helper T cell; TNF-α, tumor necrosis factor.

hydroxylation reactions (96). Through its main function as an antioxidant, it became capable to defend the body against reactive oxygen species that are the result of the activity of toxins and pollution (97).

Vitamin C is responsible for discontinuing the action of the pro-inflammatory cytokines and inhibiting the initiation of the NF-κB reaction (98). In peripheral blood cultures that are stimulated with LPS (lipopolysaccharide), after vitamin C administration, an enhancement of IL-10 and a reduction of TNF-α and IFN-γ has been observed (99). Moreover, as a result of ROS accumulation in microbial infections, vitamin C causes neutrophils displacement into infected sites (100). Additionally, vitamin C might be useful as a cofactor in the synthesis pathways for vasopressin and norepinephrine in severe infections. This has a noticeable effect on the infection response of the cardiovascular system when the pathological state represents a danger (101). It appears that vitamin C may be considered as an antioxidant protector for the skin in the fight against ROS as a result of external factors' synergistic work, particularly of pollutants (102). In this case, the effect is more pronounced if vitamin C is administrated in combination with vitamin E (103). Ellulu et al. (104) have demonstrated in hypertensive and/or diabetic adults that following C vitamin treatment a decreased inflammation associated with a moderate decline in inflammatory markers such as: the high-sensitivity C-reactive protein (hs-CRP) and IL-6 is observed. Vitamin C is also involved in the regulation of hypoxia-inducible factor 1-alpha (HIF-1α) activity, which makes neutrophil viability under hypoxic conditions possible (105), and in this way, neutrophil apoptosis is delayed (106). Furthermore, it is thought that vitamin C is involved in the fight against tumor cells through the increase in the number of NK cells (107).

Vitamin D

Vitamin D, (**Table 1**) exerts many anti-inflammatory roles (108) since receptors to this vitamin are expressed in different organs throughout the human body. The best known and established effects are linked to mineral and bone metabolism (109). Wöbke et al. (108) demonstrated that vitamin D effects are mediated through either nuclear and cytosolic signaling control pathways involving also pro-inflammatory components. Vitamin D binds its receptors (VDR) resulting in a complex of vitamin D-VDR that may contribute to the formation of homodimer with an additional VDR or formation of a heterodimer compound with the nuclear retinoid X receptor (RXR). Also, the nuclear role is demonstrated following the formation of heterodimers with steroid hormone receptors (110). Vitamin D bound

to/VDR/RXR can cross the nuclear membrane, and then binds to a response element and start its specific gene regulation action (111) through activation of expression of its responsive genes.

Vitamin D is apparently involved in the adaptive immunity since immune cells such B and T cells express a high number of VDR's (112). From the immunological regulatory aspect, vitamin D can block the secretion of the pro-inflammatory cytokines IL-6 or TNFα in monocytes (113). Additionally, the same effect has been observed in prostate cells (114). These effects are caused by the inhibition on P-38 MAP kinase (a subclass of mitogen-activated protein kinase) as a response to pro-inflammatory cytokines (113). Moreover, there is an interesting relationship between VDR/RXR and MAP kinase signaling path in terms of activation or inhibition. The results are closely related to the specificity of cells, their response, and effects of triggering factors (115). Furthermore, the complex VDR/RXR can bind to other compounds involved in the transcription process, such as glucocorticoid receptor (GCR) and NF-κB. Thus, vitamin D may be considered an anti-inflammatory micronutrient as a result of these interactions.

The vitamin D bound VDR becomes active and thus exerts inhibitory effects on NF-κB, which is also a heterodimer compound (116). Additionally, some studies suggest anti-inflammatory role of D vitamin is mediated also through the inhibition of specific pro-inflammatory Th1 cell cytokines such as TNF-α, IFN-γ, IL-6, IL-2, and IL-17 (117, 118). Additionally, vitamin D is capable of increasing the concentration of cytokines such as IL-10, IL-4, and IL-5 as a result of an increase in the activity of Th2 cells (119). At the same time, it may induce the amplification of Treg cells as well as a reduction of the number of Th17 cells (120, 121).

From a medical perspective, vitamin D has an important effect on the lung defense system against microbial pathogens. This function represents the result of the antimicrobial peptides activation expression in monocytes, epithelial cells lining the respiratory tract, monocytes, neutrophils, and NK cells (122). Lower levels of vitamin D in the serum are correlated with higher infection risks (123). Particularly, the administration of vitamin D induce a decline in acute respiratory infections (124).

The anti-cancer effect of vitamin D in tumor cells is mediated by calcitriol which is the biologically active molecule of vitamin D (125). The stimulation of apoptosis, the suppression of cancer cell proliferation, and associated delayed tumor development in cancer are the main effects mediated by calcitriol (125, 126). At a molecular level, through the suppression of pro-inflammatory cytokines and prostaglandins (PG) activity as well as by preventing the NF-κB signaling pathway, calcitriol is considered an anti-inflammatory nutrient (127). From this point of view, calcitriol may be used as a preventive and therapeutic agent in cancer (128).

The minerals-zinc and selenium

When inflammatory cytokines are maintained at a high level, chronic inflammation takes place (129). This process is closely linked to the action of some minerals. In this way, it is important to test what is the role of Zinc in this essential process. The process is mediated (**Table 2**) by the activity of several signaling pathways that are triggered due to the action of some changes produced by antigens and their metabolites. The main compound involved in inflammatory responses as a result of its role in cell proliferation, cell apoptosis, and the release of certain cytokines like IL-6, IL-8, and IL-1β are mediated by the activity of the NF-κB factor (130). The role of Zinc in this regard is controversial. *In vitro* studies demonstrate that the zinc effects can be either anti-or-pro- inflammatory (131). The NF-κB signaling pathway can be blocked by distinctive intracellular membrane chelator such as TPEN (N, N, N', N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine) (132). From one side the apoptosis effect is evident following the binding of the chelator and heavy metals (133). From the other side, other studies indicate a strong relationship between the initiation of LPS-induced NF-κB and zinc (132). Moreover, after a decline in the release of IL-1β, zinc is able to inhibit pro-inflammatory actions (134).

Furthermore, it has been reported that cytokine synthesis is dependent on Zinc status and this is closely related to chronic inflammation. In this regard, it has been observed that obese people having low zinc plasma concentrations over-express IL-1β, IL-1α and IL-6 genes (135). Zinc exerts beneficial effects on the proliferation and differentiation of T lymphocytes (46). The strong relation between a high number of cytokines and the decline in zinc plasma levels in infections and trauma-associated conditions has been demonstrated in cross-sectional studies. In patients with severe head injuries, upregulated cytokine production genes have been observed (136). In addition, the production of cytokines is elevated in patients that are in a critical state due to their decrease in plasma zinc concentration (137). Moreover, zinc antioxidant effects help the body to defend against reactive nitrogen species (RNS) and reactive oxygen species (ROS) (138). Zinc is able to induce the initiation of the CD8 + T cells proliferation (36, 139). Zinc also can be capable to support the integrity of skin and the mucous membrane (36). To summarize, zinc has an essential effect on the proliferation and development of cells belonging to the immune system, such as T lymphocytes, CD8 + T cells, etc., which are known for their quick turnover. In this regard, it was observed that deficiency of this mineral negatively impacts health by decreasing resistance to infectious diseases, dermatitis, growth diseases, and genetic disorders (140).

Another crucial micronutrient is selenium (**Table 2**), which is involved in the functioning of the thyroid metabolism and the cardiovascular system as well as in ensuring a functional immune system and preventing cancer. From the cellular point

TABLE 2 Selenium and Zinc with pro-and anti-inflammatory effects as well as anti-tumor.

Nutrients	Anti-inflammatory effects	Pro-inflammatory effects	Antitumor effects
Zinc	Zinc may block the NF-κB signaling pathway through chelating with a distinctive intracellular membrane chelator such as TPEN (132). NF-κB signaling pathways may be inhibited by Zinc through a lot of suggested mechanisms (29). Through a decline of IL-1β gene expression, Zinc is responsible for the inflammatory cytokines number reduction. It is capable to inhibit TNF-α (134). It was observed that obese persons with low plasma concentrations of zinc had overexpression of, IL-1β, IL-1α and IL-6 genes (135). The cytokine production is much higher in patients with a critical state of health who were evaluated immediately after intensive therapy due to their decrease in plasma zinc concentration (137). It is able to induce the initiation of the CD8+ T cells proliferation (36, 139)	Some information has shown that the initiation of LPS-induced NF-κB is dependent on zinc (132).	
Selenium	Could be implied in inflammatory mediators production (143, 144). It may acts as a cofactor in immunity that is mediated by the vaccine (148). After selenium treatment was observed a decline in IL-1 and TNF-α gene expression (149). Can enhance the immune response of Th1 cells and the stimulation of T cells (11). Antibody increase titers due to selenium supplementation cause an enhancer of vaccine effects (145, 146).		In patients with cancer, the supplementation of selenium increased antibody titers of IgA and IgG as well as the number of neutrophils (150).

IL, interleukin; IFNγ, Interferon γ; NF-κB, pro-inflammatory factor Kappa B; ROS, reactive oxygen species; Th, helper T cell; TNF-α, tumor necrosis factor; TPEN, N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine.

of view, there are still discrepancies regarding the exact dose that may be translated into deficiency or toxicity, even if these stages do not commonly take place in the human body (141, 142). It is well known that when selenium is present within the amino acid selenocysteine is able to control certain metabolic reactions that may lead to lipoxygenase synthesis that finally, can be involved in the production of inflammatory mediators (143, 144). In mice, selenium, due to stimulation of T cell receptor complexes (TCR) activity and conversion of Th1 from T0 cells, may improve the regulation of cellular immunity (145). Selenium can also contribute to the defense against pathogens as a result of its effects on redox signaling activities (146). It was recently demonstrated that in COVID-19 patients, selenium together with zinc exert a protective role and they are associated with a higher chance of survival (147). During vaccination against COVID-19, it has been demonstrated that the response may increase after selenium administration as well as the increase of titers antibodies. It is assumed that selenium may act as a cofactor in immunity response that is mediated by the vaccine (148).

Additionally, in women that are infertile as a result of polycystic ovary syndrome, to whom fertilization *in vitro* has been recommended, a decline in IL-1 and TNF-α gene expression was observed as a result of selenium treatment (149). This effect suggests that selenium has an anti-inflammatory role in the human body. Furthermore, in patients with cancer, the supplementation of selenium increased antibody titers of IgA and IgG as well as the number of neutrophils (150). We can say that selenium is involved in the regulation of the inflammatory mediators' synthesis and, also, it might increase the activity of phagocytic cells as well. Selenium is capable to enhance the immune response of Th1 cells and the stimulation of T cells. Selenium has a positive relationship with the number of B cells. The innate immune system may be strengthened after selenium administration. A similar effect has been observed on cellular immunity (11). Increased titers of antibodies were measured due to selenium supplements that can cause an enhancement of vaccine effects (145, 146). In the brain, both neurogenesis and hippocampal neural precursor cells are increased after selenium infusion (151).

Macronutrients: Amino acids arginine and tryptophan

Besides micronutrients, macronutrients, such as proteins and amino acids, also play an important role in the activity of the immune system. Proteins are formed from amino acids that are essential in the construction of other proteins among which antibodies and cytokines that are typical proteins belonging to the immune system (20).

Arginine (**Table 3**) contribute with the production of nitric oxide in macrophage cells. Nitric oxide (NO) resulting from arginine under the action of nitric oxide synthase (iNOS) determines the cytotoxicity of macrophages in the fight against antigens such as pathogenic bacteria and parasites. Moreover, M1 macrophages use arginine to produce NO (152). Even though that arginine was initially considered a non-essential amino acid (153), after one decade, some papers have proven that arginine is essential for embryonic outliving,

ontogenetic fetal development, and for constant hemodynamics and vascular parameters (154). Moreover, the induction of the NF-κB pathway has been linked to the arginine degradation pathway (16). As we presented previously, arginine through cations dependent mechanism can improve the release of insulin from pancreatic β cells. In addition, in β-pancreatic cells, arginine causes an increase in Ca^{2+}concentration due to electron transport through a mechanism dependent on the amino acid mCAT2A transporter. When the membrane was depolarized, the Ca^{2+} voltage-dependent channels are opened, followed by the increase in the intracytoplasmic concentration of Calcium and finally the stimulation of insulin secretion. However, clinical evaluations have shown that the beneficial effects of arginine administration are limited, probably due to the fact that it is very quickly transformed into ornithine or citrulline in epithelial cells (17). In addition, the polyamines, compounds which are also derived from arginine degradation, are involved in balanced levels of membrane, mRNA and DNA. Thus polyamines are capable to control the proliferation of cells (155). *In vitro*, polyamines can modify the inflammatory process (156). Furthermore, it has been demonstrated that higher concentrations of intracellular polyamines may change the *in vitro* cytotoxicity regulated by macrophage cells (157). The inflammation regulation and identification of pathogens are closely related to polyamines through their binding manner to receptor-ligand complexes (155).

In the last decades, it has been discovered that arginine is more beneficial than it has been supposed to be in the past, for example arginine induces the decline of oxidative stress and causes the reduction of the intestine's inflammation (158).

Arginine is capable of diminishing intestinal damage and reestablishing mucosal immune equilibrium in humans and mammalians' intestinal diseases (159). In an *in vitro* intestinal system in Caco-2 cells, arginine is able to induce the inhibition of IL-1β-mediated NF-κB pathway (160). However, the mechanism of reducing the inflammatory pathways is still unknown. Perhaps, it is linked to the activity of Arginase-1 (Arg-1), which, in this case, is stimulated by L-arginine as a substrate. The arginase-1 is an enzyme involved in the end of the urea cycle with the aim of forming l-ornithine and urea from l-arginine (161). Some studies suggest that the Arg-1 has positive effects in certain inflammatory diseases through an anti-inflammatory action (162, 163). In contrast, there are studies which have shown that higher metabolism of arginine in tumors cells, together with their particular environment, create conditions that are intermediary, and at the same time, crucial for the maintenance and development of cancer cells. The result of these actions is translated into proper immunosuppression (164, 165). One thing is certain, that the relationship between arginine and cancer cells is controversial. On the one hand there is data suggesting that arginine deprivation is correlated with a delay in the development of some tumor cells (166). On the other hand, arginine can have antitumoral actions which are observed through the enhancement of immune response (167). Furthermore, Al-Koussa et al. (168) have shown that certain kinds of cancer cells need arginine to develop. In this sense, some authors suggest that arginine deprivation may downregulate the migration of cancer cells. In physiological conditions, the movement process is useful for embryonic growth and immune function. But when it comes to cancer cells, things are different. This happens since certain kinds of cancer cells can use this property with the aim to stimulate metastasis (169, 170). Therefore, arginine deprivation in cancer cells is capable of reducing metastatic activity (168). Unfortunately, the exact mechanism remains unknown.

Tryptophan (Trp) is clearly essential for the activity of the immune system (**Table 3**). Since Trp is necessary for protein synthesis, it becomes to be indispensable for cell division and development (171). Since Trp is not synthesized by the human body, it is required to be obtained from the diet (172). Trp serves as a substrate for the biosynthesis and formation of serotonin (5-HT), kynurenine (Kyn), and indoles (173). The most useful and active Trp metabolism is the Kyn path which is related to the formation of nicotinamide adenine dinucleotide (NAD) and kynurenic acid. Of course, similarly to all pathways, this type takes place due to the involvement of two types of enzymes indoleamine 2,3-dioxygenase (IDO and IDO2) and tryptophan 2,3-dioxygenase (TDO) (174, 175). Additionaly to the Trp metabolism, we brought information on its role in the regulation of inflammation through its initiators, starting with IDO, which exerts an insignificant effect on healthy and normal conditions. Things are changed by some cytokines, including interferons which represent the result of the triggered inflammatory process (176). The highly potent and amply used cytokine interferon-gamma (IFN-γ). It is linked to the promotor-region of IDO and it is capable to express itself in many types of cells. However, the highest expressive grade is found in dendritic cells and macrophages, but there are some other places where it was manifest such as epithelial and connective tissues (177–180). When it comes to cancer, infections, auto-immune diseases, or cardiovascular problems, the serum ratio Kyn/Trp may represent a marker for inflammation which is related to IDO activity (181). As we discussed before, inflammation and chronic immune tolerance are regulated by Trp biochemistry. This being affected by the ability of IDO to change the Kyn/Trp ratio. IDO-competent cells may trigger an anti-inflammatory action through the Kyn/Trp equilibrium, which has the ability to influence some immune signaling and certain metabolic pathways (182). The last step metabolite of the Kyn pathway is represented by the NAD +, which is known for initiating CD8 + and CD4 + lymphocytes programmed death (183). In tumor cells, an important step for metabolic reprogramming is represented by amino acids metabolism. Some authors suggest that, in the case of glioma, there is a strong link between the two because the metabolic amino acid pathway could be used as a predictor for survival

TABLE 3 Macronutrients implied in mediate pro-and anti-inflammatory responses.

Nutrients	Anti-inflammatory effects	Pro-inflammatory effects	Antitumor effects	Tumor effects
Arginine	The induction of the NF-κB pathway was linked with the arginine degradation pathway (16). In Caco-2 cells, arginine was able to induce the inhibition of the IL-1β-mediated NF-κB pathway (160). Some studies that showed the Arg-1 positive effects in certain diseases that are inflammatory through an anti-inflammatory action (162, 163).		There is information that reported the antitumor role of arginine through the improvement of immune response (167).	There are studies were shown that the higher metabolism of arginine in tumors cells together with their particular environment creates conditions that are intermediary and at the same time crucial for the maintenance and development of cancer cells (164, 165). Data were shown that the arginine deprivation is correlated with a delay in the development of some tumor cells (166). (168) et al., 2020 show that certain kinds of cancer cells need arginine to develop. Some authors explained that arginine deprivation may downregulate the migration of cancer cells (169, 170).
Tryptophan (Trp)	IDO-competent cells may trigger an anti-inflammatory action through the Kyn/Trp equilibrium, which has the ability to influence some signaling immune and certain metabolic pathways (182).	Usually, IDO, the tryptophan metabolite has an insignificant effect in healthy and normal conditions. Things are changed by some cytokines, including interferons as a result of the triggered inflammatory process (176). In cases of cancer, infections, auto-immune diseases, or cardiovascular problems the blood ratio Kyn/Trp may represent a marker for inflammation which is linked to IDO activity(181).	The metabolite of tryptophan 5-methoxytryptophan (5-MTP), has the ability to suppress the development of tumors and the displacement of cancer cells in other tissues (187).	The arginine deprivation of cancer cells is capable to reduce metastatic activity (166). Kynurenine metabolism is able to stimulate an oxidative stress resistance pathway, and in this way creating an opportunity to make changes in the tumor microenvironment that help the development of the tumor (186).

Arg-1, type-I arginase; IDO, indoleamine 2,3-dioxygenase; Kyn/Trp, kynurenine/tryptophan; 5-methoxytryptophan, 5-MTP; IL, interleukin; NF-κB, pro-inflammatory factor Kappa B; Th, helper T cell.

as well as certain clinical characteristics (184). As we mentioned before, amino acids and their metabolites are responsible for both controlling malignant cells as well as for changing the microenvironment. In this way, the results are translated into the improvement of immunosuppression and malignancy state (185). Kynurenine metabolism is capable of stimulating an oxidative stress resistance pathway, and, in this way, creating an opportunity to make changes in the tumor microenvironment that helps the development of the tumor (186). However, another metabolite of tryptophan; 5-methoxytryptophan (5-MTP) has the ability to suppress the development of tumors and the displacement of cancer cells in other tissues. Wu et al. (187) consider that these effects are due to suppressing the activity of cyclooxygenase-2 (COX-2). This type of inflammation-associated enzyme is very abundant in tumor cells and also contributes to development process of cancer (187).

The role of cholesterol in the immune response

Cholesterol has a key function on cellular membranes functionality, especially in the plasma membrane of the cell where it is found at higher concentrations. Its special location at the lipid bilayer allows optimal interaction with other lipids and displays a significant role in membrane fluidity. Cholesterol points its structure mainly into the lipid bilayer leaving only the hydroxyl group facing the external environment. Thus, the steroid rings are in close vicinity to the hydrocarbon chains of adjacent lipids (188). Cholesterol is vital for the many physiological roles that the plasma membrane is involved. The cells keep their lipid bilayer appropriate functionality due to cholesterol molecules, otherwise, microenvironment, endocytosis, signaling pathways, as well as other functions,

would be altered. Cholesterol is involved in membrane integrity and it is responsible for receptors arrangement and bilayer fluidity (189, 190). For a better understanding of the information provided later-on, we will describe the cholesterol synthesis and pathway in a schematic frame. Cholesterol biosynthesis of is characterized by a complex pathway, nonetheless the pathways involved have been clearly elucidated (191). Its synthesis involves more than 20 metabolic-specific actions, which include enzymatic reactions belonging to the mevalonate pathway of and additional synthesis pathway of cholesterol. Enzymes involved in cholesterol biosynthesis are mainly detected in the membranes of the endoplasmic reticulum (ER). These enzymes are the target of several molecular reactions which, closely controlled in order to not allow cellular damage (191, 192). However, cholesterol is non-uniformly disseminated in the plasmalemma. In the cell, the plasma membrane represents the main pool/storage. It has been observed that each pool is corresponded to an exclusive function in the plasma membrane physiology (193–195).

It is clear that cholesterol equilibrium involves a transport mechanism by virtue of the concentration gradient from high concentration cholesterol places to regions where cholesterol has been lost or has a low level. The transport of cholesterol is dependent on proteins due to its hydrophobic conformation, thus it cannot be transported through the blood. Thus cholesterol binds to different proteins and forms distinct lipoprotein compounds such as low-density (LDL) and high-density (HDL). As expected, regulatory mechanisms for the formation of each lipoprotein are specific (196). The surplus of cholesterol can be transported through the efflux process or deposited as intracellular lipid droplets (191) because of the incapacity of most human cells to efficiently degrade it. The deposition of lipid droplet plaques in the bloodstream causes the release of inflammatory cytokines which create later an inflammatory process. The consequence of this event is associated with inflammation triggered by the cytokine interleukin-1β (IL-1β) (197). Furthermore, IL-1β is considered an important marker in the inflammatory process (198).

The cholesterol signaling pathway plays a role in the immune response we therefore will highlight these pathways. Sterol response element-binding protein (SREBP) exerts an essential role in the signaling pathway of cholesterol (**Figure 3**). Normally, these proteins are located in the membrane of the ER, which is capable of binding with additional two complex proteins such as the cleavage-activating protein and generating (SCAP) (199) and the insulin-inducible genes (INSIGs) (200). The shift of SCAP from ER to the Golgi apparatus plays a key role in its activity. SREBPs proteins are composed of three variants SREBP1a, SREBP1c, and SREBP2, being the latter the most important (199). SREBP2 is a protein complex structure that seems to be capable to regulate the expression of all the enzymes that are involved in cholesterol biosynthesis (201). Its most important activity is its specific response to high concentration of sterols which are able to efficiently induce a decrease in cholesterol synthesis. SREBP2 fulfills its function when the sterols concentration decrease. This change in sterol concentration due to SREBP2 activity will generate afterward the shifting the complex SCAP from ER to the Golgi apparatus (200). In this organelle, the SCAP molecule is changed (199). Once the SCAP reaches the Golgi apparatus, proteases sit 1 and 2 cut this complex (**Figure 3**). As a result, the transcription factor (TF) is created and stimulated (202). Then the TF enters the nucleus where it is responsible for the regulation of the cholesterol synthetic pathway enzymes (203). Regarding immune-related mechanisms, SREBP2 may be activated in the immune cells' T cell receptor (TCR) and B cell receptor (BCR) through their signaling pathways. All these pathways may stimulate the flux of cholesterol biosynthesis (204, 205).

Additionally, it is important to mention that all cholesterol associated pathways involving synthesis, influx, efflux, and esterification take place through mechanisms closely related to each other allowing well-adjusted whole mechanistic biochemical pathways. All these tightly controlled mechanisms highlight the crucial role of cholesterol in life span, and clarify the potential risks when the concentrations are diverted from the optimal range. In this regards, Luo et al. (191) have demonstrated that there is a correlation between certain metabolic diseases such as familial hypercholesterolemia, Schnyder corneal disease and altered cholesterol metabolism (191). Moreover, in several diseases such as various types of cancer, infections and allergies, cholesterol biochemical equilibrium is severely altered through inflammation-associated consequences. Regarding the relationship between cholesterol and macrophages, counter-regulatory mechanisms oppose macrophage inflammation and at the same time cholesterol cellular accumulation. When the concentration of cellular cholesterol increases, specific sterols are formed. With their help, the transcription factors liver X receptor (LXR)–retinoid X receptor (RXR) are activated. These heterodimers have anti-inflammatory roles, including controlling the expression of ATP-binding cassette transporters (ABC transporters), which are ABC subfamily A member 1 (ABCA1) and ABCG1. They are also involved in stimulating the efflux of cholesterol from macrophages. In this way, they can suppress the activation of TLR signaling given by the increased intracellular cholesterol concentration. When TLRs are activated, LXR genes are inhibited, thus decreasing the cholesterol efflux from macrophages. Activation of cholesterol efflux by ABCA1 and ABCG1 is via apolipoprotein A1 (APOA1), forming HDL and initiating the process of transporting cholesterol back to the liver via blood vessels and lymphatics. Therefore, as a way of amplifying the inflammatory response, the immune system can alter cholesterol homeostasis (206). When the control of cholesterol biosynthesis is disturbed resulting in high cholesterol levels it can be translated into metabolic diseases such as atherosclerosis and dyslipidemia

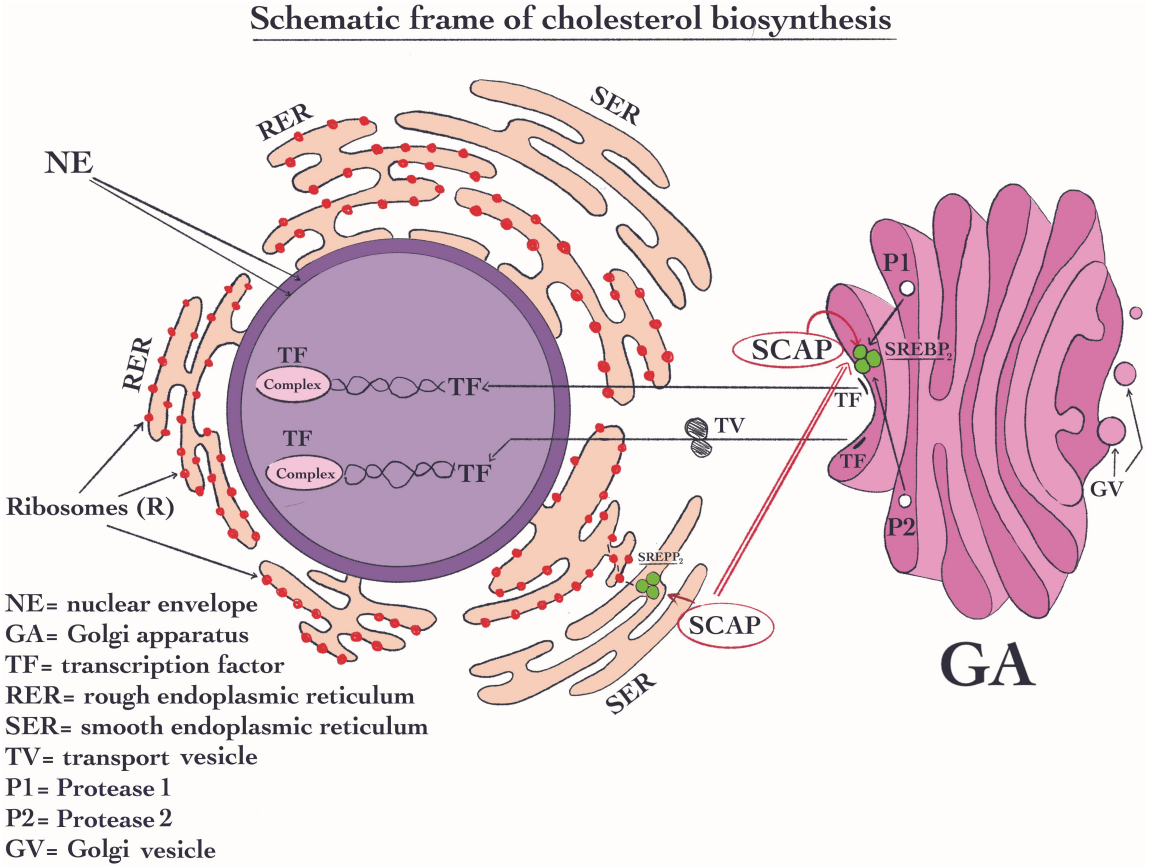

FIGURE 3
Schematic frame of cholesterol biosynthesis. In the signaling pathway of cholesterol, sterol response element-binding protein SREBP2 has an essential role. SREBP2 is located in RE, where it forms a complex with the protein like cleavage-activating protein and generating (SCAP); Its most important activity is to reduce the cellular cholesterol concentration when this is higher. Then, SCAP is shifted from ER to the Golgi apparatus. Once SCAP reaches the Golgi apparatus, proteases sit 1 and 2 digest this complex and subsequently, the transcription factor (TF) is formed and is activated (202). Then, TF moves into the nucleus where it becomes active and control the transcription of the enzymes of cholesterol biosynthetic pathway.

(Figure 4). In some of these cases, both the innate and the adaptive immune functions have the ability to regulate this phenomenon (207). In this way, ApoB-containing lipoproteins are originated immediately after atherosclerosis damages. These are generated, developed and stored in the endothelial compartment (208). Interestingly, these molecules exert pro-inflammatory effects through acetylation, oxidation, and especially induce aggregation with additional molecules (209). The modifications provoked by the accumulation of ApoB-containing lipoproteins (Figure 4) in endothelial location results in the growth of adherence, hold, and mobility in this place of immune cells (210). In summary, the inflammation is supported through the generation of ROS and certain cytokines such as (TNF) α, IL6, and (IL)1β (208, 210). When it comes to the inflammatory stage, the Treg cells exert an anti-inflammatory effect through the inhibition action of CD8 + Th and CD4 + T cells. At the same time, Th17 and Th1 are involved in the pro-inflammatory process (209, 211). Long-term inflammation (Figure 4) and the development of atherosclerosis are strongly linked with the decline of the Treg/Th17 ratio (212–214).

The polyunsaturated fatty acids effects on immunomodulation

Polyunsaturated fatty acids (PUFAs) are essential fatty acids that contain more than one double bond in their backbone. PUFAs are divided into two main groups: omega-3 and omega-6. The structural chemical difference between the two groups is represented by the location of the cis double bonds (215). Together with cholesterol, PUFAs are essential for cell membrane integrity, development and maintenance in the homeostasis of cell function. Moreover, they are used by certain structures in cells and they stimulate cell proliferation (216). Calder and Grimble demonstrated that the changes

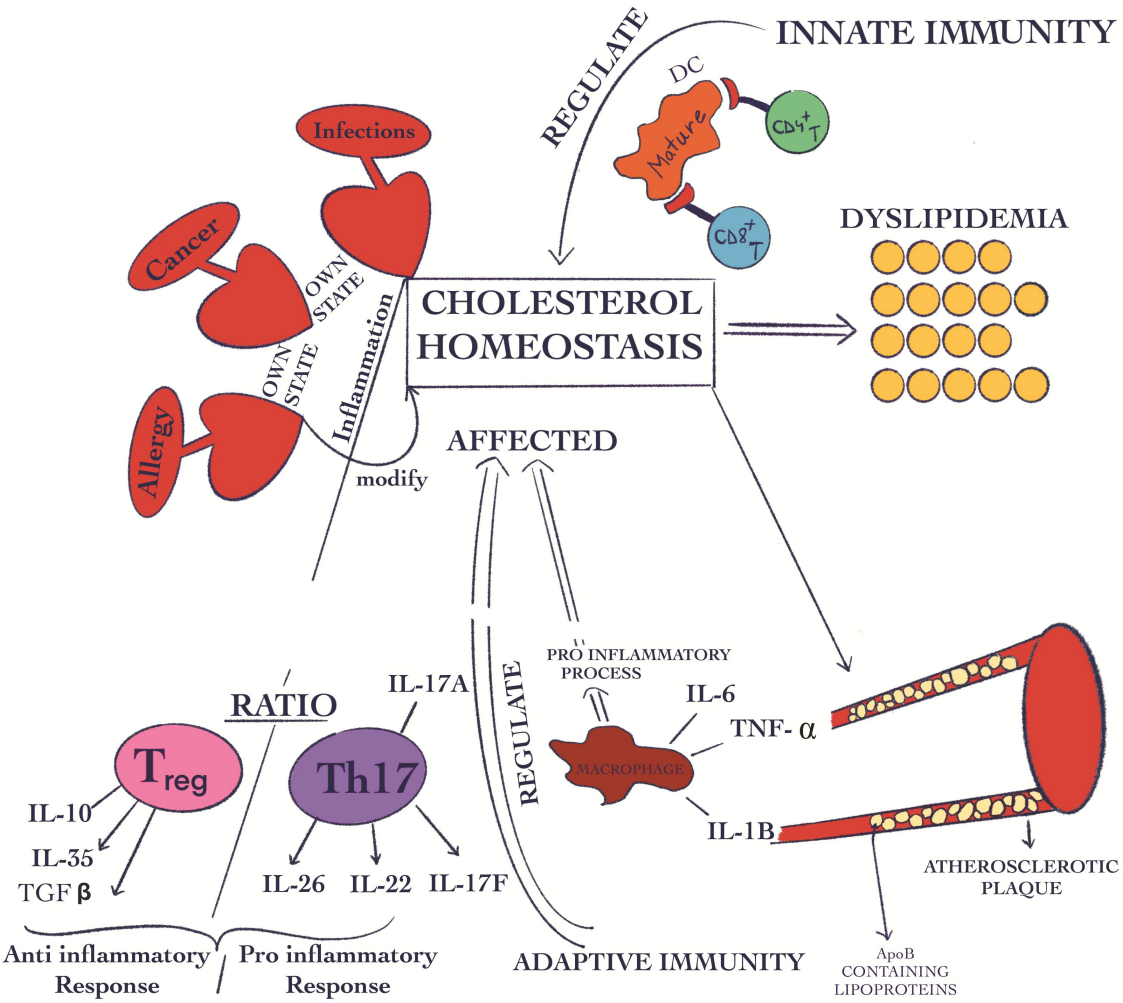

FIGURE 4
Cholesterol rate in the modulation of immune function. Some diseases, such as cancer, infections, and allergies, are capable of modifying the cholesterol biochemical equilibrium through their own state of inflammation; Atherosclerosis and dyslipidemia are negative effects of the increased cholesterol biosynthesis; The ApoB-containing lipoproteins are accumulated in endothelial place; This phenomenon has pro-inflammatory effects; The inflammation is correlated with the generation of certain cytokines like (TNF) α, IL6, and (IL)1β; The Treg cells have an anti-inflammatory effect; Th17 cells are involved in the pro-inflammatory process; The decline of Treg/Th17 ratio is linked with long-term inflammation.

in fatty acids' membrane composition can affect membrane fluidity, mostly due to their modification in the enzymes' affinity for substrates, which can change the signaling pathways. In this way, the sensitivity of the immune function can be modified (217). The most representative polyunsaturated fatty acids are eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA), and docosahexaenoic acid (DHA), all defined as omega-3 fatty acids (215). They are very intensively studied since they are involved in many essential vital activities and more interestingly in immunomodulation pathways. In addition, the ALA is important due to the fact that it is a precursor of other fatty acids (218). Omega-3 PUFAs have a role in immunomodulation by decreasing pro-inflammatory eicosanoids. They represent a substrate for AA cascade enzymes, in this way certain prostanoids and leukotrienes are produced. Some lipid mediators such as maresins have omega-3 PUFAs as precursors. They promote the ending of the inflammatory process (219). In human breast cancer cells ALA produce inhibition of cell proliferation and activate apoptosis (220). In diabetic rats, ALA increases insulin sensitivity and restored lipid and glucose metabolic abnormalities (221). ALA is considered essential because it cannot be synthesized by the human body. In these regards, from the omega-6 group, an essential is considered linoleic acid (LA). Following LA ingestion, this

fatty acid is quickly converted into arachidonic acid (ARA), which is responsible for the fluidity as well as the flexibility of the cell membrane. Free ARA is involved in the modulation of ion channels, enzymes, and receptors through stimulation or suppression of their function (222). Free unesterified ARA exerts antitumoral activity *in vitro* as well *in vivo*. It can be used as an anti-cancer drug. (223). Moreover, ARA can cause the death of tumor cells through the suppression of proliferation determining in this way, the death via stimulation of neutral sphingomyelinase (nSMase) mechanism (224).

Omega-3, as well as omega-6, participate in immunomodulation. According to Simonetto et al. (225) they have antagonist effects. Omega-3 from the PUFAs group is involved in anti-inflammatory reactions through the inhibition of ARA from the membrane, which is the main precursor for pro-inflammatory eicosanoids (225). They are capable to modulate immune and inflammatory responses through intensity and duration. On the one hand, pro-inflammatory effects are linked to fever, vasodilatation and intensification of pain. On the other hand, they could have anti-inflammatory effects by blocking natural killer activity and lymphocyte proliferation. Also, they are capable to inhibit IL-6, IL-2, and TNF-α (217). However, most importantly is the ratio between the 2 groups of PUFAs. Simopoulos, tried to shade light in this regards and proposed that a low omega-6/omega-3 ratio in women is responsible for the decrease in breast cancer risk. She additionally concluded that a lower ratio is associated with a general decrease in very common chronic diseases in the Western society (226).

Conclusion and future perspectives

The nutrients which have major effects on normal immune cell function and immune homeostasis

As mentioned throughout all this review, there is a strong and dynamic link between nutrition and immune function, as a direct consequence of the modulation of the immune function through the pro-inflammatory and anti-inflammatory effects of certain nutrients including cholesterol who exerts a crucial impact in these complex biological settings and holds a great capacity to regulate immune function, tightly related to its concentration. Certain micronutrients mentioned in this review: A, B1, B2, B3, B12, C, and D vitamins and minerals such as Zinc, and Selenium affect innate as well as adaptive immunity specifically through genetic, biochemical, and signaling pathways. All these may be translated into the modulation of proliferation, cell division, cell mobilization, and physiology of immune cells.

Additionally, we provide evidence that some macro-nutrients such as tryptophan, arginine, cholesterol and PUFAs may be involved in the prevention and therapy of some immune-related diseases. Also, is very important to note that some vitamins such as A and D are fat soluble (227). That is why when we consume fat-free (light) products, we are practically deprived of fat-soluble vitamins and the immune function can be affected. So, western diets should contain the accurate class of healthy fats, such as PUFAs, in a correct ratio, otherwise the edible products become poor in nutrients. A good example is the Mediterranean diet. In addition, the fats are much more satiating and give food a much better taste (228).

The nutrients implicated in inflammation and immunopathologies

We highlight the difference in response to micro- and macro nutrients between healthy and sick population. We provided evidence that the response in pathophysiologic stages are very different to normal physiologic stages additionally to interindividual variations. As a result, the immune response is different and variable (11, 229, 230). We also presented some evidences and speculations on the roles of some vitamins, as well as certain amino acids, in cancer patients, due to their involvement as cofactors in proliferation and energy-related pathways finally leading to the development of tumor cells. We foresee that further research needs to be done in order to clearly distinguish the possible oncogenic effects of thiamin, cobalamin, and arginine (58, 66).

We additionally provide evidence that the inflammatory responses in general, and the changes in immune functions are modified by the lack of an accurate cholesterol metabolism. The alterations in the cholesterol biosynthetic pathway may have both positive and negative immune health-related repercussions. Alterations in the cholesterol biosynthetic pathway may directly impinge and interfere with antimicrobial responses, as well as with antiviral effects (192). Thus, an immediate action is required in order to adjust the cholesterol metabolism.

Perspectives on the role of nutrients in ameliorating severity of specific inflammatory diseases, autoimmunity

Moreover, we refer to the bioavailability of macro- and micro nutrients from food. We ask whether foods contain enough amounts of macro- and micro nutrients. Does the soil and then the foods today still have the same nutritional value as before for example in fruits and vegetables? These led us to question under what conditions can artificial supplementation

with macro or micronutrients be done? And how should be done? Should they be taken alone or as a complex? The question is the synergistic and complementary action of taking supplements of vitamin complexes, results in a better or worst outcome? We surmise that the administration of nutrients (micro and macro) would exert distinct effects on each person. We know that each individual is different, and thus their immune responses will differ from each other. To add more complexity, we referred also to the absorption capability of nutrients in the different compartments of the digestive system. From all the information provided above we can establish that malnutrition and/or supplementation strongly affect the immune system.

We finally provided evidence that for each stage of the immune process, both micro and macronutrients are needed for the proper functioning of this important system.

Author contributions

BS and CM wrote the manuscript after a rigorous investigation, interpretation, systematization, and conceptualization of current data. Both authors agreed to publish the present manuscript, contributed to the article, and approved the submitted version.

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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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EDITED BY
Sergey Ponomarev,
Institute of Biomedical Problems (RAS), Russia

REVIEWED BY
Jiajia Song,
Southwest University, China
Zhigang Song,
Shandong Agricultural University, China

*CORRESPONDENCE
Yinghua Shi
annysyh@henau.edu.cn

†These authors have contributed equally to this work and share first authorship

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Pasture intake protects against commercial diet-induced lipopolysaccharide production facilitated by gut microbiota through activating intestinal alkaline phosphatase enzyme in meat geese

Qasim Ali[1†], Sen Ma[1,2,3†], Umar Farooq[4], Jiakuan Niu[1], Fen Li[1], Defeng Li[1,2,3], Zhichang Wang[1,2,3], Hao Sun[1,2,3], Yalei Cui[1,2,3] and Yinghua Shi[1,2,3*]

[1]Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, Henan Agricultural University, Zhengzhou, Henan, China, [2]Henan Key Laboratory of Innovation and Utilization of Grassland Resources, Henan Agricultural University, Zhengzhou, Henan, China, [3]Henan Herbage Engineering Technology Research Center, Henan Agricultural University, Zhengzhou, Henan, China, [4]Department of Poultry Science, University of Agriculture Faisalabad, Toba Tek Singh, Pakistan

Introduction: Diet strongly affects gut microbiota composition, and gut bacteria can influence the intestinal barrier functions and systemic inflammation through metabolic endotoxemia. In-house feeding system (IHF, a low dietary fiber source) may cause altered cecal microbiota composition and inflammatory responses in meat geese via increased endotoxemia (lipopolysaccharides) with reduced intestinal alkaline phosphatase (ALP) production. The effects of artificial pasture grazing system (AGF, a high dietary fiber source) on modulating gut microbiota architecture and gut barrier functions have not been investigated in meat geese. Therefore, this study aimed to investigate whether intestinal ALP could play a critical role in attenuating reactive oxygen species (ROS) generation and ROS facilitating NF-κB pathway-induced systemic inflammation in meat geese.

Methods: The impacts of IHF and AGF systems on gut microbial composition via 16 sRNA sequencing were assessed in meat geese. The host markers analysis through protein expression of serum and cecal tissues, hematoxylin and eosin (H&E) staining, localization of NF-κB and Nrf2 by immunofluorescence analysis, western blotting analysis of ALP, and quantitative PCR of cecal tissues was evaluated.

Results and Discussion: In the gut microbiota analysis, meat geese supplemented with pasture showed a significant increase in commensal microbial richness and diversity compared to IHF meat geese demonstrating the antimicrobial, antioxidant, and anti-inflammatory ability of the AGF system.

A significant increase in intestinal ALP-induced Nrf2 signaling pathway was confirmed representing LPS dephosphorylation mediated TLR4/MyD88 induced ROS reduction mechanisms in AGF meat geese. Further, the correlation analysis of top 44 host markers with gut microbiota showed that artificial pasture intake protected gut barrier functions via reducing ROS-mediated NF-κB pathway-induced gut permeability, systemic inflammation, and aging phenotypes. In conclusion, the intestinal ALP functions to regulate gut microbial homeostasis and barrier function appear to inhibit pro-inflammatory cytokines by reducing LPS-induced ROS production in AGF meat geese. The AGF system may represent a novel therapy to counteract the chronic inflammatory state leading to low dietary fiber-related diseases in animals.

KEYWORDS

pasture grazing, gut microbiota, intestinal alkaline phosphatase, lipopolysaccharides, oxidative stress, inflammation, Keap1-Nrf2, meat geese

Introduction

Intestinal homeostasis seems to be a defining factor for poultry health that is affected by oxidative stress either produced by heat stress or feed stress (1). The poultry birds such as broilers, layers, geese, and turkeys are continuously exposed to lipopolysaccharides (LPS) *via* different routes such as feed, water, and fine dust particles in the house that always contain some amounts of LPS. However, the major natural source of LPS is the complex community of gram-negative bacteria in the intestines (2). LPS is an outer membrane component of gram-negative bacteria such as *Enterobacteriaceae* (3), *Escherichia coli* (*E. coli*) (4), *Bacterodales* (5), and *Cyanobacteria* (6) that are recognized by toll-like receptors (TLRs), particularly TLR4 and then invade the intestinal tissues and get access to the bloodstream thereby provoking reactive oxygen species (ROS)-induced systemic diseases. This leads to a leaky gut (7) that causes diarrhea, decreased nutrient absorption, and internal fluid loss in broilers (8).

From different studies, two supporting shreds of evidence suggest that nuclear factor kappa B (NF-κB) is activated either by ROS (9) or LPS (10). Further, LPS and ROS phosphorylate NF-κB inhibitor α (*IκB-α*) and let the NF-κB migrate to the nucleus and then exert their inflammatory and apoptotic impacts by activating NF-κB pathway (11). When NF-κB pathway is established, then it consistently contributes to inducing chronic low-grade inflammation (12) through upregulating oxidative stress (N. 13), pro-inflammatory mediators i.e. inducible nitric oxide (*iNOS*) and cyclooxygenase (*COX*)-2 and pro-inflammatory cytokines (i.e. *IL-1β*, *IL-6*, and *TNF-α*) (14, 15).

Gut microbial-induced LPS-mediated NF-κB activity orchestrates chronic low-grade inflammation that is a major disease risk factor in today's animals' life (16). Thus, modifiable factors that can reduce LPS-induced inflammation may potentially modulate disease risk. Different possible modifiable factors such as feed antibiotics (17), dietary fiber and threonine (18), pectin (19), Schisandra A (12), phytochemicals (20), and oral alkaline phosphatase (21) have been used in animals to detoxify LPS. Feed antibiotics increase food-born pathogenic bacterial-induced LPS resistance (22) while phytochemicals used as anticancer drugs show minimum side effects in animals (20). Albeit different dietary fibers detoxify LPS-induced chronic low-grade inflammation (18), nobody did clearly explain the mechanisms of how dietary fibers ameliorate LPS-mediated systemic inflammation facilitated by gut microbiota in meat geese.

Different dietary fiber sources such as glucomannan, oligosaccharides, sialyl lactose, and galactooligosaccharides shape the gut microbiota to regulate intestinal ALP in rats and other animals (23, 24). Intestinal ALP is an endogenous antimicrobial peptide that is secreted from the apical enterocytes of small intestine and then moves toward the large intestine (25). Intestinal ALP has been shown to dephosphorylate LPS, CpG-DNA, and flagellin (26). In previous studies, lipid A moiety plays a role of bridge in activating myeloid differentiation factor 88 (MyD88) pathway by binding LPS with TLR4 and then activating oxidative stress (27) and NF-κB-induced systemic inflammation (12). In previous studies is not defined clearly to what extent and in which way the intestinal ALP dephosphorylates LPS by breaking TLR4/MyD88-induced ROS production and NF-κB-induced systemic inflammation. Furthermore, intestinal ALP promotes healthy homeostasis of gut microbiota (28) and gut barrier

functions (29), which has been associated with lowering systemic inflammation in several studies of healthy adults (30) and their specific health conditions (e.g. obesity, diabetes, and cardiovascular disease) (31). The increasing evidence of the salutary functions of ALP emphasizes the significance of the naturally occurring brush border enzyme. Therefore, there is a need to find optional nutritional strategies that could naturally induce and regulate the endogenous growth of intestinal ALP in poultry birds. One of the optional possible nutritional strategies is the application of dietary fiber which has been used in different studies to induce intestinal ALP production (24).

Geese are herbivorous, and because of their unique ability to use high fiber feeds, pasture was suggested to be included to promote health (32). In China, the most commonly available pasture is ryegrass, which is rich in protein, dietary fiber, fatty acids, iron, zinc, magnesium, calcium, vitamins, essential amino acids, alkaloids, steroids, flavonoids, glycosides, phenols, and tannins (33). Recently, several reports suggest that ryegrass could regulate intestinal microflora of Beijing-you chickens (34) and improve ethnomedical properties like being antioxidant, antimicrobial, and anti-inflammatory diseases in animals (35, 36). However, up to now, formal mediation analysis experiments about the association between ryegrass (high dietary fiber) intake-induced endogenous ALP-regulated kelch-like ECH-Associating protein 1-nuclear factor erythroid 2 (Keap1-Nrf2) pathway and LPS-induced oxidative stress is lacking. Moreover, the Keap1-Nrf2 system plays a central role in the regulation of the oxidative stress response, and that Nrf2 coordinately regulates cytoprotective genes (37). Here we reported that the regulation of gut microbial-induced endogenous intestinal ALP by pasture intake preserves the normal homeostasis of gut microbiota, dephosphorylates LPS/TLR4/MyD88 pathway-mediated ROS insults, and activates Keap1-Nrf2 pathway to alleviate NF-κB-induced systemic inflammation in meat geese.

Materials and methods

Ethical approval

According to animal care guidelines, the study was conducted using Wanfu geese. All used procedures were approved by the Research Bioethics Committee of the Henan Agricultural University (approval HENAU-2021).

Animals, diets, and housing

A total of 180, 25-day-old Wanfu mixed-sex geese from the commercial geese farm were purchased from Henan Daidai goose Agriculture and Animal husbandry development Co., Ltd (Zhumadian, China). The geese with an average weight of 693.6 g (± 3.32) were divided into two homogeneous groups: (1) in-house feeding group (IHF, n = 90) and (2) artificial pasture grazing group (AGF, n = 90), 12h artificial pasture grazing (06:00-18:00h) with once a day (19:00h) in-house feeding group. Each group consisted of six replicates with 15 geese per replicate. All the geese had free access to feed and freshwater *ad-libitum*. The IHF group meat geese were fed a commercial diet (Table 1). Two diets were used: a grower diet (25 to 45 days) and a finisher diet (46 to 90 days). The AGF system was established in form of grazing of meat geese at the expense of ryegrass. The nutritional composition of ryegrass was dry matter (90%), crude protein (15.47%), ash (8%), neutral detergent fiber (65%), acid detergent fiber (38%), ether extract (3.3%), calcium (0.90%), and phosphorous (0.47%). The experiment lasted for 66 days (Supplementary Figure 1).

Sample collection

Body weight and feed intake were measured every week. The pasture feed intake was measured using the method described by Cartoni Mancinelli et al. (38). On days 45, 60, and 90, we selected six healthy meat geese per replicate with a body weight range of ± 1 std. from mean 1.63-2.31 kg, 3.33-4.28 kg, and 4.38-5.39 kg respectively. Blood samples were collected in non-anticoagulant sterile blood vessels from the jugular vein. Serum samples were then obtained by centrifuging the blood samples at 4,000 × g for

TABLE 1 Nutritional composition of the diet.

	Diets	
Ingredients, %	**Grower**	**Finisher**
Wheat	57.3	59
Rice bran	5	4
Corn germ cake (exp.)	4	3.2
Corn oil	5	7
Dumpling powder	3	2
Corn distiller's grains (DDGS)	6.5	7
Spouting germ meal	3	2
Soybean meal (sol.)	7	6
Peanut meal (sol.)	1.5	1
Albumen powder	2	1.5
Stone powder	1.1	1
Liquid methionine	0.25	0.3
MuLaoDa-2	1.25	2
201/202 gunk	2.5	3
Calcium hydrogen phosphate	0.6	1
Chemical composition (%)		
Crude protein	20.12	15.54
Ash	12.89	12.86
Neutral detergent fiber	13.25	30.55
Acid detergent fiber	5.5	27.02
Calcium	1.15	1.07
Phosphorous	0.47	0.32

15 minutes at 4°C and stored at –80°C until analysis. After blood sampling, the geese were slaughtered and pH was determined from the proventriculus, gizzard, ileum, and cecum. Fresh cecal chyme was collected from the cecum using sterile 5 ml centrifuge tubes and then stored at –80°C for further analysis. The cecal tissues were immediately removed, thoroughly washed with phosphate-buffered saline (PBS), stored in liquid nitrogen, and then preserved at –80°C for further analysis. Cecal tissues were fixed by immersion in 4% and 10% neutral buffered formalin.

Measurement of LPS, ROS, and ALP levels

The serum and cecal tissues were sampled to measure LPS, ROS, and ALP activities. The kits were purchased from Shanghai Enzyme Link Biotechnology Co., Ltd (Shanghai, China) and all experimental procedures were performed according to the manufacturer's instructions.

Bacterial growth conditions

LPS is a key virulence factor of Escherichia coli (E. coli) that triggers innate immune responses *via* activation of the toll-like receptor 4 signaling pathway (39). To identify, whether *E. coli* contributes to activating the LPS, the batch cultivation for *E. coli* was carried out in Luria broth (LB) medium at 37°C with a 2-L working volume. LB medium was from recipe of Miller (5 g yeast extract, 10 g peptone tryptone, 10 g NaCl) (40). The pH was maintained at 6.95 automatically by titration with 5% H_2SO_4 or 5% NaOH. Ampicillin was added to control the growth of other bacteria. Peptone tryptone and yeast extract were from OXOID, NaCl from Sigma, agar, and ampicillin from Solarbio (life sciences). The medium was made in distilled water and autoclaved under standard conditions. Dissolved oxygen in the culture was maintained at 40% saturation automatically by varying the speed of impeller rotation. Culture growth (OD600) was monitored with a DU640 Spectrophotometer (Beckman). Further, *E. coli* was cultured onto the Petri dishes for 24h at 37°C. The CFU/g stool for *E. coli* from the Petri dishes was counted.

Measurement of gut permeability

The concentration of tight junction proteins is a widely recognized indicator known as gut permeability. For this, the tight junction proteins ZO-1, Occludin, and Claudin concentrations were determined from the cecal tissues in the present study. The kits were purchased from Shanghai Enzyme Link Biotechnology Co., Ltd (Shanghai, China) and all experimental procedures were performed according to the manufacturer's instructions. Further, mRNA expression levels of *ZO-1*, *Occludin*, and *Claudin* and 2 genes encoding tight junction proteins *dlg1* and *E-cadherin* were also measured from cecal tissues for gut barrier functions. Details related to gene expression are proposed to be harmonized in section 2.14.

Measurement of antioxidant parameters

Heme oxygenase 1 (HO-1) and glutathione reductase (GSR) were measured from serum using ELISA kits (Shanghai Meilian Biology Technology, Shanghai, China). The total superoxide dismutase (T-SOD, #A001-1), glutathione peroxidase (GSH-Px, #A005), total antioxidant capacity (T-AOC, #A015-2–1), malondialdehyde (MDA, #A003-1), and catalase (CAT, #A007-1) were measured using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, P. R. China) according to the manufacturer's instructions.

Measurement of metabolic (plasma lipid) profiles

Serum total cholesterol (T-CHO, #A111-1-1), low-density lipoprotein cholesterol (LDL-C, #A113-1-1), high-density lipoprotein cholesterol (HDL-C, #A112-1-1), triglycerides (TG, #A110-1-1), and blood urea nitrogen (BUN, #C013-1-1) was enzymatically determined using a kit from Nanjing Jiancheng Bioengineering (Nanjing, Jiangsu, P. R. China) following the manufacturer's instructions. Fasting blood glucose level was determined by a blood glucose meter (www.sinocare.com).

Hematoxylin and eosin staining

First, the cecal tissues were fixed with 10% paraformaldehyde and stained with Harris' hematoxylin solution for 6h at a temperature of 60-70°C and were then rinsed in tap water until the water was colorless. Next, 10% acetic acid and 85% ethanol in water were used to distinguish the tissue 2 times for 2h and 10h, and again the tissues were rinsed with tap water. In the bluing step, we soaked the tissue in saturated lithium carbonate solution for 12h and then rinsed it with tap water. Finally, staining was achieved with eosin Y ethanol solution for 48h.

Paraffin embedding

The tissues were dehydrated with 95% ethanol twice for 0.5h, and then soaked in xylene for 1h at 60-70°C followed by paraffin for 12h. For the cecal tissues, we used 0.5 ml of 95% ethanol in dehydration.

Slicing and imaging

The stained tissues were cut into 3 sections of 7-μm slices with well-oriented parts using a Leica RM2235 microtome and then imaged using Nikon NIS-Elements microscopy.

Localization of NF-κB and Nrf2 by immunofluorescence analysis

The tissue sections were fixed in 4% paraformaldehyde for 10 minutes. Paraffin sections were dewaxed with xylene for 15 minutes, dehydrated with gradient alcohol, and rinsed in distilled water. Dewaxed and rehydrated slides of cecal tissue sections were boiled in citrate-EDTA antigen retrieval solution for 2 minutes and were blocked with 10% fetal bovine serum for 30 minutes. The tissue sections were incubated for NF-κB and Nrf2 with primary antibody Rabbit Anti-NF-κB (Affinity, P65-AF5006, 1:200; v/v) or Rabbit anti-Nrf2 (Bioss, bs-1074R, 1:500; v/v) respectively, at 4°C overnight followed by secondary antibody (HRP Goat Anti-Rabbit IgG (SeraCare, 5220-0336, 1:400; v/v) with incubation at room temperature in the dark for 50 minutes. Tyramine salt fluorescein was added dropwise to the tissues (configured with phosphate-buffered saline with Tween 20 (PBST) containing 0.0003% H_2O_2) and incubated at room temperature for 20 minutes. 4',6-dia-midino-2-phenylindole (DAPI) was used to incubate the slides for 10 minutes at room temperature. Finally, the slides were sealed with anti-fluorescent quenching mounting medium, and the localization of Nrf2 and NF-κB was detected with a confocal fluorescence microscope (TCSSP8STED, Leica, Wetzlar, Germany). The nuclei stained by DAPI are blue under ultraviolet excitation and are positively expressed as the corresponding fluorescein-labeled green light.

Western blotting analysis of ALP

The cecal tissues were harvested and cut open longitudinally, and luminal contents were removed. The tissues were washed with phosphate-buffered saline (PBS) and homogenized with liquid nitrogen, and homogenates were mixed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Sigma Aldrich), incubated on ice for 30 minutes, and centrifuged at 12,000 rpm for 5 minutes at 4°C. Proteins (50 ng/sample) were solubilized and heating denaturation in 40 mL of sodium dodecyl sulfate (SDS) loading buffer (SolarBio, Shanghai, China) and then resolved by electrophoresis (BioRad, Hercules, CA, USA) on 10% SDS-PAGE gels prior to electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Standard markers for protein molecular masses were purchased from Thermo (Waltham, MA, USA). The membranes were blocked with 5% nonfat dry milk (NFDM) in Tris-buffered saline with 0.05% Tween 20 (TBS-T, Solarbio, Shanghai, China) for 1h at room temperature and then probed with 1:1,000 dilution of rabbit ALP primary antibodies (ET1601-21, huabio, Hangzhou, China) and GAPDH primary antibodies (MAB45855, Bioswamp, Wuhan, China) in 5% BSA at 4°C overnight. After washing three times in TBS-T, the blots were further incubated with 1:3,000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (E-AB-1003, Elabscience, Wuhan, China) or HRP-conjugated goat anti-rabbit IgG antibody (E-AB-1001, Elabscience, Wuhan, China) at 37°C for 1h. The proteins were visualized using Beyo ECL reagents (Beyotime, Shanghai, China). The intensity of the bands was quantified with a Pro Plus 6.0 Biological Image Analysis System.

RNA extraction and RT-qPCR

Total RNA from the cecal tissues (about 50 to 100 mg) was extracted by the addition of 1 mL of MagZol-reagent (#R4801-02; Magen Biotechnology, Guangzhou, Guangdong, China) according to the manufacturer's instructions. The concentration and purity of the total RNA were assessed using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). Subsequently, the RNA was reverse transcribed to cDNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, OSAKA, Japan) according to the manufacturer's instructions. The cDNA samples were amplified by real-time quantitative polymerase chain reaction with ChamQ Universal SYBR qPCR Master Mix from Vazyme Biotechnology (Nanjing, Jiangsu, P. R. China). Gene-specific primers for each gene were designed using Primer3web, version 4.1.0 (Supplementary Table 1). PCR was performed on the C1000 Touch PCR Thermal cycler (BIO-RAD Laboratories, Shanghai, China) using ChamQ Universal SYBR qPCR Master Mix from Vazyme Biotechnology (Nanjing, Jiangsu, P. R. China) and was as follows: 40 cycles of 95°C for 15 s and 60°C for 30 s. All measurements will be performed in triplicate. The messenger ribonucleic acid (mRNA) expression of target genes relative to beta-actin (*β-actin*) was calculated using $2^{-\Delta\Delta CT}$ method (41).

DNA extraction and PCR amplification

According to the manufacturer's instructions, the microbial community genomic DNA was extracted from cecal chyme samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.). The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo

Scientific, Wilmington, 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') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 minutes, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, and single extension at 72°C for 10 minutes, and end at 4°C. The PCR mixtures contain 5 × *TransStart* FastPfu buffer 4 µL, 2.5 mM dNTPs 2 µL, forward primer (5 µM) 0.8 µL, reverse primer (5 µM) 0.8 µL, *TransStart* FastPfu DNA Polymerase 0.4 µL, template DNA 10 ng, and finally ddH_2O up to 20 µL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, USA).

Illumina MiSeq sequencing

Purified amplicons were pooled in equimolar, and paired-end sequenced (2 ×300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Processing of sequencing data

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic, and merged by FLASH with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotides mismatches in primer matching. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the 16S rRNA database (e.g. Silva SSU128) using a confidence threshold of 0.7. The species composition and relative abundance of each sample were counted at the phylum level, and the composition of the dominant species of different groups was visualized by the package pie chart of R (version 3.3.1) software. PICRUSt is a software package for the functional prediction of 16S amplicon sequencing results which was used to determine the COG IDs related to LPS production. Spearman rank correlation coefficient was performed to construct a correlation heatmap among the highly abundant GO terms and cecal microbiota at phylum as well as genus levels mostly relevant to LPS production. Further, to determine the effect of microbiota interacting with apparent performance, redundancy analysis (RDA) was performed at the genus level using the R language vegan packet on Spearman correlation analysis (RDA 2014).

Statistical analysis

Data were expressed as mean ± SEM. Statistical analyses were performed using SPSS 20.0 software (=D3 SPSS, Inc., 2009, Chicago, IL, USA www.spss.com). Data from two groups were evaluated by unpaired two-tailed student T-Test. Significance was considered to be at $P< 0.05$. Spearman correlation analysis of the Euclidean distance was performed using GraphPad Prism version 8.3.0., and origin 2021. To compare host markers' relationships, Pearson's correlation analysis was performed by OECloud tools (https://cloud.oebiotech.cn).

Results

Artificial pasture grazing system modulates gut microbiota to inhibit LPS synthesis induced by in-house feeding system

The metagenome predicted functions classified using clusters of orthologous genes (COG) database in phylogenetic reconstruction of unobserved states (PICRUSt) software are performed to investigate the functional differences in the gut microbiota between the two feeding meat geese groups (in-house feeding group (IHF) and artificial pasture grazing group (AGF) meat geese) at different time points 45d, 60d, and 90d. A total of 4060, 4069, 4082, 3959, 4030, and 4060 COG IDs were identified in samples IHF45, AGF45, IHF60, AGF60, IHF90, and AGF90 respectively (unpublished data). To identify which strains contribute to LPS production in the meat geese gut, we focused on the GO terms and cecal microbiota at the phylum level mainly relevant to LPS biosynthesis (Figure 1A and Supplementary Figures 2A–F). At 45d, in the IHF meat geese, *Firmicutes* families dominated the four while *Actinobacteiota* dominated the two main COG terms related to LPS biosynthesis. At 60d, in the IHF meat geese, *Actinobacteriota* and *Proteobacteria* families dominated the four and two main COG terms related to LPS biosynthesis, respectively. At 90d, only *Bacteroidota* family dominated the three main COG terms related to LPS biosynthesis in IHF meat geese.

Overall, *Firmicutes* and *Bacteroidota* families dominate both in the IHF and AGF meat geese at 45d, 60d, and 90d, but

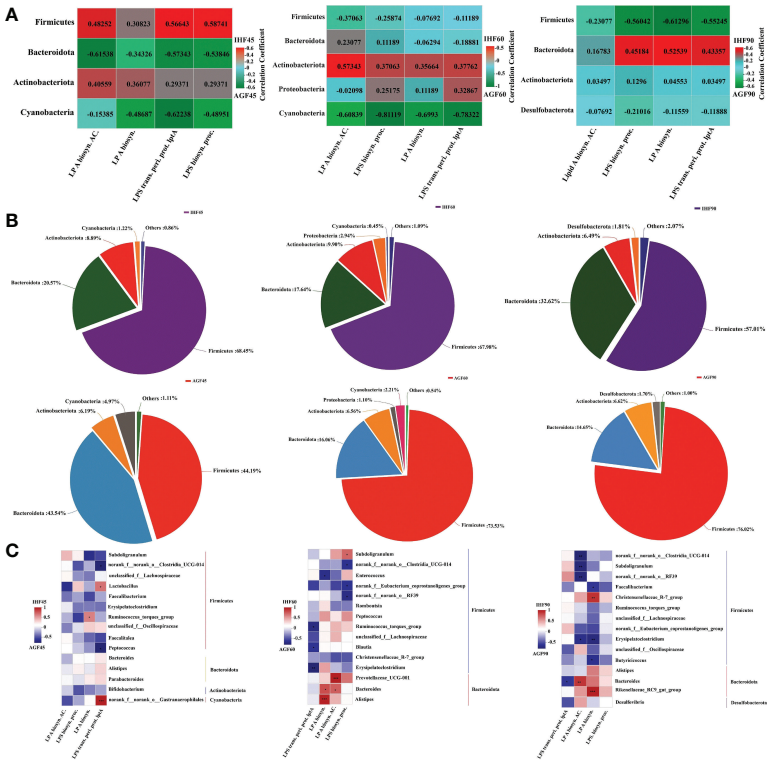

FIGURE 1
Artificial pasture grazing system modulates gut microbiota to inhibit LPS synthesis induced by in-house feeding system. **(A)** Average abundance per sample of genes related to the four main LPS biosynthesis-related functions. Red indicates a positive correlation; green indicates a negative correlation, **(B)** Relative contributions of the different phyla to the total LPS-encoding capacity of the gut microbiome, and **(C)** Contribution of individual genera to LPS biosynthesis functions. The average abundances of genes related to any of the four LPS-related GO functions are shown for individual genera within each phylum. LP A biosyn. AC.; lipid A biosynthesis acyltransferase, LP A biosyn.; lipid A biosynthesis, LPS trans. peri. prot. lptA; lipopolysaccharide transport periplasmic protein lptA, and LPS biosyn. proc.; lipopolysaccharide biosynthesis process. Red indicates a positive correlation; blue indicates a negative correlation. In-house feeding system (IHF) and artificial pasture grazing system (AGF). The asterisks symbol indicates significant differences *P < 0.05, **P < 0.01, ***P < 0.001

Firmicutes and *Bacteroidota* individually contribute 68.45% and 32.62% of the LPS biosynthesis in the IHF meat geese at 45d and 90d, respectively (Figure 1B). In contrast, *Actinobacteriota* family is the minor contributor to LPS biosynthesis, with an average of 8.89% and 9.90% of the total LPS biosynthesis in IHF meat geese at 45d and 60d, respectively. In contrast to other bacterial families, *Proteobacteria* family with a minute quantity with an average of 2.94% contributes to the total LPS biosynthesis in IHF meat geese at 60d.

The quality and types of diet make alterations in the gut microbiota in a time-dependent manner. We next examined whether the bacterial composition of the samples would correlate with the potency of activation or inhibition of these samples (Figure 1C). At the genus level, we determined the Spearman correlation between the GO terms and cecal microbiota falling in different phyla mainly relevant to LPS biosynthesis and those have been individually expressed in a supporting file (Supplementary Figures 3A–E). We identify a strong correlation between GO functions related to LPS biosynthesis and microbial composition at the genus level and could only find a moderate-to-strong correlation between a few microbial individual genera and the stimulatory potency of individual cecal LPS production in IHF meat geese (Figure 1C). However, we found that the abundance of very few bacterial genera *Lactobacillus* and *Ruminococcus_torques_group* following *Firmicutes* phylum show a moderate correlation with the activation of lipid A biosynthesis and LPS transportation periplasmic protein lptA at 45d in IHF meat geese (Figure 1C). While only single bacterial genera *norank_f:norank_o: Gastranaerophilales* following the phylum *Cyanobacteria* show a strong correlation with the activation of LPS transportation periplasmic protein lptA at 45d in IHF meat geese (Figure 1C). In contrast, we found a maximum of the bacterial genera *Prevotellaceae_UCG-001*, *Bacteroides*, and *Alistipes* following *Bacteroidota* phylum were contributing to the lipid A biosynthesis acyltransferase and lipid A biosynthesis at 60d in IHF meat geese (Figure 1C). Similarly, *Bacteroides* and *Rikenellaceae_RC9_gut_group* following *Bacteroidota* phylum

follow the same trend in activating the lipid A biosynthesis acyltransferase and lipid A biosynthesis at 90d in IHF meat geese (Figure 1C). Notably, *Bacteroides* dominate activating lipid A biosynthesis acyltransferase at 60d and 90d in IHF meat geese (Figure 1C). Our results revealed that *Bacteroidota* phylum is by far the most abundant contributor to the LPS biosynthesis functions in IHF meat geese intestinal microbiota, consistent with their high abundance relative to other Gram-negative genera in the gut.

Inhibitory effects of artificial pasture grazing system on in-house feeding system-induced ROS production *via* LPS/TLR4/MyD88 pathway in meat geese

We observed significantly increased in pH of proventriculus, gizzard, ileum, and ceca in the AGF meat geese compared with IHF meat geese (Supplementary Figures 4A–D and Supplementary Table 2). Then we measured the protein levels of intestinal ALP activity and expression levels of intestinal alkaline phosphatase gene (*ALPi*) and 2 separate alkaline phosphatase genes (*CG5150* and *CG10827*) from the meat geese. The serum ALP activity by enzyme-linked immunosorbent *assay* (ELISA) kit (Figure 2A) and mRNA expression of *ALPi* (Figure 2B) and alkaline phosphatase genes (*CG5150* and *CG10827*) (Figure 2C) increased significantly in AGF meat geese as compared to IHF meat geese at 45d, 60d, and 90d. Furthermore, intestinal ALP may contribute to maintaining the normal gut microbial homeostasis by suppressing the *E. coli* and as well as detoxifying the LPS (42, 43). To identify, whether *E. coli* contributes to activating the LPS and the suppression of intestinal ALP, we cultured the *E. coli* onto the Petri dishes for 24h at 37°C. For this, we counted the colony-forming units (CFU) for *E. coli* from the Petri dishes. We observed that the CFU/g stool was less in the AGF meat geese compared with the IHF meat geese (Supplementary Figure 5A). To further verify our results, we pick up one colony from the petri dish and incubated it in the LB medium for 48h at 37°C. Then we determined the *E. coli* cell cultures based on spectrophotometer readings at OD600 for 48h with an interval of 2h. We found a significant decline in the concentration of *E. coli* cell cultures from the AGF meat geese as compared to IHF meat geese (Supplementary Figure 5B).

The genes in rfa cluster such as *rfaK* and *rfaL* are involved in the synthesis and modification of LPS core. These two genes play a vital role in the attachment of 0 antigens to the core. This result was following the higher protein abundance of serum LPS (Figure 2D)

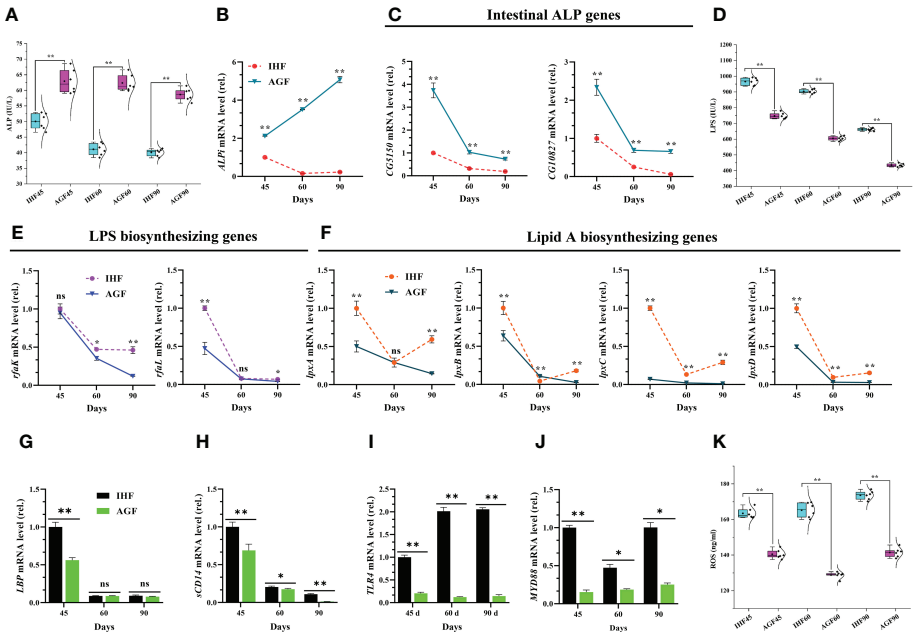

FIGURE 2
Inhibitory effects of artificial pasture grazing system on in-house feeding system-induced ROS production *via* LPS/TLR4/MyD88 pathway in meat geese. **(A)** ALP protein level in serum, **(B)** *ALPi* mRNA level in cecal tissues, **(C)** mRNA levels of intestinal ALP genes (*CG5150* and *CG10827*) in cecal tissues, **(D)** LPS protein level in serum, **(E)** mRNA levels of LPS biosynthesizing genes (*rfaK* and *rfaL*) in cecal tissues, **(F)** mRNA levels of lipid A biosynthesizing genes (*lpxA*, *lpxB*, *lpxC*, and *lpxD*) in cecal tissues, **(G)** *LBP* mRNA level in cecal tissues, **(H)** *sCD14* mRNA level in cecal tissues, **(I)** *TLR4* mRNA level in cecal tissues, **(J)** *MyD88* mRNA level in cecal tissues, and **(K)** ROS protein level in serum, normalized by *β-actin* and measured by qPCR. In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different ($P < 0.05$) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^*P < 0.05$, $^{**}P < 0.01$.

and mRNA expression of LPS biosynthesizing genes *rfaK* and *rfaL* (Figure 2E) in cecal tissues of IHF meat geese. Next, to identify whether lipid A moiety of LPS permits LPS to bind with TLR4 and then activate MyD88 pathway, first, we determined the genes related to lipid A biosynthesis. We found that the mRNA expression of *lpxA*, *lpxB*, *lpxC*, and *lpxD* was lesser in the AGF meat geese compared with IHF meat geese (Figure 2F). Next, we found decreased mRNA expression of *LBP* (Figure 2G) and *soluble cluster of differentiation 14* (*sCD14*) (Figure 2H) in AGF meat geese. This suggests that the higher mRNA expression of genes related to lipid A may able the LPS to attach with *TLR4* (Figure 2I) and then activate MyD88 dependent pathway in cecal tissues of IHF meat geese compared with AGF meat geese at 45d, 60d, and 90d (Figure 2J). The activation of TLR4/MyD88 pathway may contribute to ROS production (44). As expectedly, the higher *TLR4/MyD88* gene expression was observed to be increased in IHF meat geese concerning higher serum ROS production (Figure 2K).

Inhibitory effects of artificial pasture grazing system on in-house feeding system deteriorated nutrient absorption

In our study, we found that intestinal ALP activity (Majorbio i-Sanger cloud platform (http://rdp.cme.msu.edu/)) was decreased in IHF meat geese along with increased ROS abundances (Figure 2K) compared with AGF meat geese. Based on this scenario, next, to identify whether the IHF-induced intestinal dysbiosis may impact intestinal morphology in meat geese, we performed H&E staining of cecal tissues. The effect of AGF system on the morphology of cecal tissues is presented in Supplementary Table 3. Irrespective of a commercial diet, the villus height, villus width, surface area, and distance between villi in cecum of AGF meat geese group were improved as opposed to crypt depth compared to those of IHF meat geese group at different time points 45d, 60d, and 90d. While the villus height to crypt depth ratio (V:C) values of the cecum were not different. Further, the morphology of the cecal tissues from different feeding systems was measured and compared to one another as shown in Supplementary Figure 6. These results illustrated the nutrient absorption in cecum under different feeding systems.

Beneficial effects of artificial pasture grazing system on in-house feeding system-dependent apoptosis-induced gut permeability in meat geese

Alterations in mitochondrial membrane permeability could initiate the stimulation of cytochrome C into the cytoplasm, which activates caspases that, in turn, trigger apoptosis. Before starting apoptosis-related experiments, we confirmed that cytochrome C activity was increased with a commercial diet in the cecal tissues of IHF meat geese (Majorbio i-Sanger cloud platform (http://rdp.cme.msu.edu/)), then further we confirmed it from the mRNA expression of *cytochrome C* from cecal tissues (Figure 3A). To discover that apoptosis production is affected by feed type in meat geese, we tested mRNA expression levels of *caspase 3* and *8* in cecal tissues from IHF and AGF meat geese at different stages such as 45d, 60d, and 90d. We found a significant increase in *caspase3* (*CASP3*) and *8* (*CASP8*) activity in IHF meat geese (Figures 3B, C). Next, we performed H&E staining of cecal tissues. The upper normal limit for the number of apoptotic cells per field (Mean ± SD) in the villus at 45d, 60d, and 90d from the cecal tissues of IHF and AGF meat geese has been shown in Figure 3D and Supplementary Table 4. From different studies, this accelerated apoptosis has been involved in inducing intestinal mucosa disruption and intestinal permeability (45). To evaluate whether the mucus phenotype can be explained by altered structural organization of the mucosal barrier, we stained cecal tissues with H&E. A well-defined mucus-producing goblet cell genes (mucin2 (*MUC2*) and Mucin 5, subtype AC (*MUC5AC*)), the number of goblet cells per 20μm, and inner muscular tonic/muscularis mucosa layer thickness were observed in AGF meat geese group (Figures 3E, F; Supplementary Figures 7A; 8A, B and Supplementary Table 5). In agreement with a mucosal barrier, the mucus-producing goblet cell genes and inner muscular tonic/muscularis mucosa layer appeared less organized upon a commercial diet feeding. Furthermore, intestinal ALP is known to promote gut barrier function, and the disruption of the intestinal barrier is thought to play a critical role in gut permeability development, hence, we measured the gut permeability in IHF and AGF meat geese at 45d, 60d, and 90d. The ELISA kit method showed an IHF-dependent increase in endotoxemia (LPS) (Figure 2D), significantly influenced by intestinal ALP deficiency in IHF meat geese (previously explained in Figures 2A–C). Furthermore, expression levels of intestinal tight junction proteins were measured in cecal tissues of IHF and AGF meat geese. Again diet and loss of endogenous ALP were associated with a significant reduction in protein expression levels of zona occludin-1 (ZO-1), Occludin, and Claudin (Figures 3G–I).

Inhibitory Effects of artificial pasture grazing system on in-house feeding system-induced NF-κB pathway and its systemic inflammation

In our study first, we explained that the activation of MyD88 dependent pathway could lead to the stimulation of ROS in IHF meat geese. Along with this dependent pathway, the cellular protein LC8 (8-kDa dynein light chain) plays a role in the redox regulation of NF-κB pathway. Actually, LC8 binds to *IkB-a* in a redox-dependent manner, thereby preventing its phosphorylation by IKK (46). Here, IHF system-induced intestinal ALP disruption and the resulting ROS production oxidized LC8 which leads to the dissociation from *IkB-a* and then causes *NF-κB* activation. This

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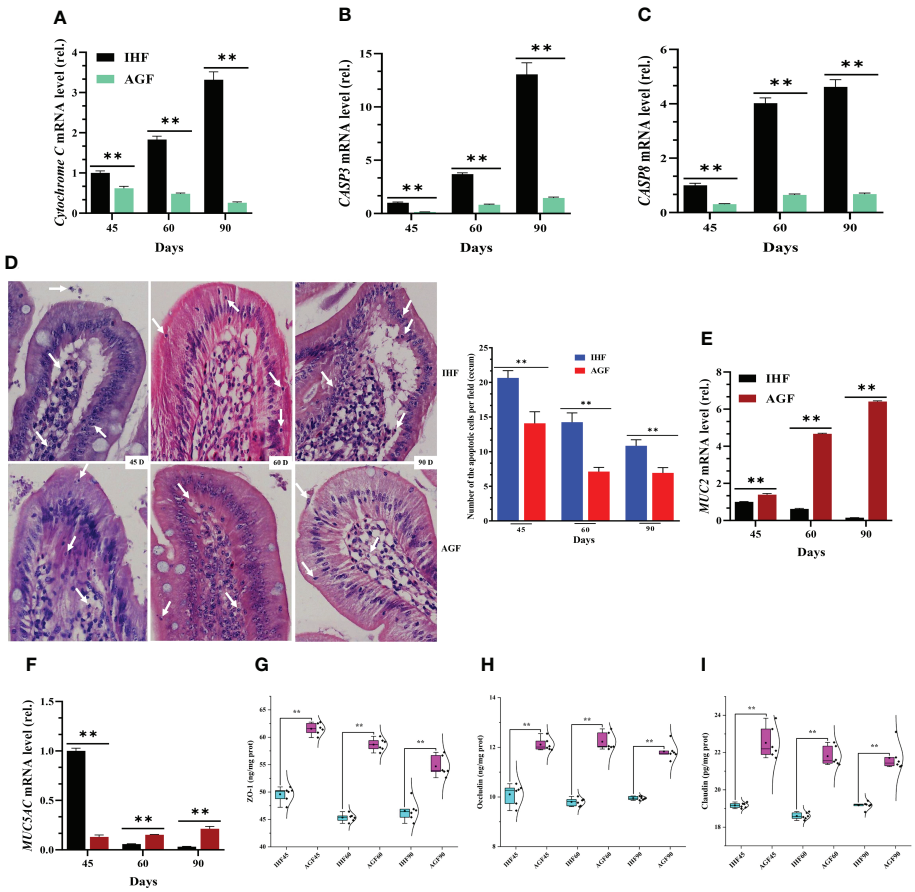

FIGURE 3
Beneficial effects of artificial pasture grazing system on in-house feeding system-dependent apoptosis-induced gut permeability in meat geese. **(A)** *Cytochrome C* mRNA level in cecal tissues, **(B)** *CASP3* mRNA level in cecal tissues, and **(C)** *CASP8* mRNA level in cecal tissues. **(D)** H&E staining of cecal tissues (magnification, 40×) for the number of apoptotic cells per field (Mean ± SD). **(E)** *MUC2* mRNA level in cecal tissues, and **(F)** *MUC5AC* mRNA level in cecal tissues, normalized by *β-actin* and measured by qPCR. **(G)** ZO-1 protein level in cecal tissues, **(H)** Occludin protein level in cecal tissues, and **(I)** Claudin protein level in cecal tissues. In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different ($P < 0.05$) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^{**}P < 0.01$.

result was under reduced mRNA expression levels of *LC8* and *IkB-a* and increased mRNA levels of *NF-κB* in IHF meat geese (Figures 4A–C). Furthermore, we investigated the genes related to regulating NF-κB pathway. The mRNA expression of *NF-κB*-regulated genes *IL-8, CCL2, PLAU*, and *BIRC3* in IHF meat geese (Figures 4D) showed that *IL-8*, *PLAU*, and *BIRC3* were by far the most abundant contributor genes involved in regulating the NF-κB pathway in the cecal tissues of IHF meat geese. Further to confirm the NF-κB pathway activation in IHF meat geese, we performed the nuclear translocation of NF-κB by the immunofluorescence analysis. As observed using a confocal fluorescence microscope, most of the NF-κB protein was translocated into the cell nucleus in the cecal tissues of IHF meat geese compared with AGF meat geese (Figure 4E). Next, a significant increase in mRNA expression of pro-inflammatory mediators *iNOS* and *COX2* and cytokines *IL-1β, IL-6*, and *TNF-a* in cecal tissues of IHF meat geese were observed instead of *IL-1β* as a function of diet composition. Here we observed that intestinal ALP deficiency was associated with significantly increased mRNA expression levels of these five cytokines (Figures 4F–J).

Effects of artificial pasture grazing system on activation of Nrf2 pathway in meat geese

When NF-κB pathway is established under the insults of ROS, then a natural immune defense mechanism is activated underpinning the Keap1-Nrf2 pathway. *Keap1* regulates the activity of *Nrf2* and acts as a sensor for oxidative stress. Upon oxidative stress, *Keap1* loses its ability to ubiquitinate *Nrf2*, allowing *Nrf2* to move in the nucleus and activate its target genes

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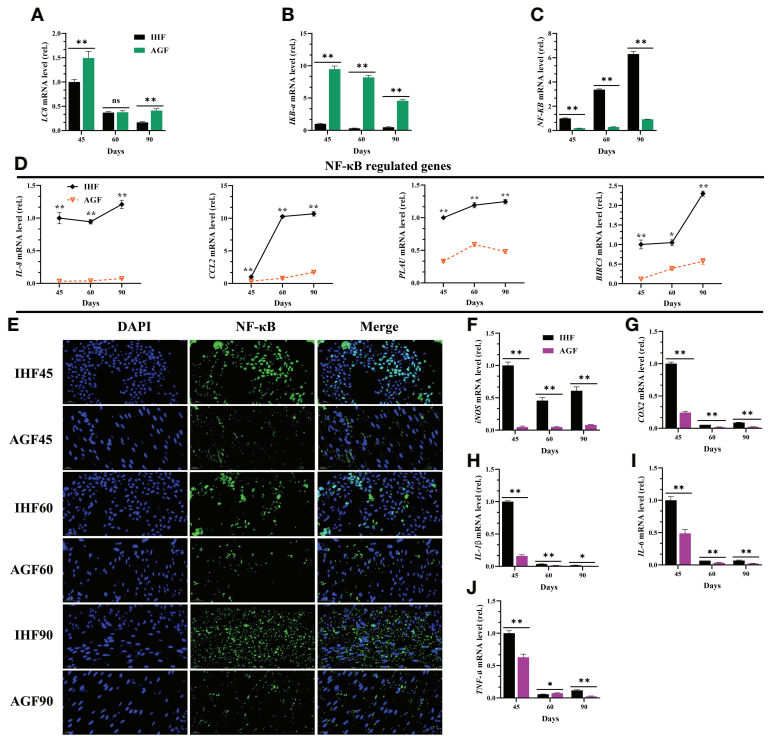

FIGURE 4
Inhibitory effects of artificial pasture grazing system on in-house feeding system-induced NF-κB pathway and its systemic inflammation. **(A)** *LC8* mRNA level in cecal tissues, **(B)** *IKB-a* mRNA level in cecal tissues, **(C)** *NF-κB* mRNA level in cecal tissues, **(D)** NF-κB-regulated genes (*IL-8, CCL2, PLAU,* and *BIRC3*) mRNA levels in cecal tissues, **(E)** Immunofluorescence (IF) analysis using Rabbit Anti-NF-κB (P65-AF5006) (1:200; v/v) showing nuclear translocation of NF-κB in the cecal tissues of meat geese. Blue: nucleus (DAPI); Green: NF-κB-staining; Cerulean blue: merge of blue and green indicating nuclear localization of NF-κB, scale bar = 20 μm. **(F)** *iNOS* mRNA level in cecal tissues, **(G)** *COX2* mRNA level in cecal tissues, **(H)** *IL-1β* mRNA level in cecal tissues, **(I)** *IL-6* mRNA level in cecal tissues, and **(J)** *TNF-a* mRNA level in cecal tissues, normalized by *β-actin* and measured by qPCR. In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different ($P < 0.05$) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^{*}P < 0.05$, $^{**}P < 0.01$. The lack of a superscript letter means that all differences were nonsignificant (ns).

(47). For these consensus, first, we found that the mRNA expression levels of *Keap1* were declined and mRNA expression levels of *Nrf2* were increased by a limited ROS production in AGF meat geese (Figure 5A). This demonstrates that the production of ROS activates Nrf2 pathway. Because Nrf2 pathway is known to promote cellular redox homeostasis, and as the impairment of *Nrf2* activity is considered to play a crucial role in cellular defense system, we measured *Nrf2* and *Nrf2*-regulated genes and the antioxidant enzymes regulated by *Nrf2* in IHF and AGF meat geese at three-time points i.e. 45d, 60d, and 90d. With AGF system, the mRNA expression levels of *Nrf2* (Figure 5B) and *Nrf2*-regulated genes *NQ01, Gclc, Gclm*, and *GSTA4* were increased in AGF meat geese (Figure 5C). Next, to evaluate the impacts of AGF system on Keap1-Nrf2 pathway, we determined nuclear translocation of Nrf2 by immunofluorescence analysis. The results revealed increased Nrf2 in AGF versus IHF meat geese (Figure 5D), which suggested that artificial pasture intake may involve in the activation of the Nrf2 pathway. It is clearly understood that the improved Nrf2 regulation should contribute in antioxidant defense mechanisms. Again we observed that the pasture intake was involved in increased protein levels of antioxidants HO-1, GSR, T-SOD, GSH-PX, T-AOC, and CAT from serum samples of meat geese at 45d, 60d, and 90d (Figures 5E–J). Further, whether these antioxidants are involved in attenuating the mediators that caused ROS insults in IHF and AGF meat geese, we measured oxidative mediator MDA from serum samples. We examined a severe increase in protein levels of MDA in IHF meat geese compared with AGF meat geese at three time points 45d, 60d, and 90d (Figure 5K).

Artificial pasture grazing system attenuates the in-house feeding system-induced endotoxemia, gut permeability, and chronic systemic inflammation of meat geese

To further explore the effects of long-term establishment of AGF system on intestinal alterations at three-time points 45d,

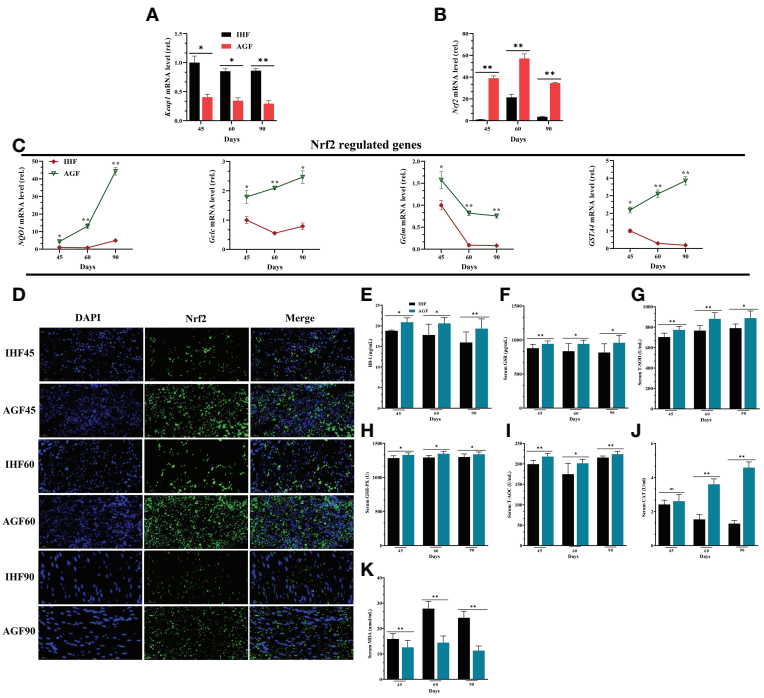

FIGURE 5
Effects of Artificial Pasture Grazing System on Activation of Nrf2 Pathway in Meat Geese **(A)** *Keap1* mRNA level in cecal tissues, **(B)** *Nrf2* mRNA level in cecal tissues, and **(C)** Nrf2-regulated genes (*NQO1, Gclc, Gclm*, and *GSTA4*) mRNA levels in cecal tissues, normalized by β-actin and measured by qPCR. **(D)** Immunofluorescence (IF) analysis using Rabbit anti-Nrf2 (bs1074R) (1:500; v/v) showing nuclear translocation of Nrf2 in the cecal tissues of meat geese. Blue: nucleus (DAPI); Green: Nrf2-staining; Cerulean blue: merge of blue and green indicating nuclear localization of Nrf2, scale bar = 20 μm. **(E)** HO-1 protein level **(F)** GSR protein level **(G)** T-SOD protein level **(H)** GSH-PX protein level **(I)** T-AOC protein level **(J)** CAT protein level and **(K)** MDA protein level. In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different ($P < 0.05$) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^{*}P < 0.05$, $^{**}P < 0.01$.

60d, and 90d, we hypothesized whether intestinal ALP may involve in activating Nrf2 pathway, and we performed correlation analysis among host markers (Figure 9B). The results obtained from this relationship showed that intestinal ALP was positively correlated with *Nrf2* and *Nrf2*-regulated genes and as well as its antioxidant enzymes. Further Nrf2 pathway including its antioxidation immune system was positively correlated with *IL-4*, *IL-10*, and tight junction proteins including 2 genes encoding tight junction proteins *Discs large 1* (*dlg1*) and *E-cadherin*. IL4 and IL-10 are known to be anti-inflammatory cytokines (48). Indeed, we also measured the protein levels of cecal ALP by western blot analysis (Figure 6A) and by ELISA kit method (Figure 6B) in meat geese. The endotoxin (LPS) and ROS measured by ELISA kit method were increased in cecal tissues of IHF meat geese compared with AGF meat geese (Figures 6C, D). Further, we measured the mRNA expression levels of TJs proteins including *ZO-1*, *Occludin*, and *Claudin* (Figures 6E–G), 2 genes encoding tight junction proteins *dlg1* and *E-cadherin* (Figures 6H, I), anti-inflammatory cytokines (*IL-4* and *IL-10*) (Figures 6J, K), and pro-inflammatory cytokines (*iNOS*, *COX2*, *IL-1β*, *IL-6*, and *TNF-α*) (Figures 4F–J) in meat geese that have received AGF and IHF environment from 45d to 90d. The results obtained from correlation analysis suggest that the activation of Nrf2 pathway by intestinal ALP enzyme was primarily involved in attenuating endotoxemia, gut permeability, and pro-inflammatory cytokines in AGF meat geese.

Long-term artificial pasture grazing system attenuates the manifestation of *KEAP1*-induced aging phenotypes in meat geese

The investigation of impact of Nrf2 pathway activation on cecal tissues aging from the expression levels of aging marker genes showed elevated level of *p19ARF, p16INK4α*, and *p21* in IHF meat geese compared with cecal tissues of AGF meat geese at 45d, 60d, and 90d of age (Figures 7A–C). The results illustrated that Nrf2 pathway activation induced by ROS-directed *Keap1* inhibition effectively suppressed the manifestation of aging phenotypes in cecal tissues of AGF meat geese.

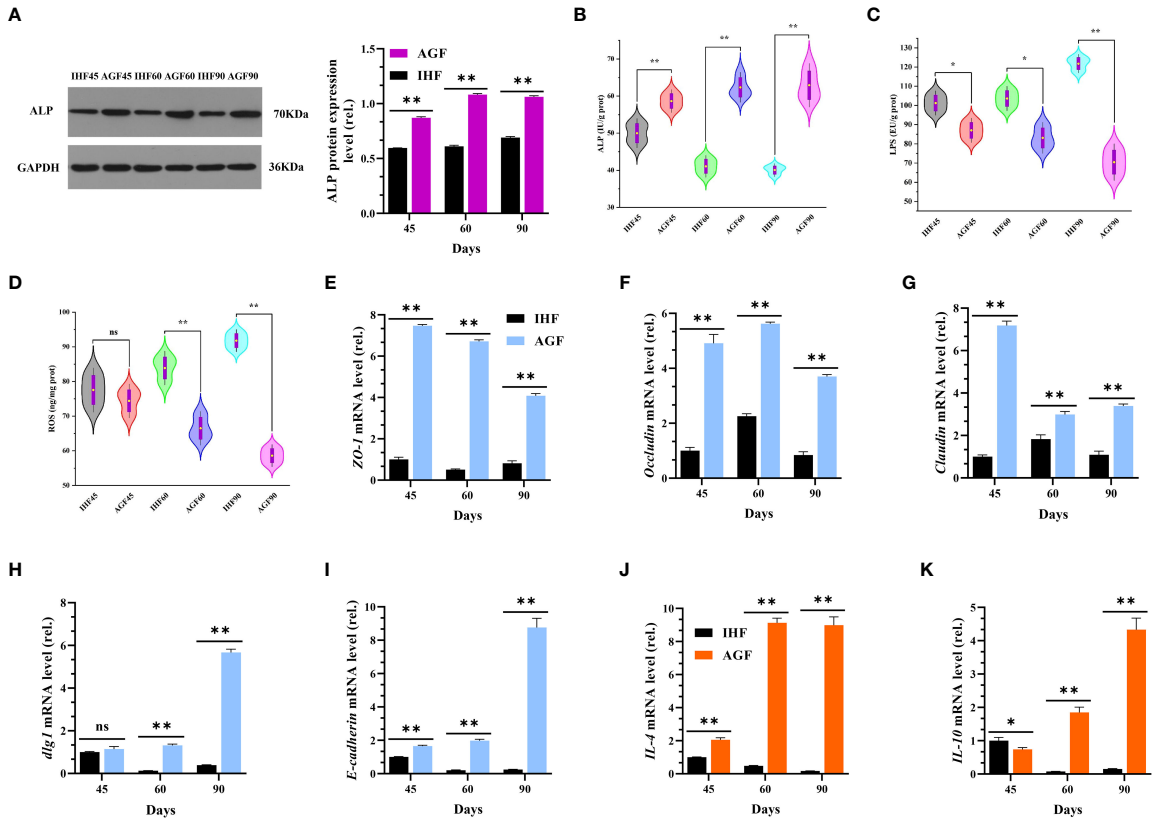

FIGURE 6
Artificial pasture grazing system attenuates the in-house feeding system-induced endotoxemia, gut permeability, and chronic systemic inflammation of meat geese. **(A)** Western blot analysis was performed to detect the protein levels of ALP in the cecal tissues, normalized by *GAPDH*. **(B)** ALP protein level in cecal tissues, **(C)** LPS protein level in cecal tissues, and **(D)** ROS protein level in cecal tissues. **(E)** *ZO-1* mRNA level in cecal tissues, **(F)** *Occludin* mRNA level in cecal tissues, **(G)** *Claudin* mRNA level in cecal tissues, **(H)** *dlg1* mRNA level in cecal tissues, **(I)** *E-cadherin* mRNA level in cecal tissues, **(J)** *IL-4* mRNA level in cecal tissues, **(K)** *IL-10* mRNA level in cecal tissues. In-house feeding system (IH) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different ($P < 0.05$) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^{*}P < 0.05$, $^{**}P < 0.01$. The lack of a superscript letter means that all differences were nonsignificant (ns).

Long-term artificial pasture grazing system improved metabolic profile in meat geese

The AGF system was effective in preventing metabolic syndrome in AGF meat geese with a significantly improved body weight (Figures 8A) and lipid profile (Figures 8B–E), as well as lowering blood glucose and urea nitrogen levels (Figures 8F, G).

Long-term Supplementation of artificial pasture grazing system impedes compositional changes in gut microbiota by stimulating intestinal ALP enzyme

The microbiota in meat geese' cecal chyme samples were analyzed at three time points (45d, 60d, and 90d) by deep sequencing of the bacteria 16S rRNA gene V3 – V4 region. As shown in Figure 9A, the Spearman correlation between microbiota and metabolic indices in the gut tract of meat geese at 45d showed significant positive association between *Bacteroides* with serum GSH-PX, and T-AOC and cecal ZO-1, Occludin, *IL-4*, *IL-10*, *LC8*, and *IKB-α* and negative association with serum LPS, ROS, T-AOC, and LDL-C, and cecal *NF-κB*, *TLR4*, *MyD88*, *COX2*, *IL-6*, *TNF-a*, *cytochrome C*, *CASP3*, *P16INK4α*, *P21*, body weight, and blood glucose. *Alistipes* were strongly positively correlated with the cecal pH, serum ALP, HO-1, GSH-PX, and T-AOC, and cecal Occludin, *IL-4*, *IL-10*, and *LC8* and negatively correlated with serum LPS, MDA, TCHO, TG, LDL-C, and ROS, and cecal *MyD88*, *NF-κB*, *TNF-a*, *cytochrome C*, *CASP3*, *p19ARF*, *p16INK4a*, *p21*, and blood glucose. *Lactobacillus* was positively correlated with serum ALP and HDL-C and cecal *Nrf2*, *IL-4*, ZO-1, Occludin, and *E- cadherin* and negatively correlated with serum LPS and TG, and cecal *LBP*, *sCD14*, *NF-κB*, *iNOS*, *IL-1β*, *cytochrome C*, *CASP3*, *Keap1*, *p19ARF*, *p16INK4a*, and *p21*. *Norank_f:norank_o:Gastranaerophilales* was positively correlated

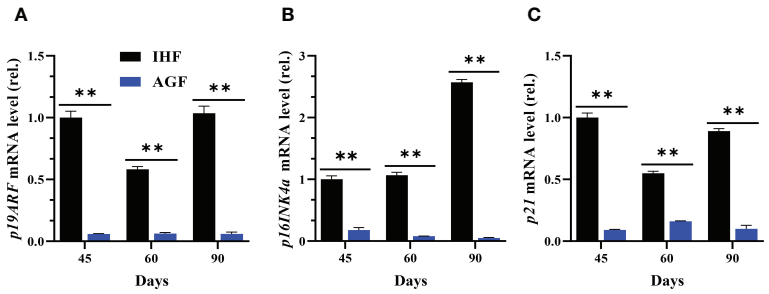

FIGURE 7
Effect of different feeding systems on KEAP1-induced aging phenotypes in meat geese. **(A)** *p19ARF* mRNA level in cecal tissues, **(B)** *p16INK4α* mRNA level in cecal tissues, and **(C)** *p21* mRNA level in cecal tissues, normalized by *β-actin* and measured by qPCR. In-house feeding system (IHF) and Artificial pasture grazing system (AGF). Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^{**}P < 0.01$.

with serum T-AOC and cecal pH, Occludin, and *IL-4* and negatively correlated with serum LPS and cecal *iNOS*, *CASP3*, *Keap1*, *p19ARF*, and *p21*. *Alistipes* and *Lactobacillus* were strongly positively correlated with intestinal ALP and Nrf2 pathway and suppress all those bacteria (*Subdoligranulum*, *norank_f:norank_o:Clostridia_UCG_014*, and *Erysipelatoclostridium*) that were the causative factors for pathogenesis in AGF meat geese.

As diet has a major influence on gut microbiota composition, richness, and diversity (49). It has been known that the origin, type, and quality of diet modulate the gut microbiota in a time-dependent manner. Based on the previous studies, we hypothesized whether AGF system as a high dietary fiber source modulates the gut microbiota at different time points or not. We further analyzed the correlation between microbiota and metabolic indices in the gut tract of meat geese at 60d. The results of which showed that the *norank_f:norank_o:RF39* was significantly positively correlated with the cecal pH, serum HO-1 and GSH-PX and cecal *Nrf2*, *IL-4*, *IL-10*, Zo-1, Occludin, claudin, *dlg1*, and *E-cadherin* and negatively correlated with serum LPS and TG and cecal *MyD88*, *COX2*, *TNF-a*, *Keap1*, *p19ARF*, and *p16INK4a* in AGF meat geese. *Romboutsia* was positively correlated with cecal *Nrf2*, *IL-4*, *IL-10*, *dlg1*, *E-cadherin*, and *LC8* and negatively correlated with cecal *MyD88*, *iNOS*, *TNF-a*, and *p16INK4a*. *Norank_f:norank_o:RF39* and *Romboutsia* were positively correlated with Nrf2 pathway that was strongly involved in attenuating the harmful impacts of *Peptococcus* and *Ruminococcus_torques_group* in AGF meat geese.

To further illustrate the impacts of long-term establishment of AGF system on gut microbial modulation with different time

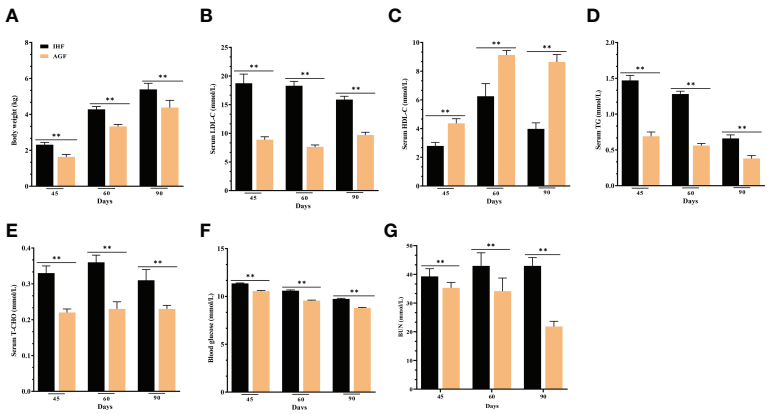

FIGURE 8
Effect of different feeding systems on metabolic profile of meat geese. **(A)** Body weight (kg), **(B)** LDL-C protein level in serum, **(C)** HDL-C protein level in serum, **(D)** TG protein level in serum, **(E)** T-CHO protein level in serum, **(F)** Blood glucose levels, and **(G)** BUN protein level in serum. In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. The asterisks symbol indicates significant differences $^{**}P < 0.01$.

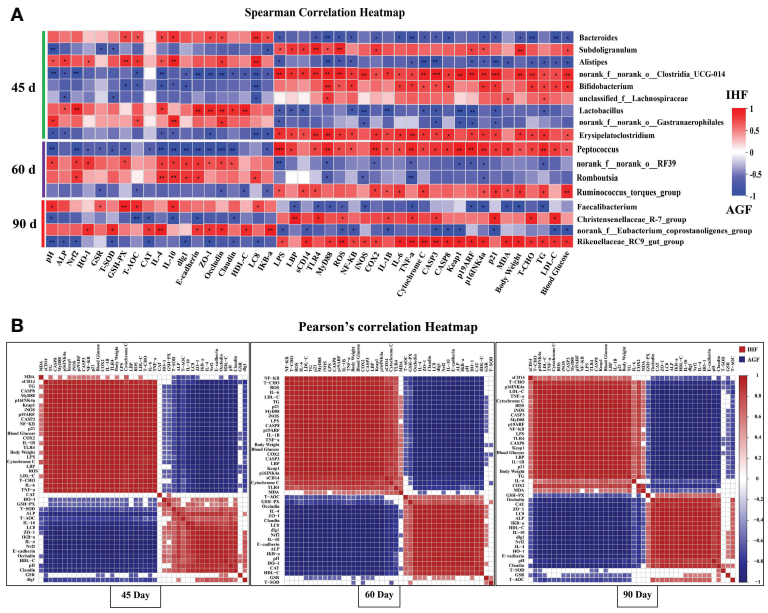

FIGURE 9
Association and model predictive analysis of the top 44 host markers with the highest correlation scores. **(A)** Correlation between gut microbiota and host markers by Spearman correlation analysis. Red squares indicate a positive correlation; whereas blue squares indicate a negative correlation. **(B)** The labels on the abscissa and the longitudinal axis represent Pearson's correlation heatmap among host markers. Red squares indicate a positive correlation and blue squares indicate a negative correlation. Deeper colors indicate stronger correlation scores. The asterisks symbol indicates significant differences *P < 0.05, **P < 0.01, ***P < 0.001.

points, we further started analyzing the correlation between microbiota and metabolic indices in the gut tract of meat geese at 90d. The results based on microbial alterations showed that *Faecalibacterium* was positively correlated with cecal pH, GSR, GSH-PX, and T-AOC and cecal *IL-10* and *LC8* and negatively correlated with serum ROS, MDA, and TG, and cecal *LBP*, *TLR4*, *MyD88*, *TNF-a*, *p19ARF*, and *p16INK4a*, and body weight. *Norank_f:Eubacterium_coprostanoligenes_group* was positively correlated with serum HO-1, T-SOD, CAT, and HDL-C and cecal *IL-4*, ZO-1, Occludin, *E-cadherin*, and *IKB-a* and negatively correlated with serum LPS, ROS, and LDL-C and cecal *sCD14*, *NF-κB*, *IL-1β*, *CASP3*, *CASP8*, *Keap1*, and *p21* and blood glucose. At 90 day we observed that *Faecalibacterium* was less positively correlated with ALP that detained the pathogenic effects of *Christensenellaceae_R-7_group* and *Rikenellaceae_RC9_gut_group* in AGF meat geese.

Next, a series of correlation analyses among endotoxemia, gut permeability, pro- and anti-inflammatory cytokines, aging phenotypes, and metabolic syndrome was shown by a Pearson's correlation heat map (Figure 9B) in meat geese at 45d, 60d, and 90d. Among them, *LBP* (P = 9.8E-08, R = 0.97351), *sCD14* (P = 1.4E-05, R = 0.92743), *TLR4* (P = 1E-08, R = 0.98324), *MyD88* (P = 3.2E-08, R = 0.97892), ROS (P = 4.6E-07, R = 0.96388), *NF-κB* (P = 3.4E-08, R = 0.97865), *cytochrome C* (P = 2.5E-07, R = 0.968042101), and *Keap1* (P = 2.8E-09, R = 0.98707) were significantly positively correlated with LPS at 45d in IH feeding meat geese. ALP (P = 0.00027, R = -0.8663), *Nrf2* (P = 2.7E-08, R = -0.9796), *IL-4* (P = 3E-07, R = -0.9669), *IL-10* (P = 2.8E-05, R = -0.9164), *dlg1* (P = 0.02022, R = -0.6572), *E-cadherin* (P = 4.4E-08, R = -0.9775), ZO-1 (P = 1.4E-06, R = -0.9546), Occludin (P = 2.6E-05, R = -0.9177), Claudin (P = 4.5E-06, R = -0.9426), *LC8* (P = 9.2E-06, R = -0.9334), and *IKB-a* (P = 1.5E-08, R = -0.9819) were negatively associated with LPS at 45d in GL feeding meat geese.

At 60d *LBP* (P = 1E-08, R = 0.98321), *sCD14* (P = 0.00011, R = 0.88818), *TLR4* (P = 0.00248, R = 0.78524), *MyD88* (P = 1.9E-11, R = 0.99523), ROS (P = 1.6E-09, R = 0.9885), *NF-κB* (P = 4.8E-07, R = 0.96351), *cytochrome C* (P = 0.00016, R = 0.88005), and *Keap1* (P = 1.2E-08, R = 0.98258) were significantly positively correlated with LPS in IH feeding meat geese. ALP (P = 9.2E-08, R = -0.9739), *Nrf2* (P = 1.3E-12, R = -0.9972), *IL-4* (P = 8E-12, R = -0.996), *IL-10* (P = 5E-13, R = -0.9977), *dlg1* (P = 5E-12, R = -0.9964), *E-cadherin* (P = 1.8E-13, R = -0.9981), ZO-1 (P = 5.1E-10, R = -0.9908), Occludin (P = 2.1E-08, R = -0.9806), Claudin (P = 5.5E-11, R = -0.9941), *LC8* (P = 1.6E-10, R = -0.9927), and *IKB-a* (P = 9.4E-07, R = -0.9582) were negatively associated with LPS at 60d in GL feeding meat geese.

At 90d *LBP* (P = 3.2E-10, R = 0.99164), *sCD14* (P = 0.01474, R = 0.68113), *TLR4* (P = 1E-10, R = 0.99336), *MyD88* (P = 4.8E-12, R = 0.99638), ROS (P = 4.6E-10, R = 0.99098), *NF-κB* (P = 2.1E-08, R = 0.98056), *cytochrome C* (P = 5.8E-09, R = 0.98501), and *Keap1* (P = 2.4E-09, R = 0.98744) were

significantly positively correlated with LPS in IH feeding meat geese. ALP (P = 1E-08, R = -0.9832), *Nrf2* (P = 2.2E-11, R = -0.9951), *IL-4* (P = 7.2E-12, R = -0.9961), *IL-10* (P = 9.6E-11, R = -0.9934), *dlg1* (P = 6.7E-10, R = -0.9903), *E-cadherin* (P = 1.3E-11, R = -0.9956), ZO-1 (P = 4.5E-12, R = -0.9964), Occludin (P = 5.5E-06, R = -0.94), Claudin (P = 1.3E-05, R = -0.9285), *LC8* (P = 9.1E-11, R = -0.9935), and *IKB-a* (P = 2.2E-08, R = -0.9803) were negatively associated with LPS at 60d in GL feeding meat geese.

Discussion

Intracellular ROS production by diet-induced gut microbiota facilitated LPS generation (50) may lead to chronic low-grade inflammation and modern chronic inflammatory diseases (51). Discovering a safe and novel means of limiting its development is urgently required for the prevention and treatment of these diseases. Diet is a primordial need for life and today, the modern poultry industry is based on grains with lower content of dietary fiber (52). Moreover, the worldwide trends of excessive low dietary fiber intake have been implicated in today's chronic inflammatory diseases including diabetes mellitus, autoimmune, cancer, cardiovascular, and chronic kidney disease (53, 54). However, the connections between the shifts in dietary fiber contents and attenuating the incidence of chronic inflammatory diseases by activating the intestinal ALP-dependent-redox signaling mechanism remain to be elucidated in meat geese. Therefore the present study demonstrates for the first time, that a long-term pasture grazing can alleviate commercial diet-induced gut microbial dysbiosis, gut barrier dysfunction and integrity, inflammatory diseases, aging phenotypes, and metabolic syndrome.

Though recent dietary supplementation studies have addressed some impacts of dietary fiber on gut microbiota (55, 56). However, despite these captivating findings, the mechanisms underlying these diverse associations and their outcomings have not been fully explored. In our study, we established artificial pasture grazing system for meat geese that supports concurrent processes to impede in-house feeding system-induced metabolic endotoxemia and systemic inflammation. Consequently, the innovative feature of the AGF system as a high dietary fiber source is to build a pathway-based mechanism by which it increased the abundance of intestinal ALP-producing bacteria, and prevented IHF system-induced LPS-producing bacteria. These changes improve the intestinal nutrient absorption, mucus layer, and mucus-producing goblet cell genes, resulting in reduced metabolic endotoxemia (LPS), LPS-induced ROS production, and gut permeability. The subsequent reduction of pro-inflammatory cytokines lead to the prevention of chronic inflammatory diseases and aging phenotypes. Spearman and Pearson's correlation analysis, including the above-mentioned findings, strongly supports the proposed mechanisms.

Metabolic endotoxemia can be determined by the abundance of bacteria affecting LPS production (57). In this study, we found that AGF intervention reduced the enrichment of genes involved in LPS biosynthesis based on the predicted function by 16S rRNA sequencing and PICRUSt analysis. Interestingly, our current findings have shown similar results with AGF intervention similar to dietary capsaicin in human subjects (58). This would indicate the possibility that a lower abundance of Gram-negative microbiota must be responsible for the low abundance of COG orthology belonging to LPS biosynthesis functions in the AGF meat geese. This could mainly be due to the prevention of members of the Gram-positive phyla *Firmicutes* (genera *Lactobacillus*, *Ruminococcus_torques_group*, *Subdoligranulum*, and *Christensenellaceae_R-7_group*) and *Actinobacteriota* (genera *norank_f_norank_o_Gastranaerophilales*) and gram-negative phyla *Bacteroidota* (genera *Prevotellaceae_UCG_001*, *Bacteroides*, *Alistipes*, and *Rikenellaceae_RC9_gut_group*) with AGF intervention because these were the key bacterial phyla and their respective genera that largely contribute to the IHF meat geese. These lipopolysaccharides are bacterium-associated molecular patterns, which act *via* TLR4/MyD88 pathway by promoting the inflammatory response (59).

The production of ROS by LPS-induced TLR4/MyD88 pathway activation (44) may depend on a diet rich in high fat, high calorie, high protein, and high carbohydrates (60, 61). The correct cellular response to ROS production is critical to preventing oxidative damage and maintaining cell survival. However, when too much cellular damage has occurred, it is to the advantage of a multicellular organism to remove the cell for the benefit of the surrounding cells. ROS can therefore trigger apoptotic cell death based on the severity of the oxidative stress (62) and may contribute to inducing NF-κB pathway (63). ROS-induced severe apoptotic cell death may accelerate intestinal mucosa disruption and result in causing intestinal permeability (64). Based on the current studies, we hypothesized how intestinal ALP would incinerate in LPS-induced TLR4/MyD88 facilitates ROS production pathway.

Intestinal ALP is a major enzyme of interest for its gut microbiota-modifying properties (65, 66). Endogenous ALP production has been shown to inhibit the overgrowth of *E. coli* by dephosphorylating LPS (28, 57, 67). It is well known that intestinal ALP capacity to dephosphorylate LPS was shown to be present in the colon and feces of mice (26) and reduces LPS-induced gut permeability and inflammation in Caco2 and T84 cell lines (68). LPS binds specifically to TLR4 and stimulates inflammation by activating two distinct pathways, namely LPS-dependent release of TNF-α and NF-κB (through MyD88-dependent and –independent pathways) (69). The data from our experiment support the notion that the AGF system as high

dietary fiber source enhanced the abundance of intestinal ALP producing *Alistipes*, and *Lactobacillus*, (45d), *Norank_f: norank_o:RF39* and *Romboutsia* (60d), and *Faecalibacterium* (90d) genera. This microbiota was further seen to involve in suppressing *E. coli* and inactivating the capacity of lipid A biosynthesizing genes (*lpxA*, *lpxB*, *lpxC*, and *lpxD*) to bind LPS with TLR4 and then inhibit the activation ability of MyD88 dependent pathway. Some reports, utilizing dietary fiber as a nutrient source in animals, support our results (24, 70).

It is well-known that endogenous ALP production enhances the expression of proteins (Zo-1, Occludin, and Claudin) involved in tight junctions, thereby preventing the translocation of endotoxins (LPS) by intestinal gram-negative bacteria (*E. coli*) across the gut barrier (21, 57). Our results were by the reports of Kaliannan et al. (57); Schroeder et al. (71); Kühn et al. (21); and Mei et al. (72), in which microbially-induced endogenous intestinal ALP production was observed to decrease the ROS production and apoptosis-related genes *CASP3* and *CASP8*, improve the mucus-producing goblet cell genes *MUC2* and *MUC5AC* as well as inner muscular tonic/muscularis mucosal layer thickness. Moreover, tight junction proteins Zo-1, Occludin, and Claudin and 2 genes encoding tight junction protein *dlg1* and *E-cadherin* were observed to be increased in AGF meat geese which gave the fact that these proteins were strongly involved in inducing nutrient absorption and overall intestinal health (Figure 10).

ROS production regulates *NF-κB* activity in a bidirectional fashion, namely, ROS may trigger activation or repression of *NF-κB* activity (73). In many studies, *NF-κB* inhibition is LC8 dependent (46). But in our study, we have shown that the activation of *NF-κB* by LPS-induced TLR4/MyD88-accelerated ROS production is collectively *LC8* and *IkB-a* dependent. Some reports, utilizing LPS as a ROS inducer, support our results (74, 75). The results of our study with a little modification from those of Cario (76) and Fukata et al. (69), evinced that the triggering of LPS facilitated MyD88 pathway and the subsequent ROS production may altogether activate the *NF-κB* signaling cascades, which played an important role in the development of inflammation by synthesizing and stimulating pro-inflammatory cytokines (*iNOS*, *COX2, IL-1β, IL-6*, and *TNF-α*) (29, 77). Dysregulated inflammatory cytokines production plays a pivotal role in developing low-grade inflammation (78). The above-mentioned so-called studies were unable to describe whether the activation of NF-κB pathway and the resulted pro-inflammatory cytokines were owing to connections between the dietary components (dietary fiber) and gut microbiota. Our results were in accordance with the report of Kyung-Ah Kim et al. (79); Eva d'Hennezel et al. (5); and Sun et al. (51) in which the microbially-induced LPS production increased the mRNA expression of *NF-κB, iNOS, COX2, IL-1β, IL-6*, and *TNF-α* in the liver of mice, pigs, and humans as that of our IHF treatment meat geese. Conversely, in our study, we discovered from the spearman correlation analysis between microbiota and host markers that AGF intervention prevented the IHF-induced upregulation of gut microbiota interacting with LPS, ROS, and pro-inflammatory cytokines by activating gut microbiota those directly interacting with intestinal ALP and Nrf2 pathway. Further, following the mechanism of Bates et al. (80) and Estaki et al. (28), we developed combined Pearson's correlation analysis among host markers to evaluate whether intestinal ALP is involved in activating Nrf2 pathway. We observed that the intestinal ALP was significantly positively correlated with Nrf2 in all stages of sample collection suggesting that intestinal ALP may contribute to activating Nrf2 pathway in AGF treatment meat geese.

In response to oxidative challenge, a stress response is activated to control ROS overproduction and provide optimal conditions for effective ROS signaling to support redox homeostasis. The Nrf2/Keap1 and nuclear factor kappa-light-chain-enhancer of activated B cells/inhibitory κB protein (NF-κB/IkB) systems were considered to be two major "master regulators" of the stress response. One of the most important ways in which *NF-κB* activity influences ROS levels is *via* increased expression of antioxidant proteins has been explained elsewhere (81). The way by which Keap1-Nrf2 responds to ROS has not been elucidated clearly in meat geese. Upon oxidative stress, *Keap1* acts as a sensor and regulates the activity of *Nrf2* thereby, *Keap1* loses its ability to ubiquitinate *Nrf2*, allowing *Nrf2* to move in the nucleus and activate its target genes (47, 82). The results of our study following this mechanism by which dietary fiber in AGF meat geese was able to activate intestinal ALP that was significantly positively correlated with *Nrf2* and *Nrf2*-regulated genes including *NQ01, Gclc, Gclm*, and *GSTA4*, and the antioxidant defense network-related enzymes such as HO-1, GSR, T-SOD, GSH-PX, T-AOC, and CAT and significantly negatively correlated with oxidative related enzyme MDA.

Several studies revealed that *Nrf2* activity is modulated with different dietary interventions such as a high-fat diet or dietary energy restriction (83, 84). The results suggest that the progression of age-related phenotypes *p19ARF, p16INK4α*, and *p21* detected in this study are primarily caused by the decline of protective function by Nrf2 pathway in IHF meat geese. This may be because of chronic smoldering inflammation which is considered one of the important factors associated with low-fiber diet-related diseases and aging phenotypes (53, 85). We indeed observed that the expression of pro-inflammatory cytokine genes *iNOS, COX2, IL-1β, IL-6*, and *TNF-a* were increased in IHF meat geese. While epidemiological evidence shows that *TNF-a* and *IL-6* are predictive of many aging phenotypes (86). Hence, we found it to be associated with more pronounced aging phenotypes *p19ARF, p16INK4α*, and

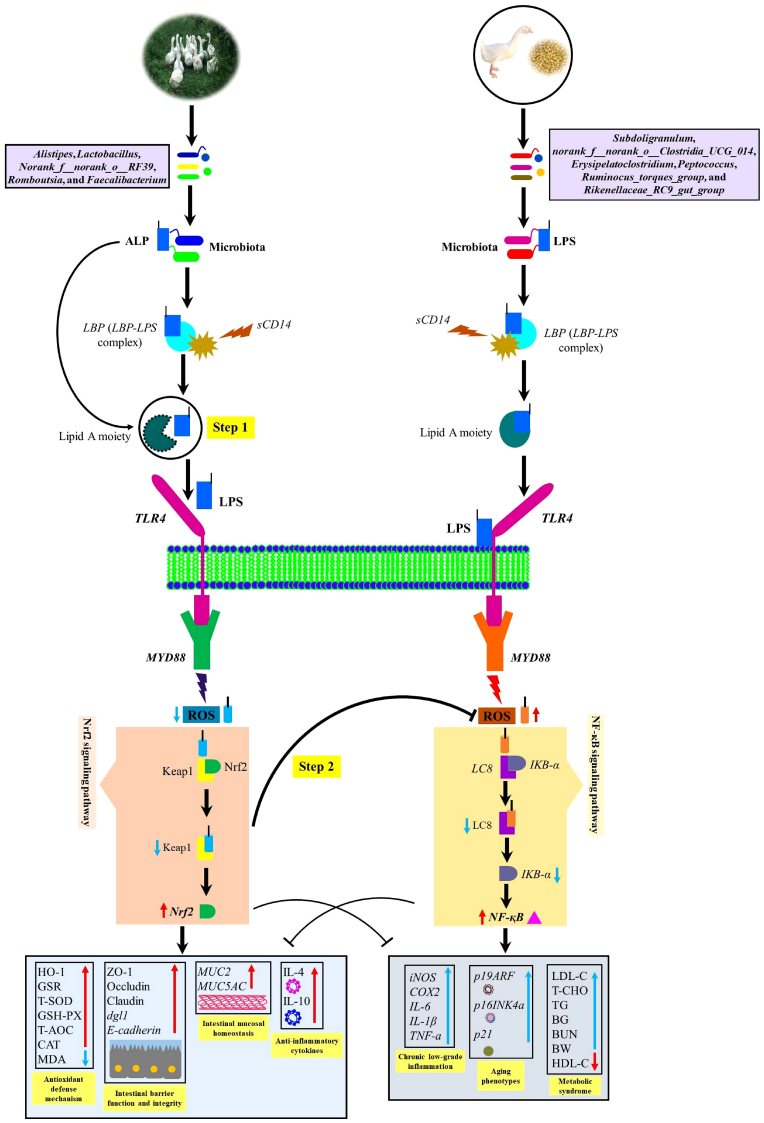

FIGURE 10
Diagram illustrating a proposed mechanism by which artificial pasture grazing system as a high dietary fiber source up-regulates intestinal ALP-producing bacteria and Nrf2 signaling pathway while downregulating LPS-producing bacteria and ROS in meat geese. The increase in intestinal ALP-producing bacteria and the activation of Nrf2 signaling pathway maintain antioxidant and anti-inflammatory mechanisms that lower LPS-producing bacteria and LPS-induced ROS generation, intestinal mucosal deterioration, gut permeability, and metabolic endotoxemia. In the first step, the intestinal ALP attack on TLR4 and let the lipid A moiety not to allow LPS to bind with TLR4 and dephosphorylate LPS by breaking TLR4/MyD88-induced ROS production. In the second step, intestinal ALP activates Nrf2 pathway which reduces oxidative stress, so that ROS could not oxidize LC8 protein and deteriorate IKB-a to activate NF-κB pathway. In this way, intestinal ALP activates anti-inflammatory cytokines and then attenuates chronic low-grade inflammation, aging phenotypes, and metabolic syndrome. BG, blood glucose; TG, triglyceride; BW, body weight; BUN, blood urea nitrogen.

p21 in geese lacking pasture intake, whereas long-term AGF system significantly induced the potent anti-inflammatory action of *Nrf2* and it may evolve in reducing the pro-inflammatory cytokines in AGF meat geese with concomitantly, aging phenotypes. Of note, our results suggest that AGF-induced intestinal ALP positively correlates with *Nrf2* and negatively correlates with *Keap1* and pro-inflammatory cytokines. This notion coincides with the fact that *Nrf2*-mediated inhibition of *iNOS*, *COX2*, *IL-1β*, *IL-6*, and *TNF-a* induction contributes to the prevention of delayed aging phenotypes (87) and the development of geese' health.

In a previous study, intestinal ALP regulation prevents and reverses the changes associated with a high-fat diet-induced metabolic syndrome (21). Furthermore, regulation of intestinal

ALP by dietary fiber-rich diets improves the lipid profile during low dietary fiber and low-fat diets (88). In the current study, we found a low dietary fiber-related spontaneous increase in the serum lipid profile and glucose levels in meat geese, significantly more pronounced in geese lacking pasture intake, underscoring the potential beneficial role of AGF-induced intestinal ALP in the prevention of metabolic diseases.

To prove the hypothesis that intestinal ALP might directly contribute to the reduction of endotoxemia, gut permeability, pro-inflammatory cytokines, and metabolic syndrome, we applied a combined correlation analysis among them. We found that ROS production owing to microbially-induced LPS was seen to be increased with IHF system and further involved in inducing intestinal mucosa deterioration, apoptosis, gut permeability, oxidants, NF-κB pathway, pro-inflammatory cytokines, aging phenotypes, and metabolic syndrome. The establishment of AGF system as a high dietary fiber source can reverse this process. Specifically, AGF system increase the abundance of ALP-producing bacteria and that intestinal ALP negatively correlates with ROS. The low production of ROS in AGF meat geese interacts with *Keap1* and diminishes its activity and then alternatively activates the Nrf2 pathway. Activation of intestinal ALP and Nrf2 pathway collectively positively correlates with *LC8*, *IKB-a*, antioxidants (HO-1, GSR, T-SOD, GSH-PX, CAT, and T-AOC), tight junction proteins ZO-1, Occludin, and Claudin, including 2 genes encoding tight junction proteins *dlg1* and *E-cadherin*, and anti-inflammatory cytokines (*IL-4* and *IL-10*). *IL-4* is produced by Th2 cells (89) whereas *IL-10* is involved in Th2 differentiation (90) and both are known to be anti-inflammatory cytokines (48). Several pieces of evidence from previous studies revealed that *IL-4* and *IL-10* depletion is associated with pronounced ulcerative colitis and Crohn's disease, type 2 diabetes, metabolic syndrome (91, 92). In our study, the activation of intestinal ALP, Nrf2 pathway, antioxidants, *IKB-a*, and anti-inflammatory cytokines potentially evolve in reducing endotoxemia, gut permeability, pro-inflammatory cytokines, aging phenotypes, and metabolic syndrome in AGF meat geese.

In summary, our data suggest that intestinal ALP – as a natural brush border enzyme – plays a critical role in animal health development through maintaining intestinal microbiome homeostasis, reducing LPS-induced ROS production, activating Nrf2 pathway, inducing anti-inflammatory immune responses, and preserving gut barrier function, decreasing low-grade inflammation, and metabolic syndrome. Further studies will focus on elucidating the precise mechanisms of intestinal ALP and Nrf2 pathways' beneficial role in different dietary patterns and aging. Given that AGF system safely induces intestinal ALP and Nrf2 pathways, targeting specific dietary fiber sources that could induce endogenous intestinal ALP production could represent a novel approach to preventing a variety of diet-induced gut microbial-related diseases in animals.

Conclusions

In conclusion, microbially-induced ALP production by AGF system appears to preserve intestinal microbial homeostasis by targeting crucial intestinal alterations, including LPS-induced ROS, gut barrier dysfunction, systemic chronic low-grade inflammation, and metabolic syndrome. By targeting specific dietary fiber sources that could induce endogenous intestinal ALP production may represent a novel therapy to counteract the chronic inflammatory state leading to low dietary fiber-related diseases in animals. If confirmed in humans, these findings may help to better understand diseases with an affected gut barrier functions, such as obesity, ulcerative colitis, cardiovascular, and Crohn's disease.

Data availability statement

Sequence data for cecal microbiome has been uploaded in Sequence Read Achrive of NCBI under accession code: SRP395138.

Ethics statement

The animal study was reviewed and approved by Henan Agricultural University (approval HENAU-2021).

Author contributions

QA and SM designed research, conducted experiments, acquired data, analyzed data, performed statistical analysis, and wrote the manuscript. JN, FL, DL, ZW, HS, and YC acquired data and conducted experiments. YS, SM, and UF designed research, analyzed data, and critically revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1041070/full#supplementary-material. The supplementary materials are available from FigShre: http://doi.org/10.6084/m9.figshare.21543024.

SUPPLEMENTAL FIGURE 1
Overview of feeding and sampling strategies.

SUPPLEMENTAL FIGURE 2
Contributions of bacteria at phylum level to LPS biosynthesis functions. **(A–F)** Relative abundances (%) of the six most dominant phyla in the cecal chyme of the IHF and AGF meat geese. Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. *P < 0.05, **P < 0.01.

SUPPLEMENTAL FIGURE 3
Contribution of individual genera within each phylum to LPS biosynthesis functions. The complete name for each genus is given below. **(A)** Relative abundances (%) of the sixteen most dominant genera (*Subdoligranulum, norank_f:norank_o:Clostridia_UCG-014, unclassified_f:Lachnospiraceae, Lactobacillus, Faecalibacterium, Erysipelatoclostridium, Ruminococcus_torques_group, unclassified_f:Oscillospiraceae, Faecalitalea, Peptococcus, Blautia, Romboutsia, Christensenellaceae_R-7_group, norank_f:Eubacterium_coprostanoligenes_group, Enterococcus, Butyricicoccus*, and *norank_f:norank_o:RF39*) within phylum Firmicutes in the cecal contents of the IHF and AGF meat geese. **(B)** Relative abundances (%) of the five most dominant genera (*Bacteroides, Alistipes, Parabacteroides, Prevotellaceae_UCG-001*, and *Rikenellaceae_RC9_gut_group*) within phylum Bacteroidota in the cecal contents of the IHF and AGF meat geese. **(C–E)** Relative abundances (%) of the most dominant genera within phylum Actinobacteriota (Bifidobacterium), Cyanobacteria (norank_f:norank_o:Gastranaerophilales), and Desulfobacterota (Desulfovibrio) in the cecal chyme of the IHF and AGF meat geese. Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. *P < 0.05, **P < 0.01.

SUPPLEMENTAL FIGURE 4
Effect of different feeding systems on pH values of meat geese gastrointestinal tract. **(A)** pH of proventriculus, **(B)** pH of gizzard, **(C)** pH of ileum, and **(D)** pH of cecum. In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. The asterisks symbol indicates significant differences *P < 0.05, **P < 0.01.

SUPPLEMENTAL FIGURE 5
Effects of different feeding systems on *E. coli* production in cecal tissues of meat geese. **(A)** Representative culture plate photos showing the difference between IHF and AGF meat geese in the growth of LPS-producing gram-negative *E. coli*. CFU/g stool. **(B)** *E. coli* cell cultures based on spectrophotometer readings at OD600 for 48h. Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. *P < 0.05, **P < 0.01.

SUPPLEMENTAL FIGURE 6
Effects of different feeding systems on cecal morphology (100μm). VH – villus height; VW – villus width; DBV – distance between two villi; CD – crypt depth. In-house feeding system (IHF) and artificial pasture grazing system (AGF).

SUPPLEMENTAL FIGURE 7
(A) Comparison of the goblet cell number (per 20μm) of meat geese with different feeding systems. H&E staining of cecal tissues (magnification, 40×). Goblet cell (GC), In-house feeding system (IHF) and artificial pasture grazing system (AGF). Data with different superscript letters are significantly different (P < 0.05) according to the unpaired student T-Test. The asterisks symbol indicates significant differences *P < 0.05, **P < 0.01.

SUPPLEMENTAL FIGURE 8
(A) Light micrograph of the wall of cecum tissues of meat geese, hematoxylin and eosin (40μm): *1* – outer layer of muscular tonic; *2* – inner layer of muscular tonic; *3* – outer layer of lamina muscularis mucosa (LMM); *4* – submucosal nerve node; and *5* – inner layer of lamina muscularis mucosa (LMM). **(B)** Comparison of the cecal membrane thickness of meat geese with different feeding systems (50μm). In-house feeding system (IHF) and artificial pasture grazing system (AGF).

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EDITED BY
Sergey Ponomarev,
Institute of Biomedical Problems, Russian Academy of Sciences (RAS), Russia

REVIEWED BY
Nathalie Guriec,
Centre Hospitalier Regional Universitaire (CHU) de Brest, France
Trim Lajqi,
Heidelberg University Hospital, Germany

*CORRESPONDENCE
Yuxiang Song
songyuxiang2018@126.com
Guowen Liu
liuguowen2008@163.com

†These authors have contributed equally to this work and share first authorship

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β-hydroxybutyrate enhances bovine neutrophil adhesion by inhibiting autophagy

Jiyuan He[1†], Kexin Wang[1†], Mingchao Liu[2], Wen Zeng[1], Dong Li[1], Zolzaya Majigsuren[3], Tugsjargal Batbaatar[4], Yunfei Li[1], Siyuan Liu[1], Xiliang Du[1], Lin Lei[1], Yuxiang Song[1*] and Guowen Liu[1*]

[1]State Key Laboratory for Zoonotic Diseases, Key Laboratory of Zoonosis Research, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, Jilin, China, [2]College of Veterinary Medicine, Hebei Agricultural University, Baoding, Hebei, China, [3]Institute of Veterinary Medicine, Mongolian University of Life Sciences, Ulaanbaatar, Mongolia, [4]State Central Veterinary Laboratory, General Authority for Veterinary Services, Ministry of Food And Agriculture, Ulaanbaatar, Mongolia

Introduction: Subclinical ketosis (SCK) in dairy cows, a common metabolic disorder during the perinatal period, is accompanied by systemic inflammation and a high concentration of blood β-hydroxybutyrate (BHB). BHB induced adhesion of neutrophils may play a crucial role in the development of systemic inflammation in SCK cows. Autophagy, an intracellular degradation system, regulates the recycling of membrane adhesion molecules and may be involved in BHB regulating adhesion and pro-inflammatory activation of bovine neutrophils. Thus, the objective of this study was to determine the relationship between BHB, autophagy, and neutrophil adhesion.

Results and discussion: Here, elevated abundance of serum amyloid A, haptoglobin, C-reactive protein, interleukin-1β, interleukin-6, and tumor necrosis factor-α were found in SCK cows, and all these pro-inflammatory factors had a strong positive correlation with serum BHB. After BHB treatment, the number of adherent neutrophils and the adhesion associated protein abundance of both total and membrane CD11a, CD11b, and CD18 was greater, confirming that BHB promoted the adhesion of bovine neutrophils. However, the mRNA abundance of *ITGAL* (CD11a), *ITGAM* (CD11b), and *ITGB2* (CD18) did not show a significant difference, suggesting that the degradation of adhesion molecules may be impaired. Transmission electron microscopy revealed a decreased number of autophagosomes and a decrease in mRNA abundance of *SQSTM1* (p62) and *MAP1LC3B* (LC3) after BHB treatment. In parallel, protein abundance of p62 increased while the ratio of protein LC3 II to LC3 I decreased after BHB treatment, indicating that BHB inhibits autophagy of bovine neutrophils. To confirm the regulatory role of autophagy in BHB promoting neutrophil adhesion, we used an autophagy activator rapamycin (RAPA). Data showed that RAPA relieved the inhibitory effect on autophagy and the promotive effect on cell adhesion induced by BHB. Importantly, BHB inhibited the colocalization of LC3 and CD11b, which was relieved by RAPA, further confirming the regulatory role of autophagy in the recycling of the

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above adhesion molecules. Furthermore, BHB treatment increased the mRNA abundance and the release of pro-inflammatory factors *IL-1B*, *IL-6*, and *TNF* of bovine neutrophils, and these effects were attenuated by RAPA. Overall, the present study revealed that BHB promotes the adhesion of bovine neutrophils by inhibiting autophagy.

KEYWORDS

dairy cows, ketosis, β-hydroxybutyrate, neutrophils, adhesion

Introduction

Due to the increase in energy demand and the decrease in dry matter intake, dairy cows were subjected to a period of negative energy balance (NEB) during the transition period. NEB intensifies fat mobilization and increases the concentration of non-esterified fatty acid (NEFA) in blood (1). NEFAs are transported into the liver and involved in β-oxidation in the hepatocytes to generate more ATP to relieve the NEB, while excess NEFAs could not be completely oxidized and metabolized into ketones, such as β-hydroxybutyrate (BHB). Subclinical ketosis (SCK) is defined as an excess of circulating BHB (1.2 m*M* -3 m*M*) without clinical signs (2). The incidence rate of SCK can be as high as 36.6% in some herds, far higher than 2% to 15% in clinical ketosis (3). SCK cows experience systemic inflammation, which is characterized by enhanced blood levels of pro-inflammatory cytokines and positive acute-phase proteins (4). The systemic inflammation during SCK is closely related to secondary and concurrent inflammatory diseases, such as mastitis, endometritis, and laminitis (3), and the reduction in production performance and welfare (2). The underlying mechanism of systemic inflammation in SCK cows is still unclear.

BHB, the most abundant ketone body in blood, is not only an energy precursor but an immuno-modulating molecule. Some previous reports have shown that BHB can significantly dampen central nervous system inflammation and exert protective effects in mice and humans (5, 6), whereas, the expression of pro-inflammatory factors significantly upregulated in BHB exposure hepatocytes (7). Therefore, the inflammation-regulatory effects of BHB seem to be context or cell type dependent. Additionally, in ruminants, the pathological concentration of BHB can cause cellular dysfunction such as inhibiting apoptosis of neutrophils, liver damage, and promoting lipolysis of adipocytes (8–10). Thus, the effect of BHB on the immune cells of dairy cows needs to be further studied.

Neutrophils are the first line against the invasion of pathogenic microbes; however, abnormal excessive neutrophil recruitment may result in chronic inflammation and tissue injury. Neutrophils adhere to endothelium or extracellular matrix in a coordinated and reversible manner to start up the chemotaxis process and pro-inflammatory activation. Firm adhesion is mediated by the interaction of integrin on neutrophils with receptors on endothelial cells before recruiting to the local inflammatory sites (11). Neutrophils express high levels of the β2 integrins which are heterodimer composed of a variable α subunit including lymphocyte function-associated antigen-1 (LFA-1, also known as CD11a), macrophage 1 antigen-1 (Mac-1, also known as CD11b), and a constant β subunit (also known as CD18) (12). Synthesis, transportation, membrane location, and intracellular degradation of adhesive molecules are in a state of dynamic circulation (13). Additionally, through an intracellular-signaling process (also known as inside-out signaling), the transformation of integrins to the active conformation can be accelerated, thereby ultimately modulating their affinity for ligands (14). In mice, enhancement of adhesion between neutrophils and vascular endothelial cells causes systemic inflammation and injury of blood vessels and peripheral organs (15). In humans and rats, hyperketonemia (elevated blood ketones) can increase the expression of CD11a in monocytes and macrophages and ICAM I in endothelial cells to promote adhesion between them, potentiating infiltration of monocytes and macrophages into the aorta and liver (16). However, the effect of BHB on the adhesion of bovine neutrophils and the underlying mechanisms are still not clarified.

In both immune and non-immunized cells, autophagy was shown involved in adhesion regulation through balancing the endocytosis, circulation, and degradation of integrins (17, 18). The number of infiltrated neutrophils in microvascular venules increased significantly in $Atg5^{-/-}$ mice (19).

In SCK cows, blood neutrophils are exposed to high concentrations of BHB. Given the potential role of BHB on the adhesion and the involvement of autophagy in regulating cell adhesion, we speculated that BHB may enhance neutrophil adhesion through inhibiting autophagy in SCK cows. The objective of this study was to determine the effect of BHB on the adhesion of bovine neutrophils and the underlying mechanisms.

Materials and methods

Animals and sampling

The protocol was approved by the Ethics Committee on the Use and Care of Animals of Jilin University (No. SY202012016). In the current study, experimental animals received humane care according to the principles and guidelines of the "Guidelines for the Care and Use of Agricultural Animals, 3rd ed" (available from FASS Inc., 1800 S. Oak St., Suite 100, Champaign, IL 61820, USA). In general, ketosis cows can be initially screened by ketone powder testing for ketosis in the milk, and the final diagnosis is confirmed by testing the serum concentration of ketosis. Based on a nitroprusside test for milk ketone bodies, we screened out 23 suspected SCK and 30 control cows with similar parity (median = 3, range = 2 to 4) and days in milk (DIM) (median = 6 d, range = 3 to 15 d) from 100 lactating Holstein cows in a dairy farm located in Changchun City, Jilin Province, China. All screened cows received a routine physical examination to ensure the absence of clinical symptoms and comorbidities. Milk samples were also collected for somatic cell count (SCC) using the Delaval Cell Counter (DeLaval International AB), bacteriological examination by inoculation and culture on bacterial medium, and identification of antimicrobial residues using the SNAP tetracycline test kit, the SNAP β-lactam test kit, and the SNAP gentamicin test kit (Idexx Laboratories). Both bacteriological and antimicrobial residue examinations were negative in the milk of selected cows. Additionally, it has been demonstrated that SCC ≤ 200,000 cells/ml could be considered a threshold for mastitis (20). Cows were divided into two groups according to the concentration of serum β-hydroxybutyrate (BHB): healthy cows (control group, n = 15) with BHB < 0.6 m*M* and SCK cows (SCK group, n = 15) with 1.2 < BHB < 3 m*M*. All selected cows were housed in a climate-controlled barn with individual tie stalls to reduce environmental interference. The selected cows were milked twice daily at 08:00 h and 15:30 h. Cows had ad libitum access to the same diet (21) that was offered twice daily (09:00 and 16:30 h) and freshwater was supplied continuously.

Blood samples were collected from the jugular vein at 06:30-07:30 h before feeding for 3 consecutive days. To obtain serum, blood samples were kept for 30 min at room temperature then centrifuged at 4°C for 3,000 × g for 15 min and stored at -80 °C immediately until use. Serum glucose (GLU) and BHB were detected using commercially available kits (GLU, Cat. No. GL3815; BHB, Cat. No. RB1008; Randox Laboratories, Crumlin, County Antrim, UK) using a Hitachi 7170 autoanalyzer (Hitachi; Tokyo; Japan). Serum metabolic indices for the selected control and SCK cows are reported in Table 1.

Detection of serum inflammatory factors

Concentrations of serum haptoglobin (HP), serum amyloid A (SAA), and C-reactive protein (CRP) were determined with bovine specific ELISA kits (HP, LS-F13229; SAA, LS-F12552, LifeSpan BioSciences Inc., Seattle, Washington, USA; CRP, CSB-E08577b, GUSABIO, China) according to manufacturer's instructions. Kit sensitivity of HP, SAA, and CRP was 7.8 ng/mL, 3.12 ng/mL, and 1.25 ng/mL, respectively. Intra-/inter-assay coefficient of variation (CV) for HP, SAA, and CRP was less than 6.5%/8.8%, 10%/12%, and 8%/10%, respectively. Concentrations of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were measured with bovine specific ELISA kits (IL-1β, LS-F7588; IL-6, LS-F9752; TNF-α, LS-F5014; LifeSpan BioSciences Inc., Seattle, Washington, USA) according to the manufacturer's instructions. Kit sensitivity of IL-1β, IL-6,

TABLE 1 Basic description of the control and SCK cows.

Item	Control cows (n = 15)	SCK cows (n = 15)	*P*-value
Serum BHB (m*M*)	0.39 ± 0.43	1.89 ± 0.12	< 0.01
Serum Glucose (m*M*)	4.19 ± 0.13	3.35 ± 0.22	< 0.01
Serum SAA (μg/mL)	42.23 ± 4.98	120.07 ± 9.47	< 0.01
Serum HP (μg/mL)	128.65 ± 13.01	443.45 ± 53.49	< 0.01
Serum CRP (μg/mL)	20.34 ± 2.65	94.75 ± 3.65	< 0.01
Serum TNF-α (pg/mL)	80.45 ± 6.52	401.02 ± 28.33	< 0.01
Serum IL-1β (pg/mL)	48.97 ± 9.22	79.88± 6.78	< 0.01
Serum IL-6 (pg/mL)	13.44 ± 2.21	49.93 ± 5.98	< 0.01

The concentrations of BHB, Glucose, SAA, HP, CPR, TNF-α, IL-1β, and IL-6 in the serum of control and subclinical ketosis cows have been shown. Values are shown as mean ± SEM. P < 0.05 was considered significant and P < 0.01 highly significant. DMI, dry matter intake; BHB, β-hydroxybutyrate; SAA, serum amyloid A protein; HP, haptoglobin; CRP, C-reactive protein; IL, Interleukin; TNF-α, Tumor necrosis factor-α.

and TNF-α was 6.5 pg/mL, 0.46 pg/mL, and 3.1 pg/mL, respectively. Intra- and inter-assay CV for IL-1β, IL-6, and TNF-α were all less than 10%. Correlations between the content of serum HP, SAA, CRP, IL-1β, IL-6, TNF-α, and BHB in serum were analyzed with the Spearman method. All samples including standards were tested in triplicate.

Cell adhesion assay

For adhesion assays, the 96-well plates were pre-coated with Collagen I (50 μg/mL, Cat. No. C8062, Solarbio Science and Technology Co. Ltd.; Beijing; China) overnight. After isolation, neutrophils were adjusted to 1×10^5 cells/mL. The 100 μL cell suspension was added to a 96-well plate and placed in a 37°C and 5% CO_2 incubator for 2 hours. The non-adherent cells were removed by washing 3 times with PBS solution. The number of remaining cells was counted under a microscope (Fluoview FV1200; OLYMPUS; Tokyo; Japan). The number of adherent neutrophils was calculated by Image J (Media Cybernetics Inc., Bethesda, Maryland, USA). For each sample, at least 5 watching zones were randomly selected for evaluation.

Isolation, culture, and treatment of neutrophils

Blood was collected by jugular puncture and kept in a sterile EDTA vacutainer tube (YA1393, Solarbio Science and Technology Co. Ltd., Beijing, China) at room temperature until transported to the laboratory, within 30 min after collection. Neutrophils were isolated by the commercial bovine peripheral blood neutrophils isolation kit following the manufacturer's protocol (Cat. No. P9400; Solarbio Science and Technology Co. Ltd.; Beijing; China). In brief, neutrophils were separated based on the density gradient centrifugation method. The whole process was carried out in a sterile room at room temperature. Sixteen mL of liquid A and 8 mL of liquid B were slowly added to the 50 mL centrifuge tube. 15 mL of fresh bovine blood was added to the upper layer of liquid B in the same way and centrifuged for 30 min at 800 × g. After centrifugation, the second layer of white turbidity layers was sucked into another sterile centrifuge tube and mixed with 20 mL of cleaning solution. After centrifugation, the supernatant was discarded and 10 mL of red cell lysate was added. neutrophils were obtained through 800 × g for 10 min. The purity of isolated neutrophils was determined by double staining of CH138A (22) and CD11b, and flow cytometry showed that the purity was 97.8%. The cells were diluted with RPMI-1640 (Cat. No. SH30809.01; HyClone; Logan, UT, United States) containing 10% fetal bovine serum (FBS; Cat. No. FB15015; Hyclone Laboratories). The cell density was adjusted to 2×10^6 cells/mL to conduct the following experiment.

BHB (Cat. No. 55397; Sigma-Aldrich) powder was dissolved in PBS, and stored under -80 °C conditions after sterilizing by filtration. The BHB concentrations used in this study were selected according to normal and pathological hematological criteria of cows with or without ketosis. Accordingly, neutrophils were maintained in RPMI-1640 and treated with 1.6 m*M* (subclinical level) for 0, 1, 2, 4, and 6 h. Two hours were selected as the treatment time for subsequent experiments according to an obvious effect on neutrophil adhesion. Neutrophils were then treated with 0, 0.8 (normal level), 1.6 (subclinical level), and 3.2 m*M* (clinical level) for 2 h, and 1.6 m*M* BHB treatment had an obvious effect on neutrophil adhesion. Thus, 1.6 m*M* was selected as the treatment concentration for subsequent experiments. To verify the regulatory role of autophagy signaling, the autophagy activator rapamycin (100 μ*M*, Cat. No. AY-22989; Selleck; Texas; USA) was preincubated for 0.5 h before BHB treatment, respectively.

Protein extraction and Western blotting

Membrane protein of neutrophils was extracted using a commercial protein extraction kit (Cat. No. P0033; Beyotime; Shanghai; China). The total protein was extracted from neutrophils using a commercial Protein Extraction Kit (C510003, SANGON Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's instructions. Protein concentration was measured *via* a BCA method (P1511, Applygen Technologies, Beijing, China). A total of 30 μg of protein from each sample was separated by 12% SDS PAGE. The target protein on the gel was transferred to 0.45-μm polyvinylidene difluoride membranes (PVDF, YA1701, Solarbio Science and Technology Co. Ltd., Beijing, China). The PVDF membranes were blocked with Tris-buffered saline solution with 0.1% Tween-20 (T8220, Solarbio Science and Technology Co. Ltd., Beijing, China) in 3% BSA (A8850, Solarbio Science and Technology Co. Ltd., Beijing, China) for 2 h at room temperature. After electrophoresis, transfer, and blocking, immunoblots were performed using the primary antibody of β-actin (1:2000; ab8226, Abcam, Cambridge, UK), CD11a (1:1000; bs-20370R, Bioss, Beijing, China), CD11b (1:1000; NB110-89474F, Novus Biologicals, Littleton, Colorado, USA), CD18 (1:1000; ab62817, Abcam, Cambridge, UK), and $Na^{+/}K^+$ ATPase (1:800; bs-23413R, Bioss, Beijing, China), p62 (1:1000; PA5-27247, Thermo Fisher Scientific, Massachusetts, USA), and LC3 (1:1000; ab128025, Abcam, Cambridge, UK) at 4°C overnight, respectively. Subsequently, the PVDF membranes were washed 3 times with Tris-buffered saline solution with 0.1% Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated anti-mouse (1:5000; SA00001-1, ProteinTech Group, Inc., Wuhan, China) or anti-rabbit secondary antibodies (1:5000; SA00001-2, ProteinTech Group Inc., Wuhan, China) for 45 min at room temperature.

After washing the PVDF membranes another 3 times with TBST, immunoreactive bands were visualized by enhanced chemiluminescence solution (ECL, WBKLS0500, Millipore, Bedford, Massachusetts, USA). β-actin was used as a reference protein for total proteins, Na^+/K^+ ATPase was used as a reference protein for membrane proteins. Lastly, all bands were imaged using a Protein Simple Imager (ProteinSimple, Santa Clara, CA, USA). All protein bands were quantified using Image-Pro Plus 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA). Western blotting was run in triplicate for each experimental group.

Transmission electron microscope

The number and the ultrastructural characteristics of autophagosomes in cells were evaluated by Transmission Electron Microscope (H-7650 electron microscope, Hitachi, Tokyo, Japan). Neutrophils isolated from SCK and control dairy cows were centrifuged at 1000 × g, 4°C for 10 min After being washed with PBS, 4% glutaraldehyde was slowly added along the tube wall to fix the cell mass. It was placed upright in the refrigerator at 4°C overnight and then fixed with 1% osmium tetroxide at 4°C for 2 h. After washing again, cells were dehydrated in a series of ethanol solutions (70%, 80%, 90%, 100%, 100%, and 100%) and permeated in Spurr's resin. Cells were sliced with an ultra-thin microtome of about 70 nm and dyed with 2% uranyl acetate for 10 min. Cell sections were then dyed with 0.3% lead citrate for 10 minutes and rinsed 3 times with distilled water. After thoroughly air-dried, the sections were observed under an H-7650 transmission electron microscope (Hitachi, Ibaraki Prefecture, Japan). The number of autophagosomes was counted from at least 5 random cell sections in each sample and expressed as the average number of each cell section.

Quantitative reverse-transcription PCR assay

Ribonucleic acid was extracted from cell samples using Trizol (Cat. No. 15596026; Invitrogen; Carlsbad; CA), according to the manufacturer's instructions, and dissolved with 20-30 μL of RNase-free water. Use a k5500 microspectrophotometer (Beijing Kaiao Technology Development Ltd.; Beijing; China) to determine the concentration and quality of RNA. One μg RNA from each sample was reverse-transcribed to cDNA using a PrimeScript Reverse Transcriptase Kit (Cat. No. 6110B; TaKaRa Biotechnology Co. Ltd.; Tokyo; Japan). The mRNA abundance was detected using an SYBR green plus reagent kit (Cat. No. 4913850001; Roche; Norwalk; CT) with the 7500 Real-Time PCR System (Applied Biosystems Inc.; Waltham; MA). Relative gene expression was normalized with *β-Actin* and *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ)*. The PCR reactions were performed in triplicate for each of the 3 individual cell preps and determined by the $2^{-\Delta\Delta CT}$ method (23). Primers of target genes, as shown in Supplement Table 1, were designed with Primer Express software (Applied Biosystems Inc.).

Immunofluorescence assay

After treatment, neutrophils were harvested and washed twice with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After incubation with proteinase K for 1 min, neutrophils were permeabilized with Triton X-100 (0.1%) for 30 min. Subsequently, neutrophils were blocked with 5% goat serum for 1 h followed by incubation with the specific primary antibodies overnight at 4 °C. After washing with PBS 3 times, neutrophils were then incubated with the secondary antibody for 1 h at room temperature. Then, samples were washed with PBS 3 times and incubated with DAPI (Cat. No. D1306; Thermo Fisher Scientific) for 10 min. Fluorescence was observed using a Confocal Microscope (Fluoview FV1200; OLYMPUS; Tokyo; Japan). Co-localization analysis was done using Image J (Media Cybernetics; Rockville; Maryland). In brief, we used Coloc 2 co-localization plugin to analyze Pearson's correlation coefficients of CD11b and LC3. The experiment was repeated 3 times, and at least 5 watching zones per sample were selected for evaluation.

Statistical analysis

The one-way ANOVA or the independent-samples t-test was used when there was only one treatment factor. The two-way ANOVA was used when there were two treatment factors (BHB and rapamycin), including a Bonferroni *post-hoc* analysis when significant interaction occurred. All analyses were performed using SPSS (Statistical Package for the Social Sciences) 19.0 software (IBM). Correlation analysis was performed using the Spearman method. Weak, moderate, and strong correlations were defined as correlation coefficients (R) of 0 to 0.39, 0.40 to 0.59, and 0.6 to 1.0, respectively. $P < 0.05$ was considered significant and $P < 0.01$ was markedly significant.

Results

Systemic inflammation of SCK cows

Compared with the control group, SCK cows had a higher concentration of serum BHB ($P < 0.01$) and lower glucose ($P <$

0.01, Table 1). SCK cows had a greater ($P < 0.01$) concentration of positive acute phase proteins including HP, SAA, and CRP, as well as a greater ($P < 0.01$) concentration of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α in the serum (Table 1). These data suggest the development of SCK may be intimately tied to inflammation. Importantly, serum concentration of the above inflammatory factors had a strong positive correlation with that of BHB, respectively (Table 2).

BHB enhances the adhesion of bovine neutrophils *in vitro*

Bovine neutrophils were treated with 1.6 m*M* BHB for 0, 1, 2, 4, or 6 h *in vitro*. After 2 h of BHB treatment, the number of adherent cells increased significantly ($P < 0.01$, Figure 1A), and the total and membrane protein abundance of CD11a, CD11b, and CD18 increased significantly ($P < 0.01$, Figures 1B-I). It indicated that BHB can play the role in the enhancement of neutrophil adhesion, and 2 h BHB stimulation was chosen as the subsequent experimental condition. However, mRNA abundance of them was not significantly affected by BHB treatment (1.6 m*M*, 2 h) (Figure 1J), suggesting that the degradation of adhesion molecules is impaired.

The results of the adherent experiment showed that the number of adherent cells increased significantly when neutrophil was stimulated with the 1.6 m*M* and 3.2 m*M* BHB ($P < 0.01$, Figure 2A). There was no significant difference between the 1.6 m*M* and 3.2 m*M* BHB treatment groups, so 1.6 m*M* BHB was chosen as the stimulated concentration for the subsequent experiment. In terms of the western blot results, membrane protein abundance of CD11a, CD11b, and CD18 increased significantly after 1.6 m*M* BHB treatment ($P < 0.01$, Figures 2B-E).

BHB impairs autophagy in bovine neutrophils

To confirm the regulatory role of BHB in autophagy, bovine neutrophils were treated with BHB (0, 0.8, 1.6, or 3.2 m*M*) for 2 h *in vitro*. The protein abundance of p62 increased while the ratio of LC3 II to LC3 I decreased after treatment with a pathological concentration of BHB (1.6 m*M* and 3.2 m*M*) ($P < 0.01$, Figures 3A-C). Consistent with this, the pathological concentration of BHB decreased the mRNA abundance of *SQSTM1* and *MAP1LC3B* ($P < 0.01$, Figure 3D). Transmission electron microscopy revealed a lower average number of autophagosomes after BHB treatment (1.6 m*M*, 2 h) ($P = 0.02$, Figures 3E, F), indicating that BHB can inhibit neutrophil autophagy in cows with SCK.

BHB enhances the adhesion of bovine neutrophils by inhibiting autophagy

To investigate the relationship between BHB-induced increase in adhesion of neutrophils and impaired autophagy, the autophagy activator RAPA was used before BHB treatment. Compared with the control group, BHB treatment (1.6 m*M*, 2 h) increased ($P < 0.01$) the protein abundance of p62 while decreasing ($P < 0.01$) the ratio of protein abundance LC3 II to LC3 I in bovine neutrophils, both of which were reversed by RAPA treatment, a widely used activator of autophagy (Figures 4A-C). Compared with the control group, the protein abundance of membrane CD11a, CD11b, and CD18 was increased ($P < 0.01$) after BHB treatment, and RAPA reversed the above effects of BHB ($P < 0.01$, Figures 4D-G). Correspondingly, after BHB treatment, the number of adherent cells increased ($P < 0.01$) in the cell adhesion experiments, and the use of RAPA relives this effect ($P < 0.01$, Figure 4H).

BHB inhibits the co-localization of CD11b and autophagosomes

Compared with the control group, the co-localization between CD11b and LC3 was lower ($P < 0.01$) after BHB treatment (1.6 m*M*, 2 h). RAPA treatment enhanced their co-localization ($P < 0.01$) and relieved the effect of BHB ($P < 0.01$, Figures 5A, B). IF results further suggest that BHB can regulate the adhesion of neutrophils by inhibiting the circulation of adherent molecules.

TABLE 2 Correlation analysis between concentrations of pro-inflammatory factors and BHB in serum.

	Pro-inflammatory factors	HP	SAA	CRP	IL-1β	IL-6	TNF-α
BHB	*R*-value	0.8005	0.7986	0.8523	0.8772	0.9105	0.8012
	P-value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Weak, moderate, and strong correlations were defined as correlation coefficients (R) of 0 to 0.39, 0.40 to 0.59, and 0.6 to 1.0, respectively. P < 0.05 was considered significant and P < 0.01 highly significant. HP, Haptoglobin; SAA, Serum amyloid A; CRP, C-reactive protein; IL, Interleukin; TNF-α, Tumor necrosis factor-α; BHB, β-hydroxybutyrate.

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Autophagy is involved in BHB pro-inflammatory activation of bovine neutrophils

Compared with the control group, BHB treatment significantly increased the mRNA abundance of *IL-1B*, *IL-6*, and *TNF* ($P < 0.01$, Figure 6A). Compared with the control group, the concentration of IL-1β, IL-6, and TNF-α in the supernatant was higher ($P < 0.01$) after BHB treatment (1.6 m*M*, 2 h), while RAPA reversed this effect ($P < 0.01$, Figures 6B-D). It's consistent with our hypothesis that BHB inhibition of autophagy can participate in pro-inflammatory activation of bovine neutrophils.

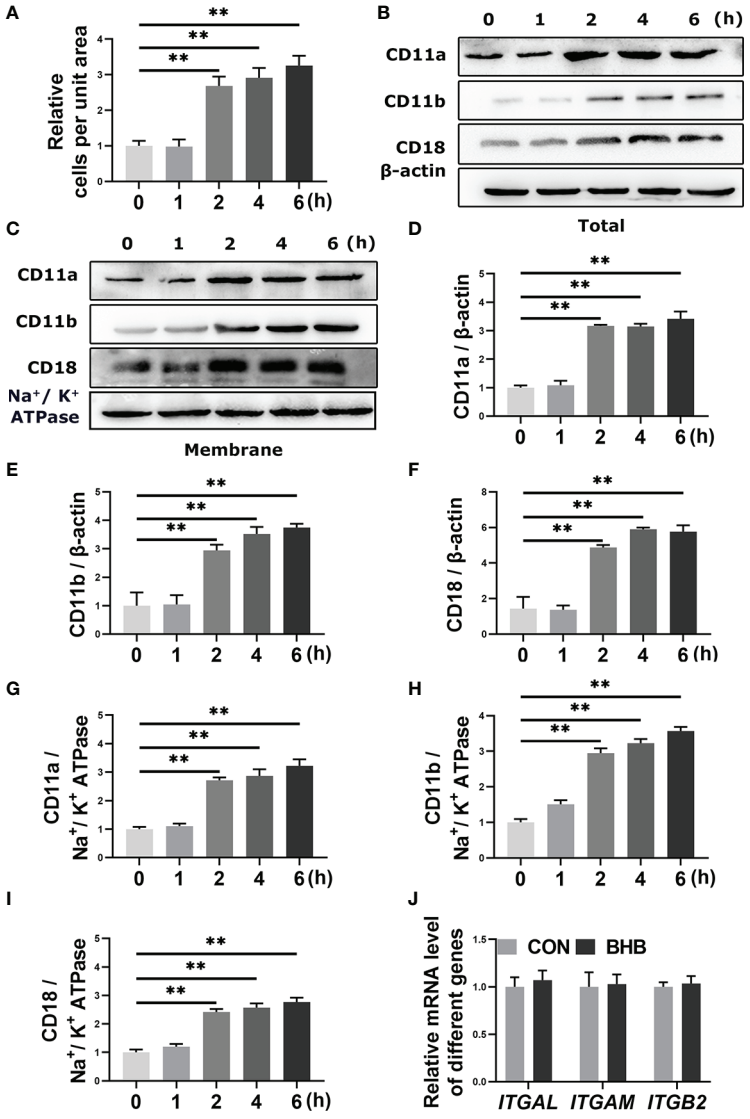

FIGURE 1
Effect of BHB with different stimulated times on the adhesion of bovine neutrophils. Neutrophils were treated with 1.6 m*M* BHB for 0, 1, 2, 4, and 6 h, respectively. **(A)** Quantitation of the number of adherent neutrophils. **(B-I)** Protein abundance of membrane and total CD11a, CD11b, and CD18. Representative blots in both groups were shown in B-C. The quantification was shown in D-I. **(J)** Neutrophils were treated with 1.6 m*M* BHB for 2h. mRNA abundance of *ITGAL*, *ITGAM*, and *ITGB2*. For WB experiments, β-actin was used to normalize the total protein abundance, while Na^{+}/K^{+} ATPase was used to normalize membrane protein abundance; for qRT-PCR experiments, *β-Actin* and *YWHAZ* were used to normalize mRNA abundance. Data were expressed as the mean ± SEM. Data were analyzed with a one-way ANOVA with subsequent Bonferroni correction or independent-samples t-tests. $^{**}P < 0.01$ as statistically highly significant. BHB, β-hydroxybutyrate; CD11b, integrin alpha-M precursor; CD11a, integrin alpha-L precursor; CD18, integrin beta-2 precursor; *ITGAL, integrin subunit alpha L; ITGAM, integrin subunit alpha M; ITGB2, integrin subunit beta 2; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta*; SEM, standard error of the mean.

Discussion

Peripartum dairy cows often experience a state of negative energy balance (NEB). Severe NEB leads to fat mobilization, elevated blood BHB, and ketosis successively. SCK cows always have systemic inflammation characterized by increased pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, as well as increased positive acute phase response proteins HP and SAA in the blood (4). In our data, serum concentration of SAA, HP, CRP, TNF-α, IL-6, and IL-1β was all greater in cows with SCK, indicating the presence of systemic inflammation. Due to systemic inflammation, ketosis cows are more likely to experience mastitis, endometritis, and laminitis, which results in additional costs (24). BHB has been shown an important role in the regulation of inflammation. In bovine hepatocytes, BHB can promote the release of TNF-α, IL-6, and IL-1β by activating the NF-κB signaling pathway, thus aggravating liver damage (7). Additionally, BHB-induced oxidative stress activated the NF-κB signaling pathway and upregulated the release of pro-inflammatory factors in bovine endometrial cells (25). The present study not only confirmed the existence of systemic inflammation but also discovered a strong positive correlation between serum BHB and systemic inflammation.

Inflammation is a double-edged sword, which not only protects the body from infections but also leads to the dysfunction of cells and tissue damage when in excess. Adhesion between neutrophils and endothelial cells mediated by adhesion molecules is the first and key step of the

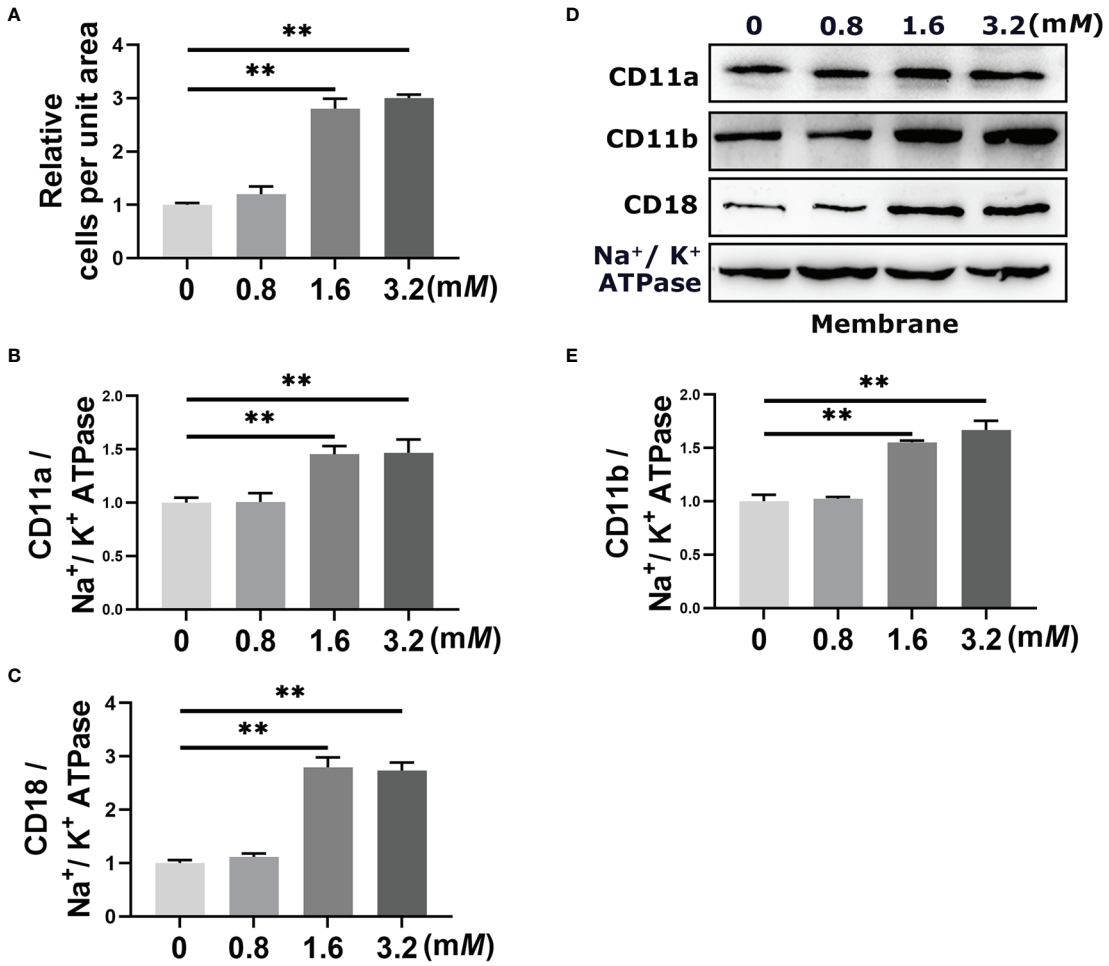

FIGURE 2
Effect of BHB with different stimulated concentrations on the adhesion of bovine neutrophils. Neutrophils were treated with 0 m*M*, 0.8 m*M*, 1.6 m*M*, and 3,2 m*M* BHB for 2 h, respectively. **(A)** Quantitation of the number of adherent neutrophils. **(B-E)** Protein abundance of membrane CD11a, CD11b, and CD18. Representative blots in both groups were shown in **(B)** The quantification was shown in C-E. For WB experiments, Na^+/K^+ ATPase was used to normalize membrane protein abundance. Data were expressed as the mean ± SEM. Data were analyzed with a one-way ANOVA with subsequent Bonferroni correction. $^{**}P < 0.01$ as statistically highly significant. BHB, β-hydroxybutyrate; CD11b, integrin alpha-M precursor; CD11a, integrin alpha-L precursor; CD18, integrin beta-2 precursor; SEM, standard error of the mean.

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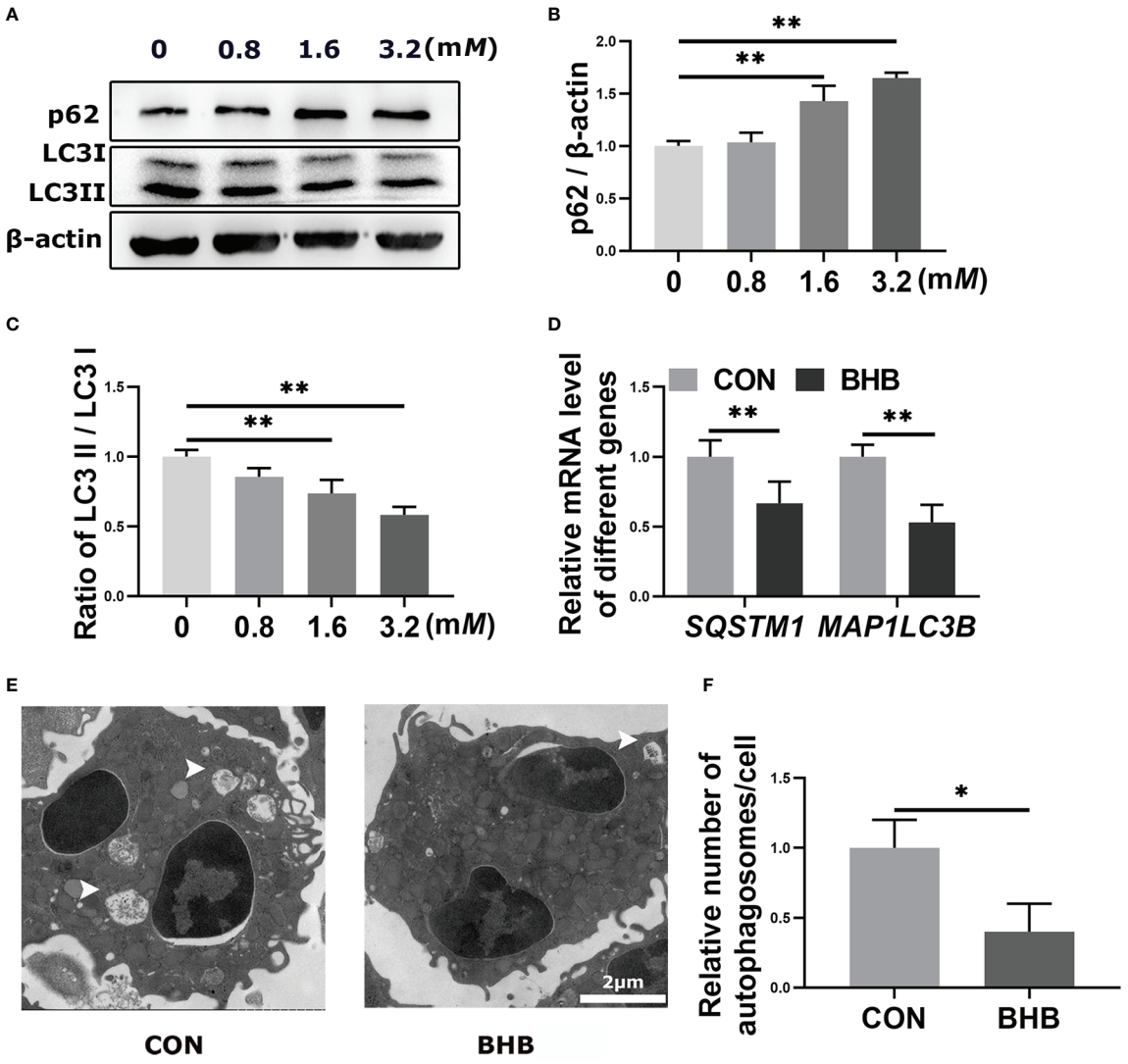

FIGURE 3
Effect of BHB on autophagy of bovine neutrophils. Neutrophils were treated with 0, 0.8, 1.6, and 3.2 m*M* BHB for 2 h, respectively. **(A-C)** Protein abundance of p62 and LC3. Representative blots in both groups were shown in A. The quantification was shown in B-C. Neutrophils were treated with 1.6 m*M* BHB for 2 h **(D)** mRNA abundance of *SQSTM1* and *MAP1LC3B*. **(E)** Representative TEM images showing the autophagosomes in neutrophils. The autophagosomes were labeled by arrowheads. Magnification, 2000× and Scale bars = 2 μm. **(F)** Relative average number of autophagosomes per cell. Autophagosomes were counted from at least 5 random complete views of cells in each sample and expressed as the number of autophagosomes per cell. For WB experiments, β-actin was used to normalize the total protein abundance. For qRT-PCR experiments, *β-Actin* and *YWHAZ* were used to normalize mRNA abundance. Data were expressed as the mean ± SEM. Data were analyzed with a one-way ANOVA with subsequent Bonferroni correction or independent-samples t-tests. $^{**}P < 0.01$ as statistically highly significant. BHB, β-hydroxybutyrate; p62, sequestosome-1; LC3, microtubule associated proteins 1A/1B light chain 3; *SQSTM1, sequestosome-1; MAP1LC3B, microtubule associated protein 1 light chain 3 beta*; *YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta*; SEM, standard error of the mean.

inflammation process (26). Adhesion between neutrophils and endothelial cells promotes the transport of neutrophils to the inflammatory site and the release of inflammatory mediators, resulting in local and systemic inflammation and tissue damage (27). Importantly, the increase of the adhesion molecule CD11a has been shown to be closely associated with the release of pro-inflammatory factors and activation of neutrophil migration and phagocytosis, thereby regulating the inflammatory response (28). In human studies, hyperketonemia increases the expression of monocyte adhesion molecules CD11a and intercellular cell adhesion molecule 1 and promotes their adhesion to endothelial cells (16). Compared with healthy subjects, concentrations of circulating inflammatory markers and adhesion molecules were higher in diabetic patients with

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hyperketonemia (29). The above evidence suggests that the over-activation of neutrophil adhesion is highly related to the development of systemic inflammation. In cows, it was confirmed that the blood content of BHB was positively correlated with the incidence rate of inflammatory diseases such as mastitis and hysterias (3, 30). In the present study, BHB treatment increased the protein abundance of membrane adhesion molecules (CD11a, CD11b, and CD18), which was

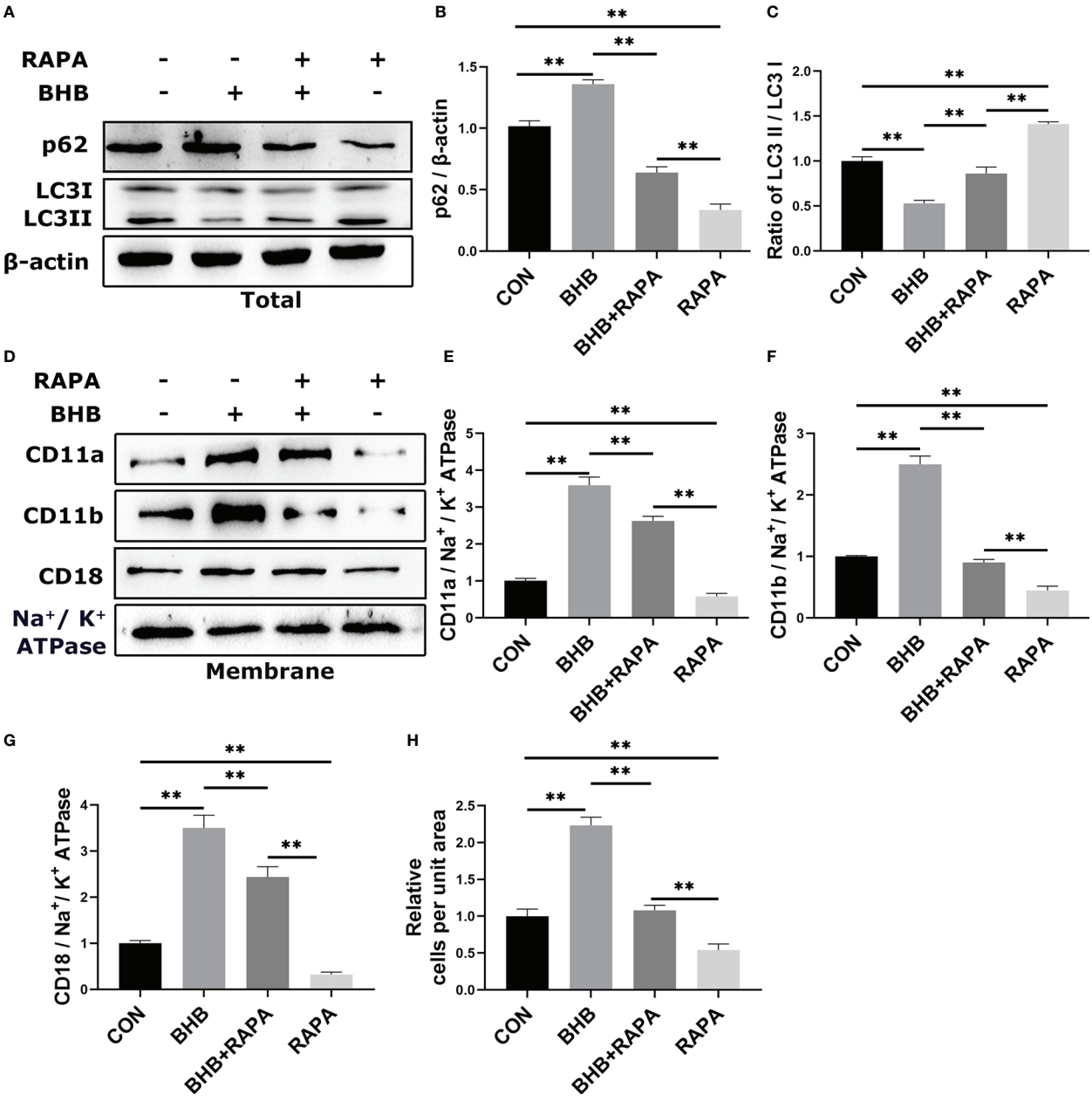

FIGURE 4
Role of autophagy in BHB promoting adhesion of bovine neutrophils. Rapamycin (100 n*M*, 2 h) was used to activate autophagy. **(A-C)** Protein abundance of p62 and LC3. Representative blots in both groups were shown in A. The quantification was shown in B-C. **(D-G)** Protein abundance of membrane CD11a, CD11b, CD18. Representative blots in both groups were shown in D. The quantification was shown in E-G. **(H)** Quantitation of the number of adherent neutrophils. For WB experiments, β-actin was used to normalize the total protein abundance, while Na^{+}/K^{+} ATPase was used to normalize membrane protein abundance. Data were expressed as the mean ± SEM. A two-way analysis of variance (ANOVA) was performed to analyze differences between BHB and RARA, including a Bonferroni *post-hoc* analysis. Two-way ANOVA showed a significant difference in BHB × RAPA interaction ($P < 0.01$), including a Bonferroni *post-hoc* analysis. $^{**}P < 0.01$ as statistically highly significant. CON, control; BHB, β-hydroxybutyrate; RAPA, rapamycin; p62, sequestosome-1; LC3, microtubule associated proteins 1A/1B light chain 3; CD11b, integrin alpha-M precursor; CD11a, integrin alpha-L precursor; CD18, integrin beta-2 precursor; SEM, standard error of the mean.

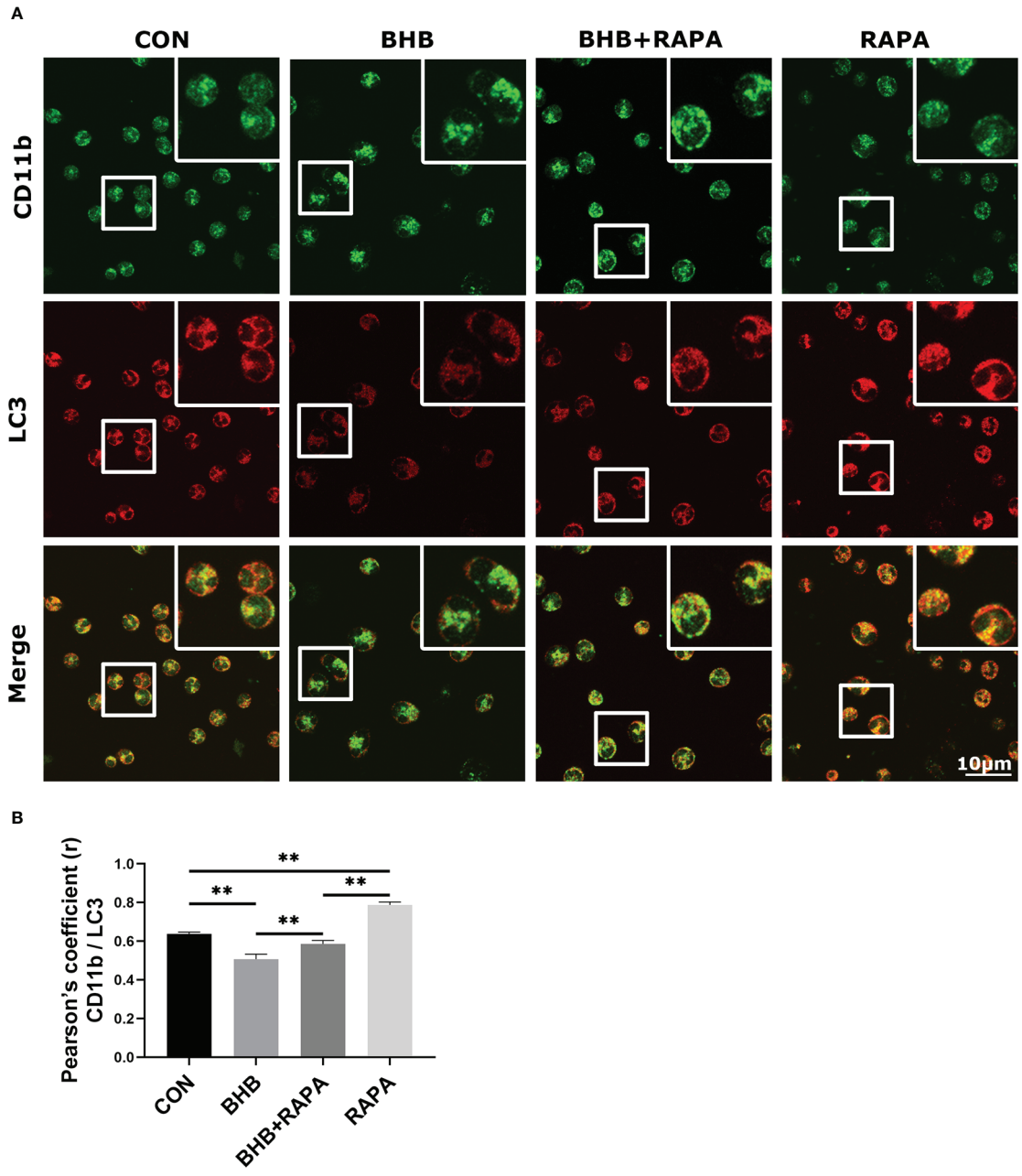

FIGURE 5
Role of autophagy in BHB induced depression of co-localization between CD11b and LC3. Rapamycin (100 n*M*, 2 *h*) was used to activate autophagy. **(A)** Immunofluorescence data showed the co-localization of CD11b with LC3 in neutrophils. Magnification 400×. Scale bar = 10 μm. Nuclei/DAPI in blue, CD11b in green, and LC3 in red. Images here represent the typical common phenotype of each group. **(B)** Pearson's correlation coefficient (r) between CD11b and LC3. Data were expressed as the mean ± SEM. A two-way analysis of variance (ANOVA) was performed to analyze differences between BHB and RARA, including a Bonferroni *post-hoc* analysis. Two-way ANOVA showed a significant difference in BHB × RAPA interaction ($P < 0.01$), including a Bonferroni *post-hoc* analysis. $^{**}P < 0.01$ as statistically highly significant. CON, control; BHB, β-hydroxybutyrate; RAPA, rapamycin; LC3, microtubule associated proteins 1A/1B light chain 3; CD11b, integrin alpha-M precursor; SEM, standard error of the mean.

consistent with the enhanced adhesion function. These data suggested the elevated blood content of BHB, at least partially, is responsible for the systemic inflammation of SCK cows. The change in protein abundance is related to both gene transcription and protein degradation. In mice, BHB can regulate intracellular signal transduction and gene transcription by activating hydroxy-carboxylic acid receptor 2 (HCA2) (31). Additionally, HCA2 may be able to interfere with

neutrophil adhesion to endothelial cells and with neutrophil migration in mice (32). Therefore, the BHB-induced increase in the abundance of adherent molecules may be caused by HCA2-mediated elevated transcription levels of these genes. However, in our data, the mRNA abundance of the above adhesion molecules was not affected significantly by BHB treatment. This suggests that the increased abundance of CD11a, b, and CD18 may be the result of the impaired degradation system in bovine neutrophils.

Autophagy is an important way of intracellular material turnover, maintains cell homeostasis, and helps their survival under stress (33). In both immune and non-immune cells, autophagy regulates cell adhesion by regulating endocytosis and the circulation of membrane integrin (17, 34). Inhibition of autophagy increases the adhesion of macrophages and promotes their migration to the renal in hyperglycemic mice (34). In Atg5 $^{-/-}$ mice, the infiltration of neutrophils in peripheral tissue increases (19). Autophagy associated adhesion strongly correlates to the metastasis of human breast cancer (35). The present study found that the autophagy of bovine neutrophils was inhibited after BHB treatment, along with the accumulation of CD11a, CD11b, and CD18. Importantly, activation of autophagy by RAPA reversed the effects of BHB enhancing adhesion, suggesting an underlying regulatory role of autophagy.

During the development of inflammation, adherent neutrophils produce inflammatory mediators such as IL-17, IL-6, and leukotriene B4. These substances induce endothelial cells, mesenchymal cells, and myeloid cells to release pro-inflammatory factors, including chemokines and matrix metalloproteinases, thereby expanding the inflammatory response and eventually leading to inflammatory injury (36). As an important participant in magnifying inflammation, the pro-inflammatory activation of neutrophils may be the driving factor of systemic inflammation in SCK cows. Some studies have

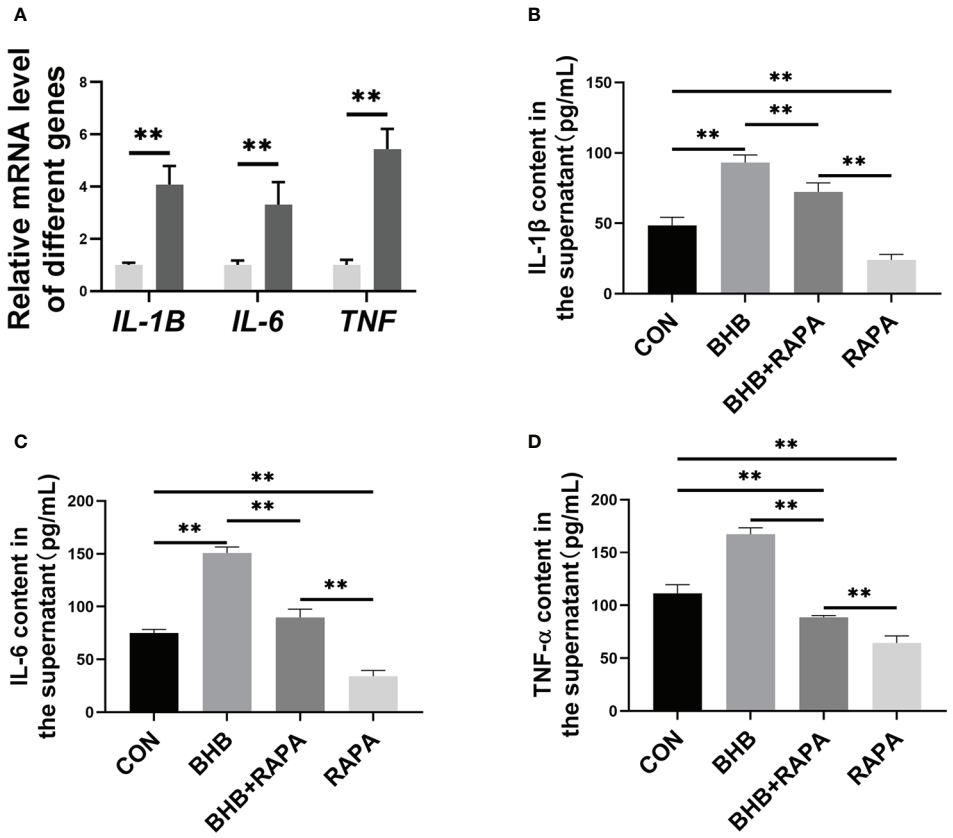

FIGURE 6
Role of autophagy in BHB promoting the production of pro-inflammatory factors in bovine neutrophils. Neutrophils were treated with 1.6 m*M* BHB for 2 h. **(A)** mRNA abundance of *IL-1B* and *IL-6*, and *TNF*. For qRT-PCR experiments, *β-Actin* and *YWHAZ* were used to normalize mRNA abundance. Data were expressed as the mean ± SEM. Data were analyzed with independent-sample t-tests. **(B-D)** Rapamycin (100 n*M*, 2 *h*) was used to activate autophagy. The concentration of IL-1β, IL-6, and TNF-α in the supernatant of the medium. Data were expressed as the mean ± SEM. A two-way analysis of variance (ANOVA) was performed to analyze differences between BHB and RARA, including a Bonferroni *post-hoc* analysis. Two-way ANOVA showed a significant difference in BHB × RAPA interaction ($P < 0.01$), including a Bonferroni *post-hoc* analysis. $^{**}P < 0.01$ as statistically highly significant. CON, control; BHB, β-hydroxybutyrate; RAPA, rapamycin; IL, Interleukin; TNF-α, Tumor necrosis factor-α; SEM, standard error of the mean.

shown that autophagy negatively regulates the pro-inflammatory activation in mouse macrophages (37), while it is still unclear in bovine neutrophils. In the present study, BHB treatment increased the mRNA abundance and the release of pro-inflammatory factors IL-1β, IL-6, and TNF-α of bovine neutrophils, while these effects were relieved when autophagy was active. Therefore, autophagy-mediated adhesion of blood neutrophils to vascular endothelial cells and the subsequent pro-inflammatory activation of neutrophils induced by a high concentration of blood BHB may contribute to the development of systemic inflammation in dairy cows with SCK. However, *in vivo* experimental data are needed to further confirm this view.

Extracellular vesicles (EVs), membrane-enclosed vesicles secreted by nearly all cell types, are enriched with cytokines, chemokines, and immunomodulatory factors which can participate in the modulation of immunity (38). They can transfer bioactive materials (e.g., proteins, nucleic acids, lipids, and metabolites) from host cells to both neighboring acceptor cells and distal recipient cells, thereby orchestrating a persistent communication network in extracellular spaces. Depending on the different host cell types and various bioactive contents, EVs can both have immunostimulatory and immunoinhibitory effects (39). In Lajqi's report, they make a perfect presentation linking small EVs to the regulation of adaptive inflammatory features in innate immune cells (40). Their data show that small EVs may increase the production of pro-inflammatory mediators (e.g., TNF-α and IL-6) and boost transmigratory as well as phagocytic properties mediated by the TLR2/MyD88 pathway in murine neutrophils. Indeed, the increased release of EVs by neutrophils has been found to induce abundance of ICAM-1 on endothelial cells and promote cell adhesion in humans (41). Additionally, neutrophils also contain a releasable membrane-bound organelle named the secretory vesicle (also named granule) which can participate in immune modulation. In our previous study, it has been demonstrated that the enhanced release of the secretory vesicle by neutrophil degranulation can contribute to systemic inflammation. (23). Importantly, a variety of studies show that neutrophil tertiary granule is rich in Mac-1, which transfers to the cell membrane during degranulation through membrane fusion (42). Thus, the granules or EVs may be involved in regulation of BHB-induced neutrophil adhesion in cows with SCK and this needs further investigation in the future.

Continuous assembly and dismantling of adhesions at the cell front and tail produce the driving force for the forward movement of migrating cells (35). There is little research on the regulation mechanism of the turnover of focal adhesion. At the end of the 1980s, scientists first discovered the endocytosis and recycling of integrin, and subsequent studies confirmed that this cycle is related to autophagy (43). Starvation-induced autophagy leads to the degradation of integrins in the lysosomes, but this process is reversible as soon as cells are returned to conditions that inhibit autophagy (17). In the present study, the co-localization between CD11b and LC3 was confirmed, suggesting that autophagy may be also involved in the regulation of adhesion molecules turnover in bovine neutrophils. BHB treatment inhibited autophagy and decreased the co-localization between LC3 and CD11b in bovine neutrophils, while RAPA alleviated this effect. These data suggested that BHB may cause abnormal turnover of adhesion molecules by inhibiting autophagy, thereby leading to their increased membrane localization and finally regulating the adhesion function of bovine neutrophils.

In conclusion, the present study demonstrated that SCK cows exist systemic inflammation, which is related to a high concentration of BHB in blood. *In vitro* experiments, it is confirmed that pathological concentration of BHB promotes the adhesion of bovine neutrophils by inhibiting autophagy. Our data provide a new understanding of the development of systemic inflammation in SCK cows.

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 authors.

Ethics statement

The animal study was reviewed and approved by Ethics Committee on the Use and Care of Animals of Jilin University.

Author contributions

JH and KW wrote the manuscript and prepared all the figures. ML, DL, YL, LL, XD and SL participated in the experiment and the analysis of data. GL, and YS were involved in the study design and funding acquisition. ZM, TB, and WZ assume responsibility for oversight and leadership in the study. All authors contributed to the manuscript and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1096813/full#supplementary-material

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EDITED BY
Sergey Ponomarev,
Institute of Biomedical Problems (RAS), Russia

REVIEWED BY
Emmanuel Asiamah,
University of Arkansas at Pine Bluff, United States
Shanshan Yang,
Jiangxi Agricultural University, China

*CORRESPONDENCE
Zhijun Cao
caozhijun@cau.edu.cn

†These authors have contributed equally to this work

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Overview of the effect of rumen-protected limiting amino acids (methionine and lysine) and choline on the immunity, antioxidative, and inflammatory status of periparturient ruminants

Muhammad Zahoor Khan[1,2†], Shuai Liu[1†], Yulin Ma[1], Mei Ma[1], Qudrat Ullah[2], Ibrar Muhammad Khan[3], Jingjun Wang[1], Jianxin Xiao[1], Tianyu Chen[1], Adnan Khan[4] and Zhijun Cao[1*]

[1]State Key Laboratory of Animal Nutrition, Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, College of Animal Science and Technology, China Agricultural University, Beijing, China, [2]Faculty of Veterinary and Animal Sciences, the University of Agriculture, Dera Ismail Khan, Pakistan, [3]Anhui Province Key Laboratory of Embryo Development and Reproduction Regulation, Anhui Province Key Laboratory of Environmental Hormone and Reproduction, School of Biological and Food Engineering, Fuyang Normal University, Fuyang, China, [4]Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China

Overproduction of reactive oxygen species (ROS) is a well-known phenomenon experienced by ruminants, especially during the transition from late gestation to successful lactation. This overproduction of ROS may lead to oxidative stress (OS), which compromises the immune and anti-inflammatory systems of animals, thus predisposing them to health issues. Besides, during the periparturient period, metabolic stress is developed due to a negative energy balance, which is followed by excessive fat mobilization and poor production performance. Excessive lipolysis causes immune suppression, abnormal regulation of inflammation, and enhanced oxidative stress. Indeed, OS plays a key role in regulating the metabolic activity of various organs and the productivity of farm animals. For example, rapid fetal growth and the production of large amounts of colostrum and milk, as well as an increase in both maternal and fetal metabolism, result in increased ROS production and an increased need for micronutrients, including antioxidants, during the last trimester of pregnancy and at the start of lactation. Oxidative stress is generally neutralized by the natural antioxidant system in the body. However, in some special phases, such as the periparturient period, the animal's natural antioxidant system is unable to cope with the situation. The effect of rumen-protected limiting amino acids and choline on the regulation of immunity,

antioxidative, and anti-inflammatory status and milk production performance, has been widely studied in ruminants. Thus, in the current review, we gathered and interpreted the data on this topic, especially during the perinatal and lactational stages.

KEYWORDS

oxidative stress, periparturient period, ruminants, antioxidants, immunity, limiting amino acids

1 Introduction

Under normal physiological conditions, the antioxidant system's capacity to neutralize and eliminate reactive oxygen species (ROS) produced during metabolic activities is usually sufficient. Metabolic alterations during pregnancy and calving have been shown to increase ROS generation above the level that the antioxidant system can cope with (1, 2). When there is an imbalance between the generation of ROS and the availability of antioxidant molecules, oxidative stress arises, exposing cattle to a variety of illnesses (3, 4). An excessive generation of ROS results in lipid peroxidation, oxidative stress, tissue damage, and changes in the quantity of reduced glutathione (GSH), a key component of glutathione metabolism (3, 5). When the pro/antioxidant balance is disrupted, damage to the structure and function of cellular macromolecules (lipids, proteins, and nucleic acids) occurs, resulting in oxidative stress. A preponderance of oxidation over reduction processes leads to metabolic disorders and diseases in dairy cows (6). Maintaining redox homeostasis in dairy cows during the periparturient and peak lactation stages is therefore critical (4, 7, 8). Parturition-related oxidative stress may contribute to immunological and inflammatory abnormalities, which increase the risk of metabolic and infectious disorders (2, 9).

Metabolic stress during the periparturient period is another key factor that exposes animals to immune depression, abnormal regulation of the inflammatory response, and oxidative stress. During the periparturient period, metabolic stress causes excessive mobilization of lipids followed by oxidative stress (10). The oxidative stress compromises the immunity and inflammatory status in dairy cattle, as shown in Figure 1.

Another critical factor is the abnormal regulation of immunity and inflammation caused by metabolic and oxidative stress (11–14), which predisposes dairy animals to various infections. In addition, the productive efficiency of animals is compromised by oxidative and metabolic stress,

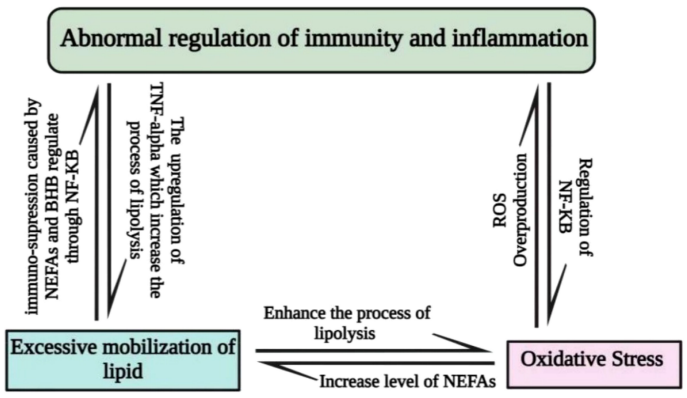

FIGURE 1
Interlink between metabolic stress, oxidative stress, immunity, and inflammation; nuclear factor kappa-B (NF-kB) signaling is used by oxidative stress to promote inflammation and immune system malfunction. Improper inflammatory regulation promotes the synthesis of tumor necrosis factor-alpha in non-phagocytic cells, leading to an excess of OS and excessive lipolysis. Oxidative stress increases the level of NEFAs, which is correlated with excessive lipid mobilization. The excessive lipid mobilization may elevate the level of NEFAs and BHB, which are the key factors in the abnormal regulation of the immune and inflammatory response.

resulting in negative energy balance, immune suppression, and low productive efficiency (15–17). Several nutritional strategies have been adopted to overcome these issues during the periparturient period in ruminants.

The role of amino acids in the regulation of intestinal immunity and inflammation has been discussed previously (18). In brief, He et al. (18) reported that amino acid supplementation regulates the expression of anti-inflammatory cytokines and suppresses the apoptosis of enterocytes, as well as the expression of pro-inflammatory cytokines in intestinal inflammation. Furthermore, it was noted that several anti-inflammatory-related pathways, such as nuclear factor-kappa-B (NF-κB), mitogen-activated protein kinase (MAPK), inducible nitric oxide synthase (iNOS), mechanistic target of rapamycin (mTOR), nuclear erythroid-related factor 2 (Nrf2), and angiotensin-converting enzyme 2 (ACE2), are regulated by amino acid supplementation (18, 19).

Rumen-protected amino acid (methionine and lysine) and choline supplementation has been widely studied for its role in the maintenance of the antioxidant status, immunity status, and anti-inflammatory ability of dairy cattle (11, 20–25). Amino acids such as lysine, choline, and methionine are considered important sources of antioxidants in the diets of ruminants because of their positive role in relieving oxidative stress, which is associated with better health and productive efficiency (22, 23, 26). Thus, in the current review, we summarize the effect of rumen-protected first-limiting amino acids (methionine and lysine) and choline on the regulation of immunity, antioxidative, and anti-inflammatory status, especially during the periparturient period in dairy cattle in which they experience severe oxidative stress followed by depressed immunity and anti-inflammatory status.

2 Effect of rumen-protected first-limiting amino acids (methionine and lysine) and choline on the antioxidant, immunity, anti-inflammatory, and health status of ruminants

2.1 Effect of rumen-protected first-limiting amino acids (methionine and lysine) and choline supplementation on the antioxidant status of ruminants

2.1.1 Effect of methionine and choline supplementation on the antioxidant status of ruminants

The availability of key methyl donors, such as methionine and choline, is limited due to extensive microbial degradation in the peripartal dairy cow (27). If, for any reason, an imbalance of negative methyl donor occurs it will compromise the synthesis of critical compounds, such as carnitine and phosphatidylcholine (PC), which are required for methyl donors in cells and tissues (28). The biosynthesis of PC from endogenous supplementation of methionine and choline could prevent the incoming fatty acids produced from the lipolysis of adipose tissue. It has been documented that the disproportionate infiltration of hepatic fatty acid can negatively influence the normal function of the liver (29). Moreover, the excessive fat infiltration in the liver may suppress intracellular antioxidants, such as glutathione (GSH) and alternatively oxidative stress, which may lead to extensive tissue damage (11, 30, 31). The methyl donors are considered the most important extracellular source of the antioxidants glutathione (GSH) and taurine (32). During the periparturient period, the increased oxidative stress enhances inflammation, resulting in suppressed leukocyte responses. To overcome these challenges and for cells to synthesize sulfur-containing antioxidants, the supplementation of methionine and choline could be beneficial.

Rumen-protected limiting amino acids, such as methionine, are necessary for the biosynthesis of S-adenosylmethionine (SAM) (33). SAM is required for many biological processes, such as transsulfuration, polyamine biosynthesis, and DNA methylation (34). Thus, the need for methyl donors, including choline and methionine, is increased at the onset of the lactation period (35, 36). It has been well-documented that DNA methylation regions are associated with the regulation of gene expression. These epigenetic changes are partly driven by betaine and methionine through SAM. A study documented that rumen-protected choline enhances the expression of genes associated with the synthesis of betaine, phosphatidylcholine, acetylcholine, muscarinic, and nicotinic acetylcholine receptors (37). Interestingly, betaine has been found to work as a methyl donor and help in the regeneration of methionine and SAM (38). In addition, phosphatidylcholine has been found to be involved in the export of low-density protein, which is followed by a decrease in hepatic fat accumulation (39). Furthermore, a study found that methionine supplementation is necessary for the regulation of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα), which plays a key role in reducing the inflammatory response, improving fatty acid oxidation, and preventing liver triacylglycerol (TAG) accumulation (40).

According to the available literature, rumen-protected choline and methionine are considered as valuable antioxidants in ruminant nutrition (41). Recent evidence suggests that methionine plays a role in glutathione formation (42), which could improve the antioxidant status of both animals and their products. Consistently, a study reported that methionine alone or in combination with choline alleviates oxidative stress by enhancing glutathione transferase (GST) activity in the plasma of periparturient ewes (43). Furthermore, another study documented that methionine in

combination with choline enhances glutathione and amino acid levels in dairy cows during the perinatal period (44).

2.1.2 Effect of methionine and lysine supplementation on the antioxidant status of ruminants

Methionine is attacked by many of the ROS generated in biological systems and is considered one of the more readily oxidized residues in proteins (45–47). Methionine has a scavenging ability, and various ROS combine with methionine to form methionine sulfoxide. The cells contain methionine sulfoxide reductases, which catalyze the thioredoxin-dependent reduction of methionine sulfoxide back to methionine (47). Methionine acts as a catalytic antioxidant and protects protein and macromolecules from the action of ROS in which they are present.

The antioxidant role of lysine and methionine has been widely studied in ruminants (48–51). Furthermore, it has been highlighted that amino acids enhance metabolism, antioxidant status, and immunity to improve production and disease resistance. Consistent with this, it has been well established that amino acids play a key role in cellular oxidative balance (52) because they participate in taurine and glutathione (GSH) biosynthesis (53–55). GSH using glutathione transferase (GST) and hydrogen peroxide neutralization *via* glutathione peroxidase (GSH-Px) causes cellular detoxification. Glutathione transferases (GSTs, EC 2.5.1.18) are some of the most important antioxidant enzymes for regulating the cell's redox state (56–58). Similarly, a reduced level of glutathione (a potent intracellular antioxidant) was reported in liver tissue in response to methionine treatment (59).

It has been reported that methionine supplementation enhances very low-density lipoprotein (VLDL) to promote vitamin E in circulation (60). Consequently, the detrimental effect of lipid peroxidation by-products, such as malondialdehyde (MDA), can be suppressed with supplementation with rumen-protected amino acids (60). Furthermore, antioxidant systems, which can be split into enzymatic and non-enzymatic (e.g., metabolites), control ROS (56, 61). The effect of methionine and lysine supplementation on oxidative stress has been studied in ewes (21). It was further noted that methionine supplementation reduces the expression of MDA and improves the level of superoxide dismutase (SOD), CAT, GPH, and GST in the plasma of ewes (21).

A few studies, through *in vitro* experiments, reported that methionine supplementation decreases the process of apoptosis and necrosis and inhibits lipid peroxidation in the bovine mammary glands (62, 63). Moreover, they documented that methionine supplementation enhances the level of antioxidant-associated genes (superoxide dismutase and glutathione peroxidase) and the cytoprotective effect against hyperthermia (62, 63).

2.2 Effect of methionine and lysine supplementation on inflammation and immunity in ruminants

Parturition has been characterized as the stage of inflammatory change during which the level of haptoglobin is elevated, thus decreasing the concentration of albumin (64). Moreover, albumin levels have been reported to decrease around parturition and are also considered as a key biomarker for inflammation in dairy cattle. The higher the level of albumin, especially during the periparturient period, the healthier the animal (65). A higher concentration of albumin has been reported in response to methionine supplementation in dairy cattle (64). Moreover, by binding to non-esterified fatty acids and bilirubin, albumin takes on a detoxifying function and alleviates inflammation (66), which is the critical factor that exposes periparturient dairy cattle to infections. Haptoglobin is another key acute-phase protein, which usually increases around parturition because of inflammatory events and parenchymal cell stimulation by fatty acid infiltration of the liver. Furthermore, it has been demonstrated that methionine supplementation significantly downregulates the level of haptoglobin, which is associated with improved antioxidant and anti-inflammatory levels during parturition in dairy cattle (64). Furthermore, Zhou et al. (64) found that dairy cattle supplemented with methionine were less susceptible to infections during the periparturient period because of suppressed inflammatory changes and improved antioxidant status.

Previous studies have proved experimentally that methionine supplementation enhances the anti-inflammation and anti-oxidative status in periparturient dairy cattle (59, 67, 68) and neonatal calves (69). In a recent study, Hu et al. reported that methionine and arginine significantly regulates milk protein synthesis, thereby alleviating a potential inflammatory and pro-oxidant state in transitioning dairy cattle (70). In addition, a few studies have reported that the supplementation of methionine with arginine plays an important role in anti-inflammation and improves the antioxidant status of transition dairy cattle (68, 71). Moreover, methionine and arginine supplementation has been associated with the regulation of immunity and relief of oxidative stress caused by bacterial lipopolysaccharide (LPS) in bovine mammary cells (71).

It has been reported that rumen-protected lysine and methionine feeding before parturition to transition dairy cows may affect the immunity of calves (69). In an experimental trial, it has been reported that the offspring (calves) of rumen-protected methionine and lysine-fed cows show higher passive immunity, including a higher concentration of immunoglobulin

G, higher serum total protein, and a higher growth rate than an unsupplemented group of dairy cows (69). Lee et al. (72) reported that supplementation with lysine and methionine positively reduces milk somatic cell count (SCC) and improves the immunity and health status of dairy cattle. Consistent with this, a study proved experimentally that methionine supplementation tends to decrease metabolic stress and milk SCC and improve udder health in goats (73).

2.3 Effect of methionine and choline supplementation on the inflammation and immunity of ruminants

It has been well established through experimental trials that treatment with a combination of choline and methionine can regulate the antioxidative state, thereby increasing the anti-inflammatory and cytoprotective effect against oxidative stress in neonatal Holstein calves (74). Consistent with this, a study reported that supplementation with choline and methionine improves the immunometabolic state, blood polymorphonuclear leukocyte phagocytosis capacity, and the anti-inflammatory effect upon pathogen challenge, and enhances the antioxidative capacity of peripartal cows (75). During the periparturient period, the liver functionality index (LFI) is the key indicator that is used to assess immune and inflammatory status, as well as metabolic profiles in dairy cattle (44, 59, 76). In addition, a study reported that a low LFI and low amino acids in circulating plasma indicates a difficult transition from gestation to lactation (30). The supplementation of a combination of choline and methionine enhances the level of the LFI, resulting in an improved immunity and anti-inflammatory status in dairy cattle (44). Moreover, ketosis was also found to be positively associated with a low LFI, while combined supplementation of choline and methionine significantly reduces the chances of ketosis by alleviating oxidative stress in dairy cattle (77).

Choline supplementation in the transition diet may be partially useful for reducing the deleterious effects of an inflammatory-like condition on the hepatic function of transition cows. The non-esterified fatty acids (NEFA) and β-hydroxybutyric acid (BHB) may lead to oxidative stress followed by an enhancement of the lipolysis process (78). Additionally, rumen-protected choline supplementation has been found to reduce the concentration of liver triacylglycerol and metabolic stress, resulting in an improved immune and antioxidative state (79, 80). Consistent with this, choline supplementation also lowers BHBH levels and the body condition score, which is the best indicator of health in dairy cattle during the periparturient period (81, 82). Furthermore, it has been reported that excessive production of NEFA and BHB may compromise immunity, resulting in abnormal regulation of immune and inflammation responses (83). The high levels of NEFA and BHB followed by oxidative stress and dysfunctional immunity have been reported as major contributory factors of mastitis in dairy cattle (10). Several studies have demonstrated that choline supplementation significantly reduces the level of NEFA and BHB, resulting in enhanced immunity and maintenance of the anti-inflammatory state in dairy cows (84–87).

During early lactation, catabolic changes are initiated and lead to the utilization of body energy reserves (non-esterified fatty acids and amino acids). The excessive utilization of NEFA is incorporated into VLDL by the liver. A high level of VLDL is associated with oxidative stress and fatty liver syndrome (88). Furthermore, supplementation of rumen-protected choline enhances the function of the liver by improving VLDL exportation from the liver and relieving oxidative stress (88). By increasing the expression of fatty acid transport protein 5 and carnitine transporter *SLC22A5* in the liver and endorsing apo B-containing lipoprotein assembly, rumen-protected choline supplementation reduces the harmful effects of hepatic lipidosis in periparturient dairy cattle (89). Consistent with this, a study reported that choline significantly decreases the level of postpartal liver TAG and enhances the biosynthesis of VLDL through the regulation of PPARα targets apolipoprotein B (*APOB*) and microsomal triglyceride transfer protein (*MTTP*) (89).

It has been well demonstrated that heat stress compromises immunity and regulates the inflammatory system in an abnormal way, affecting the production performance of dairy cattle (90, 91). Nutritional intervention with methionine and choline has been reported to enhance the immune response to heat stress and play a key role in improving the health of animals (92–94). Furthermore, choline supplementation improves lipid and energy metabolism and alleviates the inflammatory response (95). Rumen-protected choline feeding during the periparturient period is the key focus because of its key role in preventing liver lipid accumulation through VLDL export. Moreover, it has been reported that choline plays an important role in the regulation of immune function and also mitigates inflammatory changes in transition dairy cows (93), enhances immune function in calves (94), and adjusts the responses of immune cells to LPS *ex vivo* (37). Consequently, it has been documented that choline deficiency may lead to intestinal morphology and lipid metabolism impairment in rats (96). The antioxidant, anti-inflammatory and immune regulatory role of limiting amino acids (methionine and lysine) and choline has been summarized in Table 1. Based on the available data, we concluded that choline supplementation maintains the inflammatory response in immune cells and relieves metabolic stress during the periparturient period in dairy cattle.

2.4 Rumen-protected limiting amino acids (methionine and lysine) and choline-regulated genes are associated with the immunity, antioxidant, and anti-inflammatory status of ruminants

The molecular response associated with health regulation in response to rumen-protected limiting amino acids in dairy

TABLE 1 Summary of studies in dairy ruminants investigating the influence of rumen-protected limiting amino acids (methionine and lysine) and choline on immune function and the oxidative and anti-inflammatory status of ruminants.

Amino acid supplementation	Main outcomes	Species/ tissues/ cells	Author
Rumen-protected methionine supplementation	Alleviates inflammation and oxidative stress, resulting in improved liver function Enhances oxidative burst and neutrophil phagocytosis	Plasma (cattle)	Batistel et al. (68)
Methionine and choline supplementation	Methionine is utilized by cells for the synthesis GSH and taurine synthesis Increases metabolism and decreases oxidative stress, inflammation, and enhanced immunity Improves liver function Improves oxidative burst and neutrophil phagocytosis	Liver and plasma (Dairy cattle)	Zhou et al. (59); Zhou et al. (64)
Methionine supplementation	Enhances antioxidant status and improves the immune and anti-inflammatory response in periparturient dairy cattle	Dairy cattle	Zhou et al. (44)
Methionine supplementation	Decreases inflammation and enhances immunity during a transition period and reduces the chances of infection	Dairy calves	Jacometo et al. (97)
Methionine and choline supplementation	Decreases inflammation and enhances immunity during a transition period, and reduces the chances of infection	Dairy calves	Abdelmegeid et al. (74)
Methionine or lysine supplementation	Enhances innate immunity Decreases inflammation and relieved oxidative stress	Sheep	Tsiplakou et al. (43); Tsiplakou et al. (98)
Zinc and methionine supplementation	Decreases inflammation, lowers milk SCC, and prevents mastitis by enhancing immunity and alleviating inflammation	Goats	Salama et al. (73)
Choline and methionine supplementation	Enhances $CD4^+/CD8^+$ T lymphocyte ratio and improves immunity and anti-oxidative status Regulates hepatic lipid metabolism and relief of metabolic stress Improves immunity	Dairy cattle	Sun et al. (60)
Hydroxyselenomethionine supplementation	Increases antioxidant response followed by the relief of oxidative status and improved anti-inflammatory response in dairy cattle during the periparturient period	Dairy cattle	Li et al. (99)
N-acetyl-l-methionine supplementation (NALM)	Relieves oxidative stress in lactating dairy cows Increases concentrations of total protein and globulin in plasma and significantly reduces plasma malonaldehyde concentration	Dairy cattle	Liang et al. (100)
NALM supplementation	Improves metabolism and enhances milk production	Dairy cattle	Fagundes et al. (101)
Methionine supplementation	Upregulates the expression of genes involved in the metabolism of antioxidants Increases the expression of NFE2L2 (a major antioxidant transcription factor) and improves the immune and anti-inflammatory response	Dairy cattle	Han et al. (48)
Methionine supplementation	Enhances mRNA expression of genes related to antioxidative status and GSH metabolism	Dairy cattle	Liang et al. (100)
Methionine and choline supplementation	Enhances the expression of genes associated with the immune and anti-inflammatory response and reduces oxidative stress	Dairy cattle	Lopreiato et al. (102)
Methionine supplementation	Improves whole-blood neutrophil phagocytosis and decreases oxidative stress, resulting in enhanced immunity	Dairy cattle	Osorio et al. (103)
Methionine supplementation	Enhances 1-carbon metabolism and increases the antioxidative status. In addition, methionine increases liver GSH and decreases concentrations of plasma biomarkers of inflammation.	Dairy cattle	Osorio et al. (42); Osorio et al. (67)
Methionine and choline supplementation	Blocks the hyper response of IL-1β during LPS challenge, resulting in improved antioxidant ability	Dairy cattle	Vailati-Riboni et al. (104)
Methionine supplementation	Increases the expression of genes associated with immunity and antioxidant response	Dairy cattle	Zhou et al. (105)
Methionine supplementation	The expression of inflammation- and oxidative stress-associated genes is significantly reduced by methionine supplementation	Dairy cattle	Zhou et al. (75)

(Continued)

TABLE 1 Continued

Amino acid supplementation	Main outcomes	Species/tissues/cells	Author
Methionine supplementation	Enhances the metabolism of carnitine and β-oxidation of fatty acids and improves cholesterol and lipoprotein metabolism Improves 1-carbon metabolism of cystathionine Beta-synthase activity of cystathionine followed by enhancement of antioxidant synthesis	Dairy cattle	Zhou et al. (105); Alharthi et al. (106)
Choline supplementation	Alleviates fatty liver and relieves metabolic stress	Dairy cattle	Guretzky et al. (107)
Rumen-protected choline and methionine supplementation	Decreases oxidative stress, followed by enhancement of antioxidant capacity and improved immunity	Sheep	Tsiplakou et al. (43)
Choline supplementation	Decreases liver triacylglycerol concentration of plasma and improves immunity Reduces the incidence of subclinical hypocalcemia	Dairy cattle	Bollatti et al. (108)

ruminants has been well documented (21). It has been reported that heat stress upregulates miR-34a, miR-92a, miR-99, and miR-184. In addition, the upregulation of microRNA in the mammary gland by heat stress is related to cell growth arrest and apoptosis and inhibition of fat synthesis (109). Consistent with this, it has been documented that miR-34a overexpression induces apoptosis in Hep2 cells (110) and mammary cells (111) and inhibits cell proliferation (110). Salama et al. proved through experimental trials that methionine and arginine supplementation downregulates the expression of miR-34a, miR-92a, miR-99, and miR-184 in the mammary gland. Furthermore, they documented that methionine and arginine treatment regulates most of the genes that are associated with insulin signaling (*AKT2* and *IRS1*), transcription and translation (*MAPK1*, *MTOR*, *SREBF1*, *RPS6KB1*, and *JAK2*), amino acid transport (*SLC1A5* and *SLC7A1*), and cell proliferation (*MKI67*) in the mammary glands of dairy cattle (109). Heat stress also upregulates genes associated with apoptosis (*BAX)*, translation inhibition (*EIF4EBP1*), and lipogenesis (*PPARG*, *FASN*, and *ACACA*) and decreases the expression of the anti-apoptotic gene *BCL2L1* in the mammary glands of dairy cattle (109). The above effects caused by heat were reversed with methionine and arginine supplementation in dairy cattle (109).

71, reported that bacterial LPS significantly downregulates the expression of *SLC36A1* and *SLC7A1* and genes associated with antioxidant response (*NFE2L2*, *NQO1*, *GPX1*, *ATG7*, and *GPX3*), and upregulates *SOD2* and *NOS2* (71). Furthermore, they noticed that arginine and methionine supplementation enhances antioxidative gene expression and increases the signaling level of *NFE2L2* in mammary gland cells. Ma et al. (112) found that *NFE2L2* signaling plays a critical role in the cellular antioxidant defense system. It might be possible that arginine and methionine treatment significantly enhances the antioxidative response in mammary gland cells *via* the *NFE2L2* pathway (112). Consistent with this, it has been found that methionine-treated mammary gland cells show increased expression of *CSN1S1*, *CSN1S2*, *CSN2*, *CSN3*, *LALBA*, *JAK2*, *STAT5*, and *MTOR*, which are positively linked to milk protein synthesis (113, 114). Methionine supplementation significantly regulates the transsulfuration pathways that play a key role in taurine and glutathione biosynthesis. Taurine and glutathione biosynthesis alleviates the oxidative stress that is caused by negative energy balance in neonatal Holstein calves (115), as demonstrated in Figure 2.

Garcia et al. experimentally proved that choline supplementation regulates the genes that are associated with muscarinic and nicotinic acetylcholine (37). The muscarinic and nicotinic acetylcholine receptors have been detected in innate and adaptive immune cells (116). Garcia et al. identified several genes involved in choline metabolism (*SLC5A7, CHDH, CHKA, ACHE, CHRM5*, and *CHRNA7*) and inflammatory responses (*TLR4, NFKB1, TNFA, ELANE, H2A, CASP3*, and *CASP7*) in the neutrophils and monocytes of lactating dairy cattle, which are regulated in response to rumen-protected choline supplementation (37). Inflammatory genes (*TLR4*, *NFKB1*, and *TNFA*) are significantly upregulated in LPS-challenged immune cells; however, the opposite trend is observed for these genes in choline-treated immune cells (37).

Methionine supplementation elevates the expression of genes associated with inflammation (*IL1B*, *TLR2*, *NF-κB*, and *STAT3*) and oxidative stress (glutathione synthase, *GPX1*, *CBS*, and *SOD2*) in polymorphonuclear leukocytes (PMNLs) and enhances taurine in the plasma of dairy cattle (75). These findings suggest that methionine supplementation improves the anti-inflammatory and antioxidative status of periparturient dairy cattle. Furthermore, PMNLs treated with LPS reveal the hyper response of IL-1β, resulting in oxidative stress (104). Rumen-protected methionine supplementation suppresses the hyper response of IL-1β, resulting in a decreased inflammatory response and oxidative stress (104). Consistent with this, it has been documented that the combination of methionine and choline significantly upregulates the expression of genes involved in pathogen recognition mechanisms (*TLR2* and L-selectin [*SELL*]) and lowers the expression of genes associated with inflammation (cysteine sulfinic acid decarboxylase [*CSAD*],

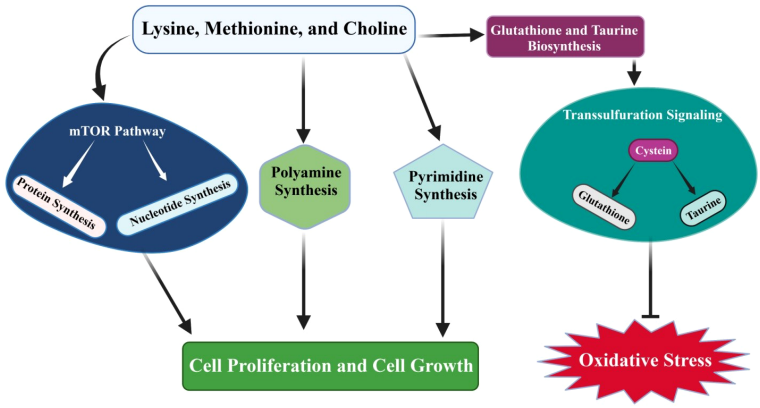

FIGURE 2
Rumen-protected limiting amino acids (lysine and methionine) and choline supplementation regulate mTOR signaling, which controls protein and nucleotide synthesis. In addition, amino acids mediate transsulfuration pathways that further facilitate taurine and glutathione biosynthesis. Taurine and glutathione biosynthesis plays a key role in alleviating oxidative stress in dairy cattle. Additionally, amino acid supplementation regulates mTOR signaling and pyrimidine and polyamine synthesis, and thus plays an essential role in controlling cell proliferation and growth processes.

cystathionine gammalyase [*CTH*], myeloperoxidase [*MPO*], glutathione reductase [*GSR*], *GSS*, *IL6*, *IL10*, and *IL1B*), resulting in an improved anti-inflammatory and antioxidative status in dairy cattle (102). Additionally, methionine downregulates the expression of several genes potentially associated with oxidative stress (*SOD1*, *GSS*, and *GCLC*) (42). Furthermore, Osorio et al. (42) reported higher expression of haptoglobin (*HP*), *S*-adenosylhomocysteine hydrolase (*SAHH*), adenosyltransferase 1A (*MAT1A*) cytosine-5-methyltransferase 3 alpha (*DNMT3A*), and *DNMT1* in response to methionine supplementation. Furthermore, they reported lowered oxidative stress and a mild inflammatory status in dairy cattle (42). Consistent with this, methionine and lysine combination treatment significantly reduces the level of TLR4, pro-inflammatory cytokines (TNF-α and IL-1β), chemokine (CXCL-16), and BHBA content in ewes (117). The summary of the genetic response to rumen-protected limiting amino acids (lysine and methionine) and choline is shown in Table 2.

TABLE 2 Summary of studies that investigated the rumen-protected limiting amino acid (methionine and lysine)- and choline-regulated genes associated with the immunity, antioxidative, and anti-inflammatory status of dairy ruminants.

Treatment	Genetic effect	Biological function	Species/ tissues	Author
Methionine supplementation	Increases expression of *NFE2L2*, *NQO1*, *GPX1*, *GPX3*, *SLC36A1*, *SLC7A1*, *SOD2*, and *NOS2* and decreases expression of *RELA*, *IL1B*, *NF-kb*, and *CXCL2*	Induces anti-inflammation and anti-oxidative responses against LPS	BMECs in dairy cattle	Dai et al. (71)
Methionine supplementation	Upregulates the expression of AKT1 and mTORC1 signaling, *PPARG*, *FASN*, *ACACA*, *BCL2L1*, *MAPK1*, *MTOR*, *SREBF1*, *RPS6KB1*, *JAK2*, and *MKI67* and downregulates the expression of *HSPA1A*, *BAX*, and *EIF4EBP1*	Alleviates oxidative status and regulates immunity and anti-inflammatory status	BMECs in dairy cattle	Salama et al., (109)
Methionine and lysine supplementation	Upregulates JunD and downregulates the expression of genes associated with inflammation (*IL-1α*, *MyD88*, *STAT-3*, and *IL-10*)	Enhances innate immunity, resulting in a reduction of inflammatory changes	Ewes	Tsiplakou et al. (98)
Methionine and lysine supplementation	Lowers the expression of pro-inflammatory cytokines (IL-1β and TNF-α), the chemokine CXCL-16, and pathogen recognition receptor-4 (TLR-4) and suppresses β-hydroxybutyric acid (BHBA) content	Reduces inflammatory changes and enhances the immune response, resulting in the alleviation of mastitis	Ewes	Tsiplakou et al. (117)

(Continued)

TABLE 2 Continued

Treatment	Genetic effect	Biological function	Species/ tissues	Author
Methionine and choline supplementation	Unregulates *GCLC, GPX1*, and adenosylhomocysteinase (*SAHH)* and lowers the expression of *CXCR1*, *IL10*, *IL6*, *IRAK1*, *NFKB1*, *NR3C1*, *SELL*, *TLR4*, and *TNFA* in polymorphonuclear leukocytes	Increases homocysteine synthesis, resulting in enhanced antioxidative status, and reduces inflammatory changes and cytoprotection against oxidative stress	Neonatal Holstein calves	Abdelmegeid et al. (74)
Choline supplementation	Decreases the expression of pro-inflammatory cytokines *IL1B*, *CXCL8*, and *TNF* caused by LPS in peripheral blood leucocytes Enhances the level of blood neutrophils undergoing phagocytosis and oxidative burst	Decreases inflammation and improves immunity	Holstein cows	Zenobi et al. (93)
Methionine supplementation	Reduces the level of oxidative stress associated with *MDA*, *CAT*, and *SOD* Enhances the level of antioxidant genes (*GST* and *GPH*)	Plays a critical role in the regulation of antioxidant activity against oxidative stress	Ewes	Mavrommatis et al. (21)
Methionine supplementation	Lowers the expression of genes related to inflammation (*IL1B*, *TLR2*, *NF-κB*, and *STAT3)* Reduces the expression of genes associated with oxidative stress *(*glutathione synthase, *GPX1 CBS*, and *SOD1)*	Improves anti-inflammatory and antioxidative status	Dairy cattle	Zhou et al. (75)
Methionine and choline supplementation	Enhances the expression of *SELL* and *TLR2* Lowers the expression of *CSAD*, *CTH*, *MPO*, *GSR*, *GSS*, *IL6*, *IL10*, and *IL1B*	Improves the antioxidant ability and suppresses inflammatory changes	Dairy cattle	Lopreiato et al. (102)
Rumen-protected choline supplementation	Decreases the expression of genes associated with inflammation (*TLR4, NFKB1*, *TNFA*, *ELANE*, *H2A*, *CASP3*, and *CASP7*) in neutrophils and monocytes treated with LPS	Decreases inflammation Improves the health of periparturient dairy cattle and alleviates mastitis	Dairy cattle	Garcia et al. (37)
Methionine supplementation	Reduces the level of inflammation-associated genes (*SELL*, *CXCR2*, *NFKBIA*, *MYD88*, *TLR4*, *TLR2*, *GSS*, *GPX1*, *TNF*, and *IL1B*), and suppresses genes involved in the oxidative stress (*MPO* and *SOD1*) of polymorphonuclear leukocytes Enhances the level of antioxidant-linked genes (*NFE2L2* and *NOS2*)	Improves immunity and antioxidative ability and reduces inflammatory alterations	Holstein calves	Jacometo et al. (97)
Methionine supplementation	Enhances the level of methionine adenosyltransferase 1A (*MAT1A*), glutamate-cysteine ligase (*GCLC*), glutathione reductase (*GSR*), adenosylhomocysteinase (*AHCY*; also known as *SAHH*), and DNA (cytosine-5-)-methyltransferases (*DNMT1*, *DNMT3A*, and *DNMT3B*) Lowers the expression of cysteine sulfinic acid decarboxylase (*CSAD*)	Decreases oxidative stress, enhances immunity, and reduces metabolic stress, which is responsible for abnormal regulation of immunity and inflammation	Holstein calves	Jacometo et al. (115)
Methionine supplementation	Upregulates peroxisome proliferator-activated receptor alpha (PPARα)-associated genes (*ANGPTL4*, *FGF21*, and *PCK1)*	Upregulation of hepatic PPARA has been reported to be associated with lipid metabolism and immune function	dairy cattle	Osorio et al. (118)
Rumen-protected methionine	Regulates PPARα, fatty acid Desaturase 2 (*FADS2*), CBS, glutathione S-transferase omega 1 (*GSTO1*), *GPX1*, MAPK and MTOR activator *LAMTOR2*, mammary serum amyloid (*SAA*), peroxisome proliferator-activated receptor gamma (*PPARG*), and forkhead box O1 (*FOXO1*)	Enhances cell metabolism, reduces metabolic and oxidative stress, relieves inflammation and enhances immunity in Holstein cows	Holstein cows	Palombo et al. (119)
Methionine supplementation	Upregulates the expression of genes associated with amino acid transport (*SLC38A1*, *SLC38A2*, and *SLC7A1*) and valyl-tRNA (*VARS*), isoleucyl-tRNA synthetases (*IARS*), glucose transport solute carrier family 2 member 3 (*SLC2A1*), glucose transport solute carrier family 2 member 3 (*SLC2A3*), and casein α-s1 (*CSN1S1*) Elevates the expression of Janus kinase 2 (*JAK2*) and the phosphorylation status of AKT, protein phosphatase 1, and regulatory subunit 15A (*PPP1R15A*)	Enhances metabolism, increases the level of plasma amino acids, and decreases metabolic stress and inflammation Improves milk production	Holstein cows	Ma et al. (112)

3 Conclusions

Based on published data, we conclude that rumen-protected limiting amino acids (lysine and methionine) and choline supplementation alleviate oxidative stress, which is the primary cause of several diseases in ruminants. Moreover, oxidative stress, particularly during the periparturient period, compromises immunity, production performance, and metabolism in dairy ruminants. The supplementation of rumen-protected amino acids and choline, especially during the periparturient period, enhances antioxidative ability, resulting in the regulation of immunity and anti-inflammation status in dairy ruminants. Thus, supplementation with a sufficient quantity of rumen-protected amino acids (lysine and methionine) and choline is highly recommended for protecting animals from diseases and enhancing their productive abilities.

Author contributions

MK, MM, and ZC designed the study and wrote the manuscript; ZC supervised the manuscript; MM, YM, JW, QU, JX, TC, SL, IMK and AK helped in collection of data resources and editing of final version of manuscript. All authors contributed to the article and approved the submitted version.

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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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Isabelle Mack,
Eberhard Karls University of Tübingen, Germany

REVIEWED BY
Christopher Peter Corpe,
King's College London, United Kingdom
Moetaza M. Soliman,
Mansoura University, Egypt

*CORRESPONDENCE
Cheuk-Kwan Sun
✉ researchgate000@gmail.com

†These authors have contributed equally to this work

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Impact of intravenous vitamin C as a monotherapy on mortality risk in critically ill patients: A meta-analysis of randomized controlled trials with trial sequential analysis

Kuo-Chuan Hung[1,2], Min-Hsiang Chuang[3], Jen-Yin Chen[2], Chih-Wei Hsu[4], Chong-Chi Chiu[5,6,7], Ying-Jen Chang[2], Chia-Wei Lee[8], I-Wen Chen[9†] and Cheuk-Kwan Sun[6,10*†]

[1]School of Medicine, College of Medicine, National Sun Yat-sen University, Kaohsiung City, Taiwan, [2]Department of Anesthesiology, Chi Mei Medical Center, Tainan City, Taiwan, [3]Department of Internal Medicine, Chi Mei Medical Center, Tainan City, Taiwan, [4]Department of Psychiatry, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung City, Taiwan, [5]Department of General Surgery, E-Da Cancer Hospital, I-Shou University, Kaohsiung City, Taiwan, [6]School of Medicine, College of Medicine, I-Shou University, Kaohsiung City, Taiwan, [7]Department of Medical Education and Research, E-Da Cancer Hospital, I-Shou University, Kaohsiung City, Taiwan, [8]Department of Neurology, Chi Mei Medical Center, Tainan City, Taiwan, [9]Department of Anesthesiology, Chi Mei Medical Center, Liouying, Tainan City, Taiwan, [10]Department of Emergency Medicine, E-Da Hospital, I-Shou University, Kaohsiung City, Taiwan

Background: This meta-analysis aimed at investigating the pooled evidence regarding the effects of intravenous vitamin C (IVVC) on mortality rate in critically ill patients.

Methods: Databases including Medline, Embase, and Cochrane Library were searched from inception to October, 2022 to identify RCTs. The primary outcome was the risk of overall mortality. Subgroup analyses were performed based on IVVC dosage (i.e., cut-off value: 100 mg/kg/day or 10000 mg/day). Trial sequential analysis (TSA) was used to examine the robustness of evidence.

Results: A total of 12 trials including 1,712 patients were analyzed. Although meta-analysis demonstrated a lower risk of mortality in patients with IVVC treatment compared to those without [risk ratio (RR): 0.76, 95% CI: 0.6 to 0.97, p = 0.02, I^2 = 36%, 1,711 patients), TSA suggested the need for more studies for verification. Moreover, subgroup analyses revealed a reduced mortality risk associated with a low IVVC dosage (RR = 0.72, p = 0.03, 546 patients), while no beneficial effect was noted with high IVVC dosage (RR = 0.74, p = 0.13, I^2 = 60%, 1,165 patients). The durations of vasopressor [mean difference (MD): −37.75 h, 404 patients) and mechanical ventilation (MD: −47.29 h, 388 patients) use were shorter in the IVVC group than those in the controls, while there was no significant difference in other prognostic outcomes (e.g., length of stay in intensive care unit/hospital) between the two groups.

Conclusion: Although intravenous vitamin C as a monotherapy reduced pooled mortality, durations of vasopressor use and mechanical ventilation, further research is required to support our findings and to identify the optimal dosage of vitamin C in the critical care setting.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42022371090.

KEYWORDS

vitamin C, sepsis, septic shock, critically illness, mortality

1. Introduction

Vitamin C, also known as ascorbic acid or ascorbate, has been reported to play a beneficial role in critical illnesses including sepsis as well as life-threatening cardiovascular diseases including coronary heart disease and stroke (1–5). Not only have previous studies demonstrated its ability to restore vasopressor sensitivity and preserve vascular endothelial integrity (2, 6–8), but vitamin C is also known to reinforce both innate and adaptive immunity (9). Therefore, the potential therapeutic benefits of vitamin C supplementation have been investigated in patients subjected to a variety of critical care settings including those with sepsis, those undergoing cardiac surgery, and those with COVID-19 infection (10–13). Nevertheless, evidence regarding the efficacy of vitamin C against risk of mortality remains to be clarified. Several meta-analyses reported no beneficial effect of vitamin C on the risk of mortality in cardiac surgical patients and those with COVID-19 infection (14, 15). For patients with sepsis/septic shock or critical illnesses, the association between mortality and the use of vitamin C was also controversial (16–22). Such conflicting findings may be attributed to the variations among individual studies including study design (i.e., prospective or retrospective), the choice of study population (e.g., critical vs. non-critical), the use of vitamin C as monotherapy or part of a combined regimen, as well as the selected dosage and route of administration.

Focusing on the critically ill population receiving intravenous vitamin C (IVVC) as a monotherapy, previous meta-analyses suggested that the use of IVVC may reduce the risk of mortality without a positive impact on the length of stay (LOS) in hospital or intensive care unit (ICU) (16, 17). However, the inclusion of a limited number of patients for analysis in the two meta-analyses [i.e., 467 patients (16) and 755 patients (17)] may impair the robustness of evidence. In contrast, a recent large-scale randomized controlled trial (RCTs) involving 872 patients with sepsis even reported a slight increase in the risk of mortality/persistent organ dysfunction at 28 days in those with IVVC monotherapy compared to those without (23).

In an attempt to provide clinical guidance based on updated information from RCTs, this meta-analysis aimed at evaluating the impact of IVVC as a monotherapy on the risk of mortality in critically ill adult patients and examining the robustness of evidence through performing trial sequential analyses (TSA).

2. Methods

2.1. Protocol and registration

Preferred Reporting Items for Systematic Reviews and Meta-Analyses have been followed in this meta-analysis, and the protocol has been registered with the International Prospective Register of Systematic Reviews (CRD42022371090).

2.2. Data sources and search strategy

We searched the databases of MEDLINE, EMBASE, and Cochrane Central Register of controlled trials to retrieve RCTs that fit our eligibility criteria from inception to October, 2022 without language restriction. As part of this analysis, only RCTs examining IVVC and prognostic outcomes in critically ill adult patients were included. The search strategies using subject headings and keywords are available in **Supplementary Table 1**. The reference lists of the retrieved articles and meta-analyses were also manually searched to identify potentially eligible articles that may have been overlooked during the initial digital search. The authors of articles with missing data of interest were emailed up to three times in an attempt to acquire the information.

2.3. Inclusion and exclusion criteria

Randomized controlled trials that met the following screening criteria were considered eligible for inclusion in the current study: (a) Population: Adults (age $\geq$ 18 years) who were critically ill [defined as those requiring ICU admission or those with a high rate of mortality (i.e., >5%) in the control arm as previously described (16)], (b) Intervention: IVVC as a monotherapy irrespective of the duration or the dosage of the treatment (IVVC group), (c) Comparison: Use of placebo or standard care (i.e., control group), (d) Outcomes: Availability of mortality risk. Other prognostic outcomes (e.g., duration of vasopressor use) were also analyzed if available.

Exclusion criteria were: (1) IVVC as combined therapy with other agents (e.g., hydrocortisone or thiamine); (2) studies without

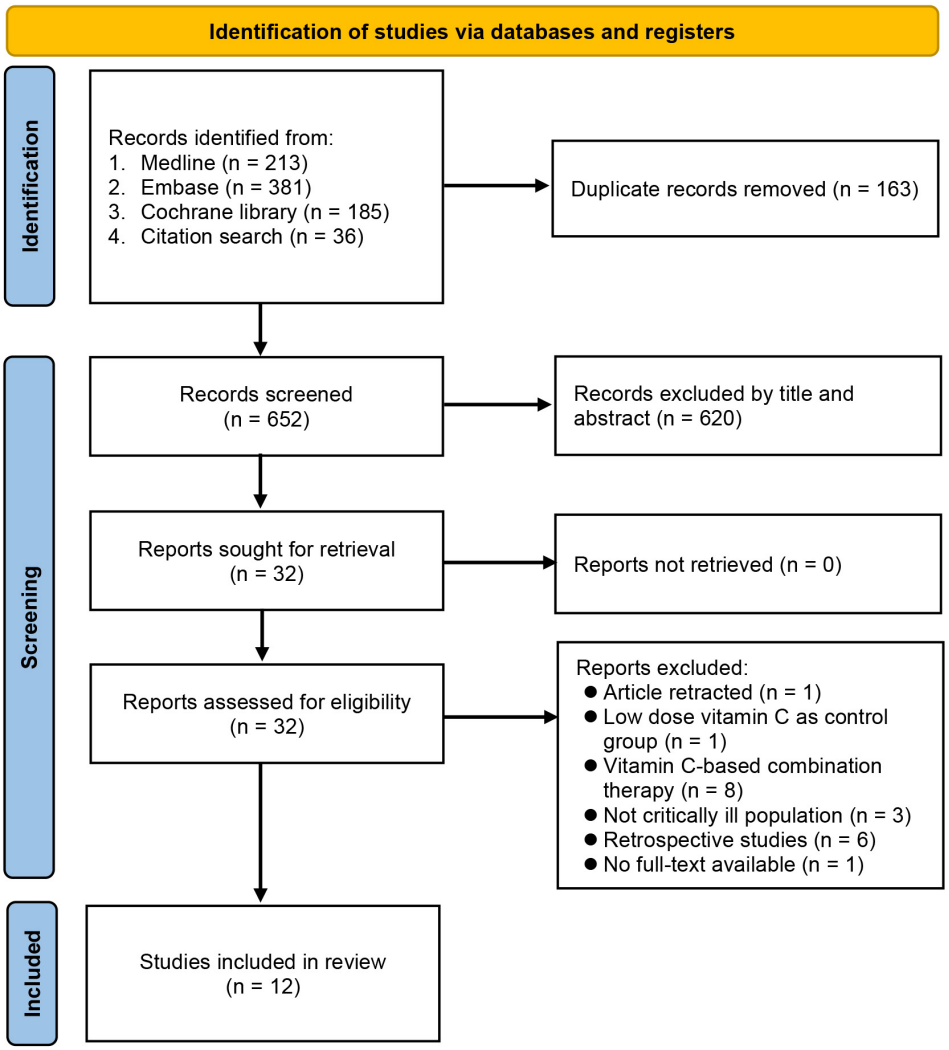

FIGURE 1
PRISMA flow diagram of study selection for the current meta-analysis.

control group; (3) those adopting oral vitamin C supplementation as an intervention group; (4) those published without peer-review or as abstracts only; (5) those with research designs other than RCT (e.g., case reports or reviews); and (6) those focusing on patients undergoing cardiac surgery.

2.4. Study selection and data extraction

The records obtained from database search were screened as follows. After independent assessment of the titles and abstracts of the articles by two independent reviewers, the same reviewers read the full texts to determine their eligibility. Other reviewers then independently extracted relevant data including country, first author's name, publication year, participant-related information (e.g., age, gender, and sample size), information regarding pharmacological therapies (e.g., dosage and treatment duration), and outcome-associated details (e.g., mortality rate, hospital LOS). In the event that missing information was needed, we emailed the corresponding authors in an attempt to get access to the data.

2.5. Clinical outcomes

The primary outcome was the risk of overall mortality. Secondary outcomes included durations of vasopressor use and mechanical ventilation time, risk of renal replacement therapy, ICU/hospital LOS as well as changes in circulating C-reactive protein (CRP) concentration and sequential organ failure assessment (SOFA) score. Subgroup analyses were performed based on the indications of IVVC treatment and dosage of IVVC (i.e., low vs. high) with cut-off value being set at 100 mg/kg/day or 10000 mg/day as previously reported (17).

2.6. Assessment of methodologic quality

Each trial was assessed for bias using the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions by two authors (24). Disagreements were resolved through discussion. The potential risks of bias of individual studies were rated as "low," "high," or "unclear."

2.7. Data synthesis

We used RevMan 5.4 (Cochrane IMS, Oxford, United Kingdom) with a random effects model as the basis for all analyses. The pooled risk ratios for dichotomous data are presented as risk ratios (RR), while continuous variables are presented as weighted mean differences (MD). All estimates are provided with 95% confidence intervals (CI). A funnel plot was generated to assess the possibility of publication bias if an outcome was reported in ten or more studies. Egger regression test was used in case of funnel plot asymmetry if required. The potential influence of the findings of an individual trial on the overall results was evaluated with sensitivity analysis using a "leave-one-out" approach. For all analysis, *p*-value of less than 0.05 was considered statistically significant.

To assess the strength of the results and to guard against statistical errors of type I and type II, we performed trial sequential analysis (TSA) as previously described (25, 26). We calculated the diversity-adjusted information size using 80% power while maintaining an overall two-sided type I error of 5%. Using this method, we could determine whether the conclusion was sufficient or if further studies are required to detect a predefined 20% reduction in the risk of overall mortality. The analysis was conducted using TSA software, Copenhagen Trial Unit version 0.9.5.10 Beta.

3. Results

3.1. Characteristics of trials and risk of bias

Through electronic (n = 779) and manual (n = 36) searches, 815 records were identified. Following deletion of duplicates (n = 163) and reports deemed unsuitable after title and abstract screening (n = 620), 32 articles were considered eligible for full-text reading that further excluded 20 studies. Finally, 12 studies with 1,712 critically ill patients published between 2014 and 2022 were included in the current meta-analysis (23, 27–37) (**Figure 1**). Among the included studies, one RCT had two intervention arms (i.e., 50 or 200 mg/kg/day); therefore, the results of that study were analyzed separately [i.e., Fowler (28) and Hill et al. (15)].

The characteristics of the eligible studies are shown in **Table 1**. The study population included 1,712 patients with sepsis/septic shock (eight trials) (23, 27–29, 33–36), severe COVID-19 (three trials) (30, 31, 37), and severe pneumonia (one trial) (32). The number of patients in the included studies ranged from 28 to 863. Of all patients, the male proportion was 50–80% and the average or median age was 30–92 years. Nine studies provided detail on the baseline SOFA score (23, 28, 29, 32–37), while related information was not available in two trials (30, 31) and in another study that assessed the risk of mortality using the Physiological and Operative Severity Score for the enumeration of Mortality and morbidity (POSSUM) score (27). High-dose IVVC (i.e., ≥100 mg/kg/day or 10000 mg/day) was used in six studies (23, 28, 29, 34, 36, 37), while low-dose IVVC was applied in seven trials (27, 28, 30–33, 35). The overall mortality rates were 29% (range: 9–60%) and 32.6% (range: 10–64%) in the IVVC and control groups, respectively.

No high-risk study was noted in any of the seven domains of bias assessment. All studies were deemed to have a low risk of selection bias because of their providing details about the methodology of randomization, while unclear risks of allocation concealment, performance bias, detection bias, reporting bias, other bias were considered in three, one, three, one, and eight trials, respectively (**Figure 2**).

3.2. Outcomes

3.2.1. Primary outcome: Risk of mortality

Forest plot demonstrated a lower risk of mortality in patients receiving IVVC as a monotherapy compared to those in the control group (RR: 0.76, 95% CI: 0.6 to 0.97, p = 0.02, I^2 = 36%, 1,711 patients) (**Figure 3**) (23, 27–37). Sensitivity analysis revealed an inconsistent finding when three studies were removed one at a time (29, 35, 36). There was a low risk of publication bias (**Supplementary Figure 1**). TSA showed that the current evidence regarding the impact of IVVC on mortality rate remains inconclusive (**Figure 4**).

Despite the absence of subgroup difference based on dosage, our analysis demonstrated an association of a low dosage (i.e., <100 mg/kg/day or 10000 mg/day) of IVVC with a reduction in mortality risk (RR = 0.72, 95% CI: 0.53 to 0.97, p = 0.03, I^2 = 0%, 546 patients), but without a positive impact for a high dosage (RR = 0.74 95% CI: 0.5 to 1.09, p = 0.13, I^2 = 60%, 1,165 patients) (**Figure 5**). On the other hand, albeit statistically non-significant after being separated according to indications, the findings demonstrated a trend in favor of using vitamin C to reduce the risk of mortality in both subgroups based on treatment indications (i.e., sepsis/septic shock vs. COVID-19/pneumonia) (**Supplementary Figure 2**).

3.2.2. Secondary outcome: Impact of vitamin C on vasopressor use, mechanical ventilation time, and risk of renal replacement therapy

The use of IVVC was associated with a shorter duration of vasopressor use than that in the controls (MD: −37.75 h, 95%: −70.77 to −4.73, p = 0.03, I^2:94%, 404 patients) (**Figure 6A**) (28, 32–36). Nevertheless, the findings were inconsistent on sensitivity analysis. The duration of mechanical ventilation was also shorter in the IVVC group compared to that in the control group (MD: −47.29 h, 95% CI: −90.13 to −4.45, p = 0.03, I^2 = 95%, 388 patients) (**Figure 6B**) (32, 33, 35–37) with inconsistent results on sensitivity analysis. The risk of renal replacement therapy was comparable between the two groups (RR = 1.56, 95% CI: 0.91 to 2.68, p = 0.11, I^2 = 28%, 1,143 patients) (**Figure 6C**) (23, 33, 35, 37) with consistent findings on sensitivity analysis.

3.2.3. Impact of IVVC on CRP level, SOFA score, and serum vitamin C level

There was no difference in CRP levels (MD: −0.76, 95% CI: −1.76 to 0.23, p = 0.13, I^2 = 92%, 236 patients; sensitivity analysis: consistent) (**Figure 7A**) (32, 33, 37) and SOFA score (MD: −0.72, 95% CI: −1.67 to 0.23, p = 0.14, I^2 = 68%, 1,156 patients) (**Figure 7B**) (23, 32, 34, 35, 37) between patients receiving IVVC and those subjected to control treatments. Sensitivity analysis revealed a lower SOFA score in the IVVC group compared to that in

TABLE 1 Characteristics of studies (n = 12).

References	Population	Age (years)[#]	Male (%)[#]	Baseline SOFA score[#]	Vitamin C group	Placebo group	Total *N*	Mortality (%)	Country
Ferrón-Celma et al. (27)	Patients with sepsis	68 vs. 65	50 vs. 60	50 vs. 50†	450 mg/day for 6 days	5% dextrose	20	60 vs. 40	Spain
Fowler et al. (28)	Patients with severe sepsis	30–70 49–92 54–68¶	63 50 50¶	10 11 13¶	50 or 200 mg/kg/day for 4 days	5% dextrose	24	44 vs. 63	USA
Fowler et al. (29)‡	Patients with sepsis and ARDS	54 vs. 57	54 vs. 54	9.8 vs. 10.3	200 mg/kg/day for 4 days	5% dextrose	167	30 vs. 46	USA
JamaliMoghadamSiahkali et al. (30)	Patients with severe COVID-19	58 vs. 61	50 vs. 50	NA	6 g/day for 5 days	Standard therapy§	60	10 vs. 10	Iran
Kumari et al. (31)	Patients with severe COVID-19	52 vs. 53	56.90%	NA	50 mg/kg/day and standard care	Standard therapy	150	9 vs. 15	Pakistan
Lamontagne et al. (23)	Patients with sepsis	65 vs. 65	65 vs. 60	10.2 vs. 10.1	200 mg/kg/day for 4 days	5% dextrose	863	35 vs. 32	Canada
Mahmoodpoor et al. (32)	Patients with severe pneumonia	57 vs. 58	57 vs. 58	12.5 vs. 10.7	60 mg/kg/day for 4 days	Normal saline	80	15 vs. 28	Iran
Nabil Habib and Ahmed (33)	Patients with septic shock	43 vs. 42	56 vs. 60	10.2 vs. 11.4	6 g/day until ICU discharge	Conventional sepsis treatment	100	24 vs. 36	Egypt
Rosengrave et al. (34)	Patients with septic shock	69 vs. 66	80 vs. 55	8.5 vs. 9	100 mg/kg/day for 4 days	5% dextrose	40	30 vs. 35	New Zealand
Wacker et al. (35)‡	Patients with septic shock	69 vs. 73	50 vs. 52	10 vs. 9	1000 mg bolus followed by 250 mg/h for 4 days	Normal saline	124	27 vs. 41	USA
Zabet et al. (36)	Patients with septic shock	64 vs. 64	71 vs. 79	11.8 vs. 12.4	100 mg/kg/day for 3 days	5% dextrose	28	14 vs. 64	Iran
Zhang et al. (37)‡	Patients with severe COVID-19	66 vs. 67	56 vs. 76	14 vs. 13	24 g/day for 7 days	Bacteriostatic water	56	22 vs. 34	China

‡Multicenter trial; ¶placebo group; †Physiological and Operative Severity Score for the enumeration of Mortality and morbidity (POSSUM); §lopinavir/ritonavir/hydroxychloroquine; ICU, intensive care unit; [#]presented as vitamin C vs. control groups.

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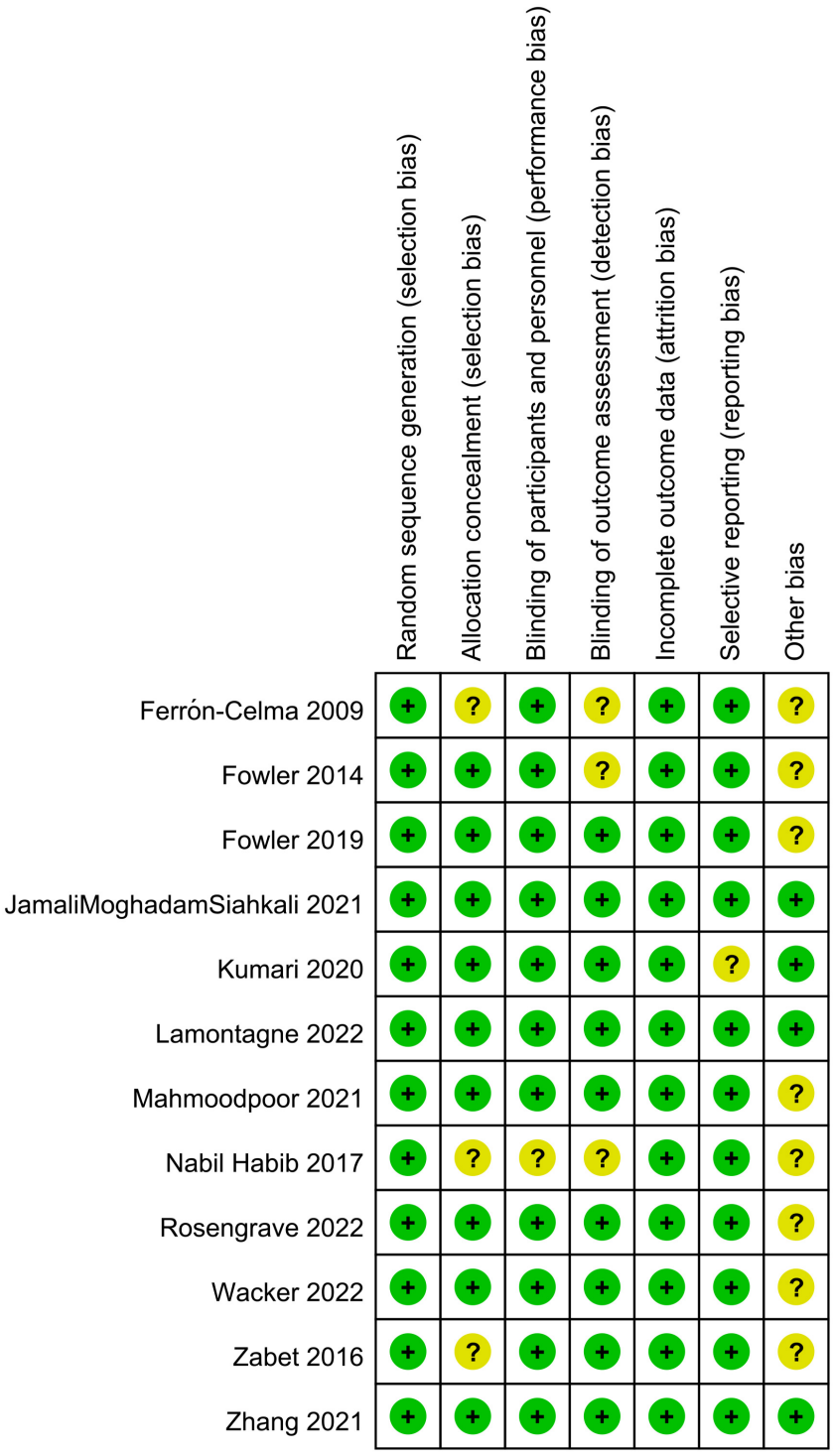

FIGURE 2
Risks of bias of the included randomized controlled trials.

the control group when one study (23) was removed (MD: −1.12, 95% CI: −1.98 to −0.25, p = 0.01, I^2 = 31%, 295 patients). The analysis of four studies providing information about serum vitamin C level demonstrated a higher serum vitamin C level in the IVVC group than that in the control group (SMD:1.7, 95% CI: 0.83 to 2.58, p = 0.0001, I^2 = 87%, 319 patients; sensitivity analysis: consistent) (**Figure 7C**) (28, 29, 32, 34).

3.2.4. Secondary outcomes: Hospital and ICU length of stay

There were no differences in ICU (MD: −0.36 days, 95% CI: −1.63 to 0.92, p = 0.58, I^2 = 62%, 1,382 patients) (**Figure 8A**) (23, 28, 30, 32–37) and hospital (MD: 0.21 days, 95% CI: −2.39 to 2.82, p = 0.87, I^2 = 78%, 1,292 patients) LOS (**Figure 8B**) (23, 30, 31, 34, 35, 37) between the IVVC and control groups. Sensitivity analysis

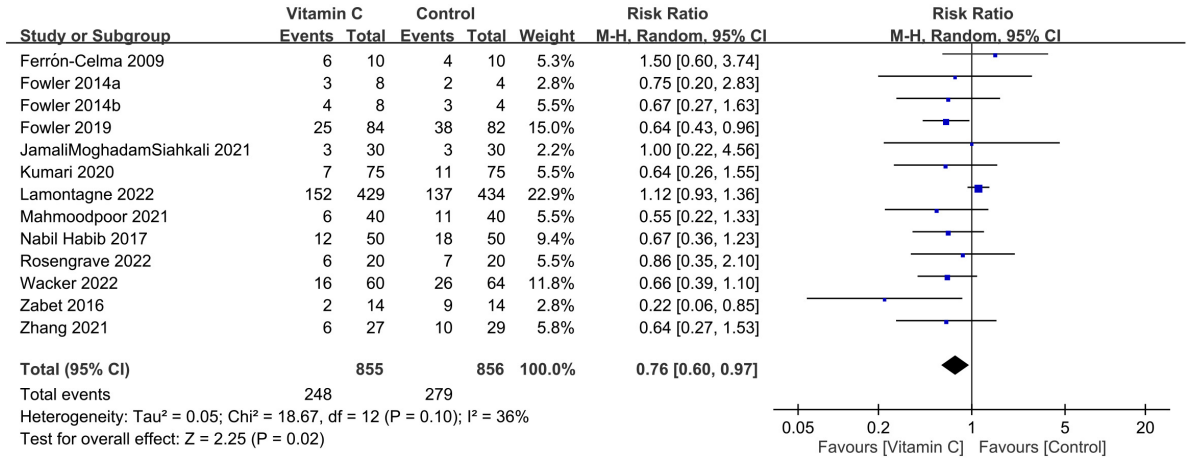

FIGURE 3
Forest plot comparing risk of mortality between intravenous vitamin C and control groups. M-H, Mantel-Haenszel; CI, confidence interval.

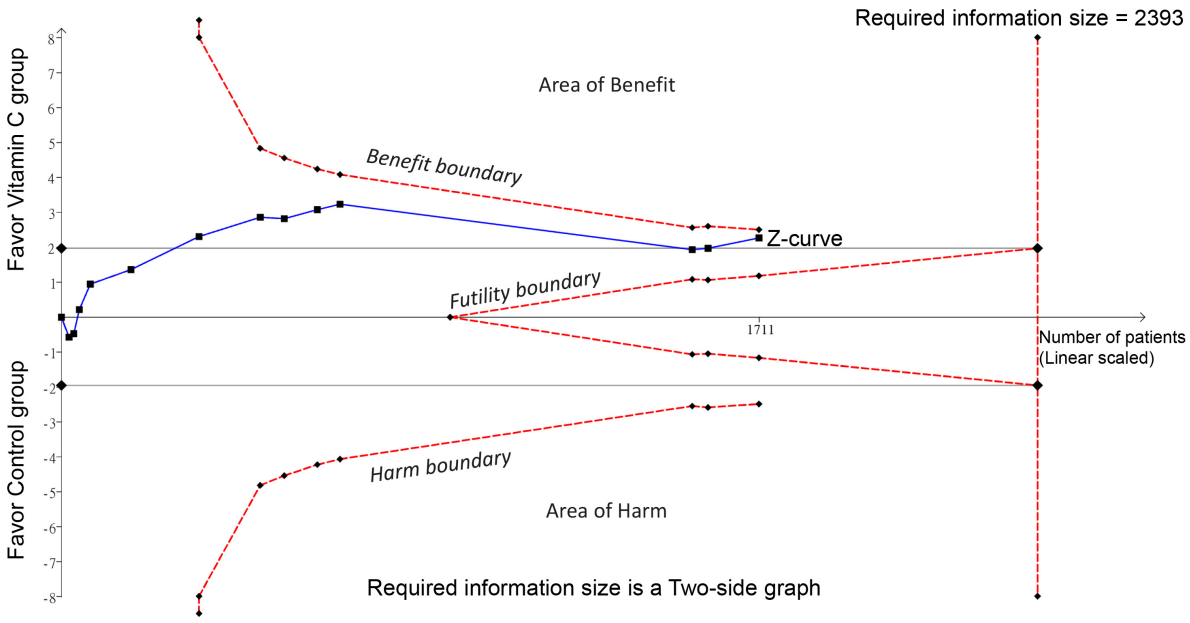

FIGURE 4
Trial sequential analysis (TSA) showing no crossing between the cumulative Z curve and the trial sequential monitoring boundary or the required information size, indicating insufficient and inconclusive evidence supporting the association of intravenous vitamin C supplementation with a decreased risk of mortality.

showed consistent findings on these two outcomes. There was a low risk of publication bias on ICU LOS (**Supplementary Figure 3**).

4. Discussion

Our updated meta-analysis of 12 trials including 1,712 critically ill patients demonstrated an association between the use of IVVC and a reduced mortality rate. Nevertheless, TSA analysis suggested that further studies are required to provide conclusive evidence. Interestingly, we found that the correlation between mortality and IVVC may be influenced by the dosage of IVVC with a favorable outcome being noted with a low dosage of IVVC (i.e., <100 mg/kg/day or 10000 mg/kg/day). In addition, the durations of vasopressor and mechanical ventilation use were shorter in the IVVC group compared to those in the control group, while there was no significant difference in other prognostic outcomes (i.e., risk of renal replacement therapy, CRP level, SOFA score, hospital/ICU LOS) between the two groups.

The unexpected finding in a recent large-scale RCT showing a slightly higher risk of mortality and persistent organ dysfunction in patients with sepsis receiving IVVC (23) highlighted the need for updating and re-analyzing the pooled evidence. Compared with the control group, the current meta-analysis showed that IVVC monotherapy was associated with a lower mortality risk in critically ill patients. Although our TSA suggested the requirement for more

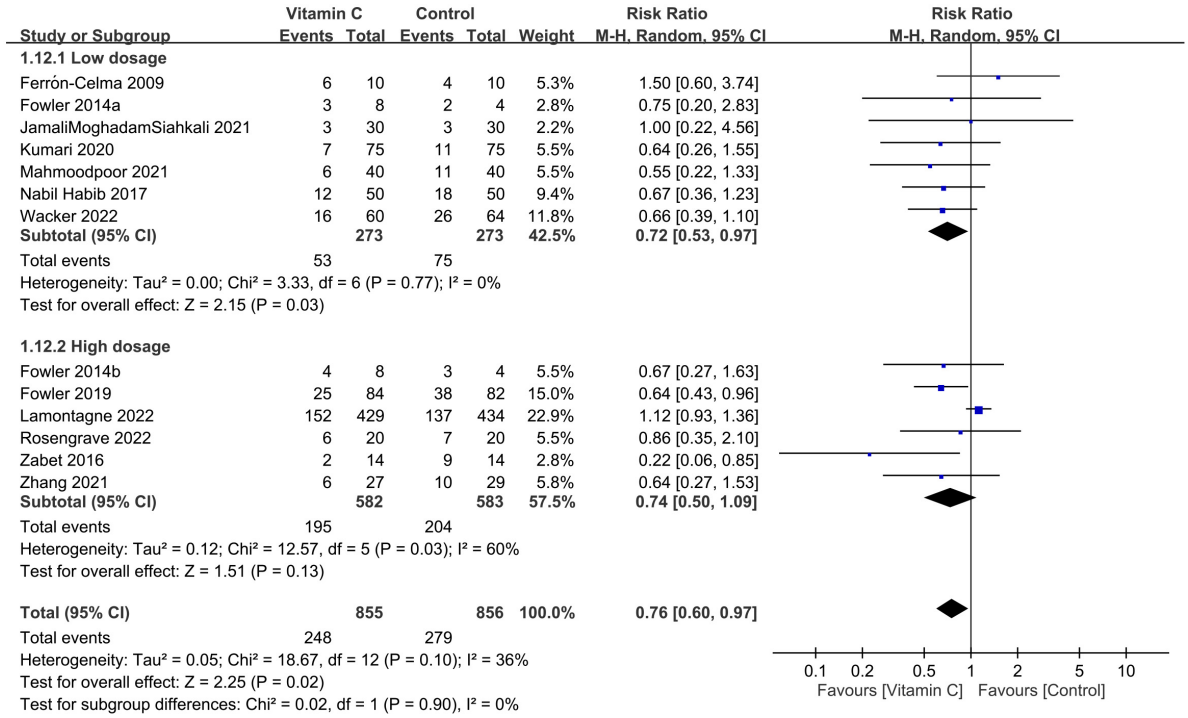

FIGURE 5
Subgroup analysis based on vitamin C dosage (cut-off value: 10000 mg/day or 100 mg/kg/day). M-H, Mantel-Haenszel; CI, confidence interval.

trials to achieve a conclusive finding, our results demonstrated that the benefits of IVVC could outweigh the reported risks in the critically ill patients (23). Because previous meta-analyses reported no beneficial effect on the risk of mortality in septic patients subjected to vitamin C-based combination therapy and in those who underwent cardiac surgeries receiving intravenous/oral vitamin C monotherapies (15, 18), we only included the critically ill population (i.e., mortality rate in the control group was more than 5%) receiving IVVC as a monotherapy with the exclusion of cardiac surgical patients to minimize the potential bias. The I^2 was only 36% in our primary outcome, suggesting a low heterogeneity in the current meta-analysis. Overall, our research provided additional information that could be used to guide clinical practice in the critical care setting.

Our subgroup analysis revealed that a low dosage of IVVC was associated with a reduced risk of mortality, while this benefit was not noted with a high dosage of IVVC. The current meta-analysis is the first to unveil this novel finding in the critically ill population. Although the underlying mechanism for this finding remains unclear, several previous observation studies and meta-analyses in different clinical settings have shown that a low-to-moderate dose of vitamin C may be more effective for decreasing the mortality rate than a high dose (38–40). For instance, a recent meta-analysis involving 19 studies on adults with COVID-infection reported that a low dosage (i.e., $\leq$1 g/day) of vitamin C could effectively decrease 1-month mortality, while no beneficial effect was observed with a large dosage of vitamin C (i.e., >1 g/day) (38). Another previous meta-analysis focusing on the use of IVVC monotherapy or vitamin C-based combination therapy found that vitamin C treatment for 3–4 days significantly improved the mortality rate in septic patients, while the risk of mortality in patients treated for 1–2 or >5 days was not reduced (40). Consistently, another large prospective cohort study (i.e., 28,945 participants) investigating the relationships between individual-level dietary intakes of vitamins C and all-cause mortality in the general population reported a lower all-cause mortality in individuals with moderate vitamin C intake (e.g., third and fourth quintiles) compared with those with the highest quintile of vitamin C intake (i.e., fifth quintiles) (39). Therefore, the lack of a dose-dependent effect between vitamin C supplement and the reduction in risk of mortality in those studies (38–40) indirectly supported the findings of the current meta-analysis. Regarding the impact of treatment indications, our findings of subgroup analysis suggest that while there may be some benefits of taking vitamin C, the effect was not statistically significant when each subgroup (i.e., sepsis/septic shock vs. COVID-19/pneumonia) was considered separately, probably due to a relatively small number of participants in each subgroup after being split.

Our results of shorter durations of vasopressor use in the IVVC group compared to those in the control group were consistent with those of previous meta-analyses (17, 19, 41). In contrast with the findings of other studies (19, 41, 42), we demonstrated no improvement in SOFA score associated with the monotherapeutic use of IVVC. Nevertheless, because of the high heterogeneity and inconsistent findings on sensitivity analysis of the three secondary outcomes, we considered our findings to be inconclusive. Further studies are required to address these issues. Another interesting finding of the current study was a non-significant trend toward a higher risk of renal replacement therapy linked to the use of IVVC compared with that in the control group based on data from four available trials that adopted relatively large IVVC dosages (i.e.,

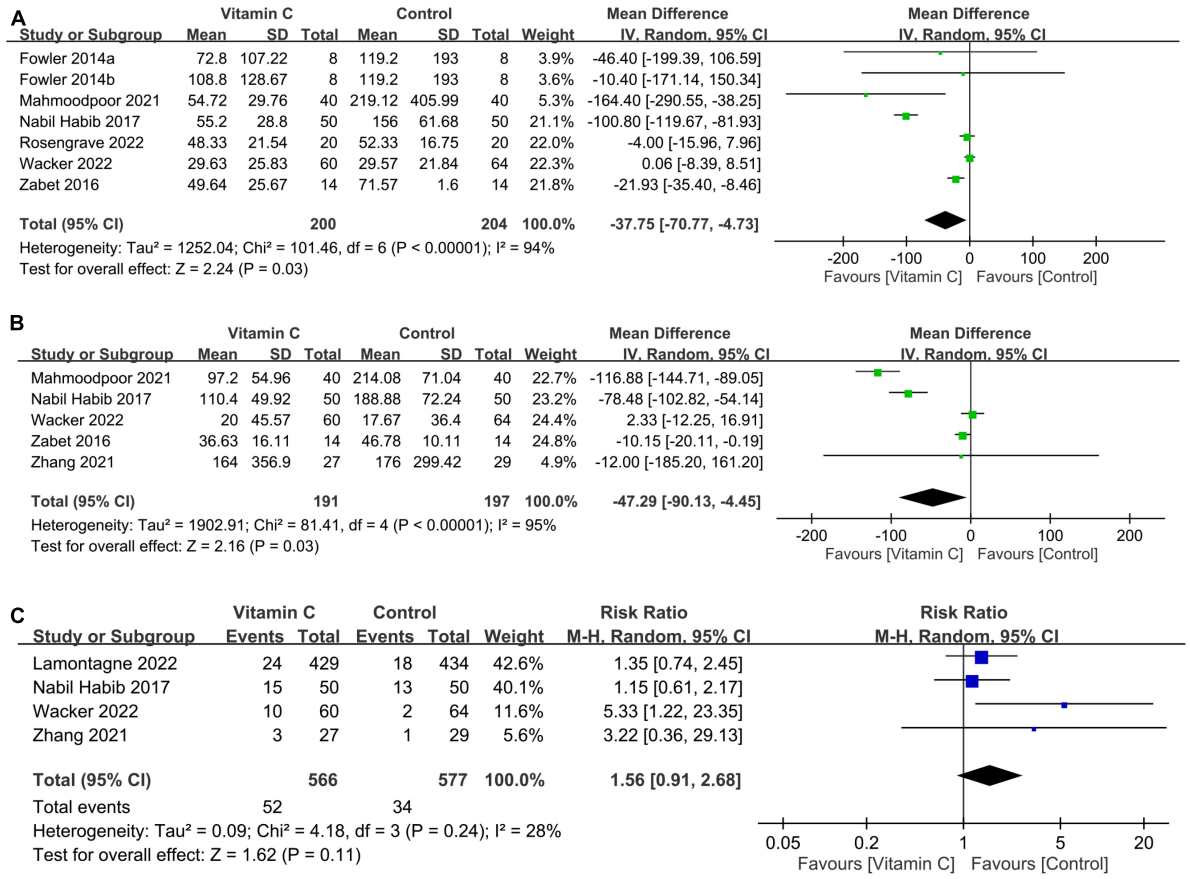

FIGURE 6
Forest plot comparing **(A)** duration of vasopressor use; **(B)** mechanical ventilation time; and **(C)** risk of renal replacement therapy between the intravenous vitamin C and control groups. CI, confidence interval; IV, inverse variance; M-H, Mantel-Haenszel.

200 mg/kg/day, 6 g/day, 24 g/day) (23, 33, 35, 37) despite not fitting the criteria for a high dosage in this study. Despite the lack of robustness of evidence based on the small number of studies, our finding may be in line with that of a previous cohort study on 1,390 critically ill patients showing that IVVC with a dosage ≥1.5 g four times a day was associated with an increased risk of acute kidney and in-hospital mortality compared with those receiving no treatment or a single dose less than 1.5 g (43).

Consistent with the findings of several recent meta-analyses focusing on patients with sepsis/septic shock or critical illnesses that showed no difference in hospital/ICU stay with or without the use of vitamin C (16, 18, 19), the current meta-analysis focused on a similar population also demonstrated no difference in hospital/ICU stay with the use of IVVC as a monotherapy. In contrast, a previous meta-analysis on ICU patients reported a reduced ICU LOS with the use of intravenous or orally administered vitamin C (44). Such inconsistencies in findings may be attributed to the choice of study population. In that meta-analysis (44), 13 out of 18 trials enrolled patients undergoing cardiac surgery and the length of ICU stay was relatively short compared with that of the present study, suggesting a lower disease severity among patients in that study than that in the current investigation. In fact, we only included a population in whom the mortality risk was more than 5% in the control group. In support of this suspicion, a previous meta-analysis on cardiac surgical patients revealed an association of vitamin C use with a lower incidence of atrial fibrillation as well as shorter ventilation time and ICU/hospital LOS without a positive impact on in-hospital mortality (15). Therefore, such findings may underscore a potential variation in the beneficial effects of vitamin C in different populations.

A meta-analysis of RCTs and that focusing on retrospective studies can sometimes produce conflicting outcomes. For instance, a meta-analysis that included 27 RCTs and 21 observational studies reported no significant impact of vitamin C on in-hospital mortality based on the meta-analysis of the RCT subgroup (45). However, the use of vitamin C was found to be associated with a lower risk of in-hospital mortality compared to the control group in the same meta-analysis based on the subgroup of observational studies. Regarding 1-month mortality risk, although that study found a borderline positive effect on the risk of mortality with the use of vitamin C based on the meta-analysis of RCTs, there was no significant difference based on the pooled data of observational studies (45). Therefore, to better evaluate the causality by avoiding confounding factors, only RCTs were included in the current meta-analysis.

Judicious interpretation of the findings of the current meta-analysis is required because of its limitations. First, the heterogeneity from our inclusion of patients with and without sepsis/septic shock as well as our definition of critically ill individuals as those whose mortality rate was higher than 5% in

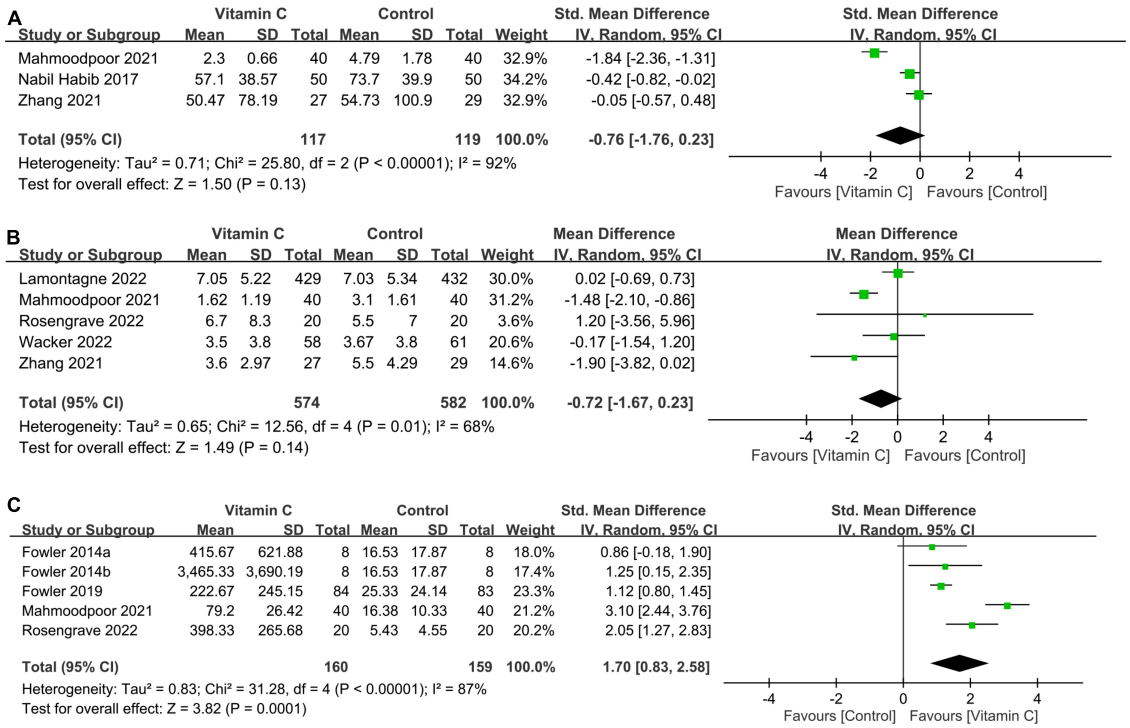

FIGURE 7
Forest plot comparing **(A)** circulating C-reactive protein concentration; **(B)** sequential organ failure assessment (SOFA) score; and **(C)** serum vitamin C level between intravenous vitamin C and control groups. CI, confidence interval; IV, inverse variance; Std, standardized mean difference.

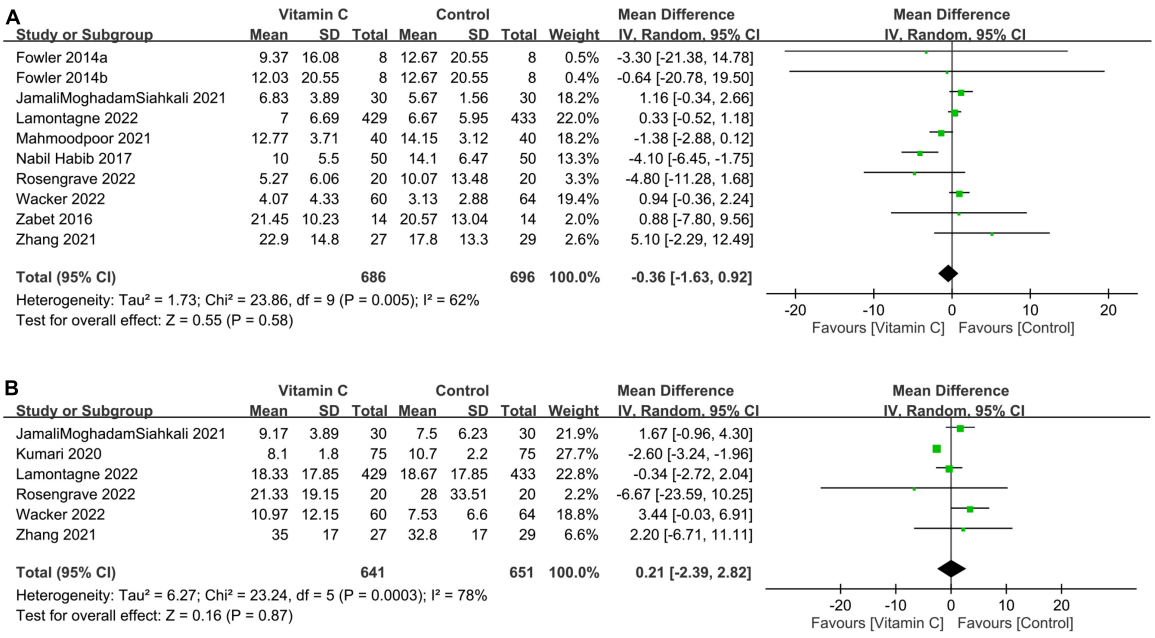

FIGURE 8
Forest plot comparing the **(A)** length of stay in the intensive care unit (ICU) and **(B)** length of hospital stay between the intravenous vitamin C and control groups. M-H, Mantel-Haenszel; CI, confidence interval.

the control group based on a previous study (16) may bias our findings. Nevertheless, the heterogeneity was low in our mortality rate, indicating a low risk of bias. Second, the wide variation in vitamin C dosage and frequency across our included study not only may bias our results but may also explain the lack of a universal guideline for vitamin C use in the critical care setting. Our subgroup analysis focusing on dosage suggested a favorable outcome associated with a low dosage of vitamin C, highlighting a

need for further research on this issue. Third, we did not investigate the efficacy of vitamin C-based combination therapy as several meta-analyses have addressed this issue (16). Finally, despite the demonstration of a gender difference in the beneficial impact of vitamin C on disease severity (i.e., acute respiratory tract infections) (46), we are unable to analyze gender-related outcomes because of a lack of relevant data.

In conclusion, this meta-analysis focusing on critically ill adults showed a lower risk of mortality in those receiving intravenous vitamin C as a monotherapy compared to those in the placebo or standard care groups. Besides, monotherapeutic use of intravenous vitamin C was associated with shorter durations of vasopressor use and mechanical ventilation. Nevertheless, the correlation between vitamin C supplementation and a reduction in mortality rate remained inconclusive on trial sequential analysis, suggesting the need for further research to verify our findings and to identify the optimal dosage for decreasing patient mortality rate in the critical care setting.

Data availability statement

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

Author contributions

K-CH and M-HC: conceptualization. J-YC: methodology. C-WH: software. K-CH and C-CC: validation. K-CH: formal analysis. Y-JC and C-WL: investigation. I-WC: resources. I-WC and K-CH: data curation. K-CH, I-WC, and C-KS: writing – original draft preparation and writing – review and editing. C-KS: visualization and supervision. All authors have read and agreed to the published version of the manuscript.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1094757/full#supplementary-material

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EDITED BY
Alexander Chouker,
LMU Munich University Hospital, Germany

REVIEWED BY
Lanzhou Li,
Jilin Agricultural University, China
Peijun Tian,
Jiangnan University, China

*CORRESPONDENCE
Min Xu
della.xumin@shsmu.edu.cn

†These authors have contributed equally to this work

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Dissecting the causal effect between gut microbiota, DHA, and urate metabolism: A large-scale bidirectional Mendelian randomization

Tianzhichao Hou[1,2†], Huajie Dai[1,2†], Qi Wang[1,2†], Yanan Hou[1,2], Xiaoyun Zhang[1,2], Hong Lin[1,2], Shuangyuan Wang[1,2], Mian Li[1,2], Zhiyun Zhao[1,2], Jieli Lu[1,2], Yu Xu[1,2], Yuhong Chen[1,2], Yanyun Gu[1,2], Jie Zheng[1,2], Tiange Wang[1,2], Weiqing Wang[1,2], Yufang Bi[1,2], Guang Ning[1,2] and Min Xu[1,2*]

[1]Department of Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2]Shanghai National Clinical Research Center for Metabolic Diseases, Key Laboratory for Endocrine and Metabolic Diseases of the National Health Commission of the PR China, Shanghai Key Laboratory for Endocrine Tumor, State Key Laboratory of Medical Genomics, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Objectives: Our aim was to investigate the interactive causal effects between gut microbiota and host urate metabolism and explore the underlying mechanism using genetic methods.

Methods: We extracted summary statistics from the abundance of 211 microbiota taxa from the MiBioGen (N =18,340), 205 microbiota metabolism pathways from the Dutch Microbiome Project (N =7738), gout from the Global Biobank Meta-analysis Initiative (N =1,448,128), urate from CKDGen (N =288,649), and replication datasets from the Global Urate Genetics Consortium (N gout =69,374; N urate =110,347). We used linkage disequilibrium score regression and bidirectional Mendelian randomization (MR) to detect genetic causality between microbiota and gout/urate. Mediation MR and colocalization were performed to investigate potential mediators in the association between microbiota and urate metabolism.

Results: Two taxa had a common causal effect on both gout and urate, whereas the *Victivallaceae* family was replicable. Six taxa were commonly affected by both gout and urate, whereas the *Ruminococcus gnavus group* genus was replicable. Genetic correlation supported significant results in MR. Two microbiota metabolic pathways were commonly affected by gout and urate. Mediation analysis indicated that the *Bifidobacteriales* order and *Bifidobacteriaceae* family had protective effects on urate mediated by increasing docosahexaenoic acid. These two bacteria shared a common causal variant rs182549 with both docosahexaenoic acid and urate, which was located within *MCM6/LCT* locus.

Conclusions: Gut microbiota and host urate metabolism had a bidirectional causal association, implicating the critical role of host-microbiota crosstalk in hyperuricemic patients. Changes in gut microbiota can not only ameliorate host urate metabolism but also become a foreboding indicator of urate metabolic diseases.

KEYWORDS

gout, uric acid, docosahexaenoic acids, mendelian randomization, mediation, gut microbiome

1 Introduction

Gout is a prevalent inflammatory condition characterized by a sustained high serum urate concentration and intermittent episodes of severely painful arthritis (gout flares) (1). It is the second most common metabolic disease after type 2 diabetes and leads to an increased rate of subsequent cardiovascular complications (2). Epidemiological investigations showed gout prevalence ranging from <1% to 6.8% worldwide, which was highly related to patients' genetics, lifestyles, and social and economic status (3). Recently, gut microbiota was reported to be associated with the pathogenesis of hyperuricemia. The abundance and composition changes in gut microbiota might increase serum uric acid levels through the dysfunction of uric acid degradation and increased uric acid production (4). Meanwhile, gut microbiota also plays a crucial role in treating metabolic diseases using probiotics and prebiotics (5, 6). Therefore, the human microbiota is gradually recognized as a novel target for a better understanding of the pathogenesis of gout and hyperuricemia.

However, previous studies focusing on the bacteria-urate interplay faced several obstacles, including inadequate participants, cross-sectional design, and less evidence of causality, as well as the fact that most of the studies were conducted within the Asian population (7–10). In addition, the underlying mechanism linking gut bacteria with urate metabolism has not been well established. Gut microbiota and its metabolites were associated with rheumatic diseases including gout, rheumatoid arthritis, and osteoarthritis *via* fatty acids (11). An experimental study investigated the impact of polyunsaturated fatty acids (PUFAs) in the handling of urate by inhibiting urate transporters *in vitro* (12). Some observational studies also suggested that ω-3 PUFAs, especially DHA were highly related to hyperuricemia (13, 14). However, a recent clinical trial found no significant difference in the serum urate level between the 24-week ω-3 PUFAs supplement group and the control group (15). Since gut microbiota might confer an effect on the host health status by metabolizing PUFAs (16), we hereby hypothesize a potential mediation effect of gut microbiota on urate metabolism *via* PUFAs and their important subtypes, such as DHA.

Given the ambiguous connection between gut bacteria and urate metabolism, we used several genetic methods to investigate the bidirectional causal effects between gut microbiota and urate metabolism and further explored potential mediators. Lifelong exposure to genetic methods could draw stable conclusions about the association and provide more evidence for probiotics treatment on urate metabolic disorders.

2 Methods

2.1 Study design

Firstly, we extracted summary statistics of gut microbiota, microbiota metabolism pathways, gout, urate level, and docosahexaenoic acid percentage (DHA, a kind of polyunsaturated fatty acid previously mentioned in clinical trials) from the respective consortiums. Secondly, we performed a large-scale bidirectional Mendelian randomization (MR) and bivariate linkage disequilibrium score regression (LDSC) to explore the genetic causality and correlation between microbiota phenotypes (gut microbiota abundance and microbiota metabolism pathways) and urate phenotypes (host gout and urate level). Finally, we used mediation analysis and colocalization to investigate the interaction of gut microbiota, DHA percentage, and urate metabolism (Figure 1). The MR analysis was reported as per the STROBE-MR guidelines, and we adopted several methods to follow the three fundamental assumptions of MR (Table S1) (17).

2.2 Instrument variables selection

Summary statistics for gut microbiota abundance were extracted from a genome-wide association study (GWAS) of host genetic variation in 18,340 multiple-ancestries participants (85% European-ancestry) from the MiBioGen consortium (Table S2) (18). It included 211 taxa: 9 phyla, 16 classes, 20 orders, 35 families, and 131 genera determined by the 16S ribosomal RNA gene sequencing. Summary statistics for microbiota metabolism pathways were extracted from the Dutch Microbiome Project (DMP) in 7738 European-ancestry participants, in which the metabolism pathways were determined by shotgun metagenomic sequencing (19). Included in the subsequent analysis were 205 bacteria metabolism pathways.

Summary statistics for gout were from the Global Biobank Meta-analysis Initiative (GBMI) (20). It is a collaborative network of 19 biobanks from four continents representing more than 2.1 million

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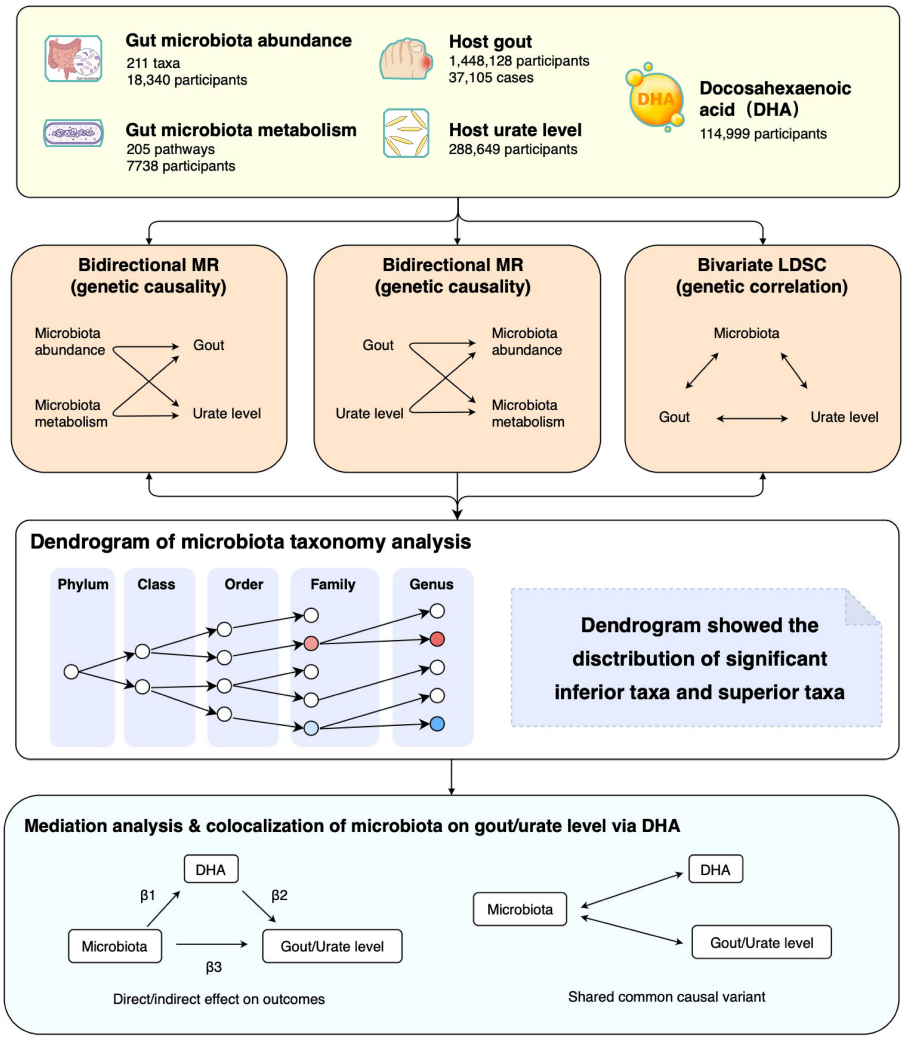

FIGURE 1
Study design and flowchart. MR, Mendelian randomization; LDSC, Linkage disequilibrium score regression.

consenting individuals with genetic data linked to electronic health records. We used the gout data of both multi-ancestry (N = 1,448,128, 72% European-ancestry, and N cases =37,105) and European-ancestry (N = 1,069,839; N cases = 30,549). Summary statistics for urate level were from the CKDGen Consortium, including 288,649 European participants (21). We also used phenotypes of both gout (N =69,374 participants) and urate level (N =110,347 participants) from GUGC Consortium as an independent replication analysis (22). Summary statistics for blood DHA and PUFAs were from the largest GWAS for metabolites in the UK Biobank, including 114,999 European participants (23). However, the summary statistics of eicosapentaenoic acid or other specific subtypes of PUFAs were not available for this study.

The IV selection of exposures in bidirectional MR followed the following criteria. For each microbiota taxon and pathway, variants with genome-wide significance P $<1\times10^{-5}$ and effect allele frequency (EAF) >0.01 were included. All these genetic variants were clumped to a linkage disequilibrium threshold of r^2 <0.001 using the 1000 Genomes European reference panel. We also calculated the F-statistics to avoid weak instrument bias. Due to the different genotyping platforms of the GWAS, some single nucleotide polymorphisms (SNPs) representing gut microbiota might be missing in the GWAS outcome, which might cause statistical bias if we simply discarded those missing SNPs. Therefore, we used proxy SNPs with LD r^2 >0.8 as substitutes for these missing SNPs (24, 25). Finally, the remaining genetic variants were used as IVs to model the effect of specific taxa and pathways of gut microbiota. For gout and urate level, SNPs with genome-wide significance P $<5\times10^{-8}$ and EAF >0.01 were included. All SNPs were clumped to an LD threshold of r^2 <0.001 using the 1000 Genomes European reference panel (26).

2.3 Genetic causality and correlation of microbiota and urate metabolism

We obtained the MR estimates for the causal effect using the inverse-variance weighted (IVW) method (27). The estimate was

provided as effect size (β) with a 95% confidence interval (CI). MR results of a specific taxon or pathway which were significant (P <0.05) in both gout and urate level with the same direction were defined as "common". To control type 1 error, a significant taxon (P <0.05) that could be replicated in the independent GUGC cohort was defined as "replicated"; other significant MR results (P <0.05) were defined as nominally significant. Results of taxa were mapped in the dendrograms to investigate the distribution of significant inferior and superior taxa. The heterogeneity of effects was assessed by Cochran's Q test. We performed several sensitivity analyses using the weighted median, mode-based, MRPRESSO, and contamination mixture methods to validate the results from the IVW method (28–32). We also used the MR-Steiger filtering to determine an actual causal direction. The MR-Egger intercept was used to assess the horizontal pleiotropy. Any results whose P value of Egger intercept was <0.05 were excluded.

We also used single-variate LDSC to estimate the heritability of 211 microbiota taxa, gout, and urate level. LD scores were calculated for all high-quality genetic variants (i.e., INFO score > 0.9 and EAF > 0.01) from each GWAS. To further understand the genetic correlation, we conducted a pair-wise genetic correlation analysis of the 211 microbiota taxa and both gout and urate level using bivariate LDSC based on the GWAS summary statistics. The genetic correlation between gout and urate level was also calculated to estimate the genetic similarity in two independent cohorts.

2.4 Mediation analysis and colocalization of microbiota, DHA, and gout/urate level

To estimate the effect of DHA acting on gut microbiota, we performed mediation analysis using multivariable MR. Firstly, we conducted an MR of DHA on gout and urate level. Secondly, we performed MR of 19 microbiota (significant effect on gout or urate level) on DHA (β1). Finally, we performed multivariable MR to determine the mediation effect of the DHA in bacteria on gout and urate. The multivariable Mendelian randomization (MVMR) estimated the effect of DHA on gout and urate adjusting for bacteria (β2) and the effect of bacteria on gout and urate adjusting for bacteria. To calculate the indirect mediation effect of bacteria on disease outcomes, we used the product of coefficients method as our primary method, which is the casual effect of bacteria on outcomes *via* DHA (β1×β2). Thus, the proportion of the total effect mediated by DHA was estimated by dividing the indirect effect by the total effect (33, 34).

We then conducted colocalization analysis to test whether the significantly mediated microbiota shared a common causal variant with both DHA and gout/urate level. The posterior probability of the specific variant (± 5000 bp) was colocalized with summary statistics of DHA and gout/urate levels.

All MR analyses were based on the TwoSampleMR package in R, version 4.1.2 (24); LDSC was based on LDSC software in Python, version 1.0.1 (35); the colocalization test was performed by the coloc package in R, version 5.1.0.1 (36).

3 Results

For 211 taxa in the MiBioGen consortium, the genetic variants used as IVs for each taxon exposure ranged from four to 26 SNPs (median 13 SNPs; F-statistics =21). For 205 metabolism pathways in the DMP, the genetic variants ranged from one to 20 SNPs (median 11 SNPs; F-statistics =25). Additionally, we used 54 SNPs as IVs for gout in GBMI and 88 SNPs for urate level in CKDGen (Tables S3–S5).

3.1 Bidirectional causal association of gut microbiota and urate metabolism

In the MR analysis, we identified 13 and 9 taxa that causally affected gout and urate level, respectively, whereas two taxa were common in both gout and urate level (Tables S6, S7). Conversely, 16 taxa were causally affected by gout and 19 taxa by urate level, whereas 6 taxa were common (Tables S8, S9).

In evaluating the causal effect of microbiota on gout and urate level, we found that the increase in abundance of the *Lachnospiraceae ND3007 group* genus commonly had a positive causal effect on both gout and urate level; the *Victivallaceae* family commonly had a negative causal effect on both phenotypes (Table 1). The MR-Steiger test supported the direction of significant MR estimates. Other taxa with significant causal effects on gout or urate level are shown in Figure S1. We then mapped the MR results of 203 taxa (except for eight unknown taxa) into dendrograms. According to the bacteria taxonomy, Figures 2A, B showed that genetically predicted 20 gut microbial taxa had causal effects on urate metabolism. We found that both the *Bifidobacteriaceae* family and its superior *Bifidobacteriales* order had significant causal effects on urate level (since they had the same IVs).

In evaluating the causal effect of gout and urate level on microbiota, we found that both gout and urate level commonly had a positive causal effect on the *Lachnoclostridium* genus and *Ruminococcus gnavus group* genus; gout and urate level commonly had a negative causal effect on *Coriobacteriia* class, *Coriobacteriales* order, *Coriobacteriaceae* family, and *Lachnospiraceae FCS020 group* genus. The MR-Steiger test supported the direction of significant MR estimates (Table 1). Other taxa affected by gout or urate level such as the *Bacteroides* genus, *Lachnoclostridium* genus, and *Eubacterium hallii group* genus can be found in Tables S8, S9 and Figure S2. Figures 2C, D show that urate metabolism had a causal effect on 29 gut microbial taxa.

Finally, replicated MR analysis in the independent GUGC cohort further validated our finding. In all common taxa, the effect of the *Victivallaceae* family on both gout and urate level could be replicated in GUGC; the effect of both gout and urate level on the *Ruminococcus gnavus group* genus could also be replicated in GUGC (Table 2).

Bivariate LDSC supported the genetic correlation of the *Eubacteriumruminantium -group* genus with gout (regression

TABLE 1 Bidirectional MR analysis of the causal effect between gut microbiota and gout/urate level.

Taxonomy	Gout				Urate level			
	Effect size β (95% CI)	*P* value	*P* for pleiotropy	Steiger test	Effect size β (95% CI)	*P* value	*P* for pleiotropy	Steiger test
Direction: microbiota → gout/urate level								
family.Victivallaceae	-0.04 (-0.08, -0.01)	0.033	0.074	True	-0.02 (-0.04, -0.01)	0.025	0.519	True
genus.Lachnospiraceae ND3007 group	0.25 (0.06, 0.43)	0.009	0.942	True	0.09 (0.01, 0.19)	0.044	0.843	True
Direction: gout/urate level → microbiota								
class.Coriobacteriia	-0.04 (-0.08, -0.01)	0.039	0.836	True	-0.07 (-0.14, -0.01)	0.025	0.524	True
order.Coriobacteriales	-0.04 (-0.08, -0.01)	0.039	0.836	True	-0.07 (-0.14, -0.01)	0.025	0.524	True
family.Coriobacteriaceae	-0.04 (-0.08, -0.01)	0.039	0.836	True	-0.07 (-0.14, -0.01)	0.025	0.524	True
genus.Lachnoclostridium	0.05 (0.01, 0.09)	0.015	0.725	True	0.07 (0.01, 0.14)	0.028	0.113	True
genus.Lachnospiraceae FCS020 group	-0.05 (-0.09, -0.01)	0.038	0.999	True	-0.08 (-0.15, -0.01)	0.039	0.326	True
genus.Ruminococcus gnavus group	0.09 (0.02, 0.15)	0.012	0.649	True	0.12 (0.01, 0.23)	0.028	0.275	True

The definition of "common" means a significant (P <0.05) result of a specific taxon that could be found in both gout and urate level in the same direction. P for pleiotropy was from the inverse-variance weighted method and meant less pleiotropy in MR analysis if P >0.05. The MR Steiger test was used to ensure the right causal direction (not confounded) from microbiota to gout/urate or gout/urate to microbiota.

coefficient [Rg]: 0.28, P = 0.041) and the *Lachnoclostridium* genus with urate level (Rg: 0.28, P = 0.008), which were consistent with the MR results. Gout and urate level were highly genetically correlated (Rg: 0.89, P <0.001) (Tables S10, S11).

Since few significant taxa appeared in the phylum, class, and order level, we further compared the results of gout with the urate level of each taxon in the family and genus level (Figure 3). In the genus level, five genera (one was common) in the *Lachnospiraceae* family had a significant causal effect on either gout or urate level; gout or urate level had a significant causal effect on five genera in the *Lachnospiraceae* family (two were common) (Figure S3). Totally, nine genera of the *Lachnospiraceae* family had a significant causal association with gout or urate level, whereas the *Lachnospiraceae NC2004 group* genus could significantly affect host urate level and be affected by host urate level.

3.2 Bidirectional causal association of microbiota metabolism pathways and urate metabolism

Based on the MR results (Tables S12, S13), we identified that a per unit increased abundance of eight pathways had causal effects on gout and 14 pathways had causal effects on urate level, including 1,4-dihydroxy-2-naphthoate biosynthesis (gout: β: 0.07, 95% CI: [0.01, 0.12], P = 0.014) or flavin biosynthesis (urate level: β: -0.06, 95% CI: [-0.10, -0.01], P = 0.013) (Figure 4).

For gout and urate level on microbiota, we found that both gout presence and urate level had a causal effect on bacterial L-rhamnose degradation (gout: β: 0.08, 95% CI: [0.02, 0.14], P = 0.008; urate level: β: 0.09, 95% CI: [0.01, 0.17], P = 0.043) and glycogen degradation (gout: β: 0.08, 95% CI: [0.01, 0.15], P = 0.031; urate level: β: 0.11, 95% CI: [0.02, 0.19], P = 0.013). Other five bacterial metabolism pathways affected by gout and nine pathways affected by urate level can be found in Tables S14, S15.

Among all 1654 analyses, 80 non-significant results with Egger intercept P <0.05 were excluded. Little pleiotropy remained in the remaining significant results (Table S16). Among all 3318 analyses, 196 non-significant IVW results with Cochrane's Q test P <0.05 were excluded. Little heterogeneity remained in the significant results (Table S17).

3.3 Mediation analysis of microbiota, DHA, and gout/urate level

Firstly, DHA had a significant causal effect on both gout (β: -0.09, 95% CI: [-0.17, -0.01], P =0.021) and urate level (β: -0.07, 95% CI: [-0.11, -0.03], P <0.001). Secondly, given the protected effect of DHA, among 19 taxa causally associated with gout or urate level, *Bifidobacteriales* order and *Bifidobacteriaceae* family (both β: 0.06, 95% CI: [0.01, 0.13], P =0.044) were positively associated with DHA level and negatively associated with urate level (β: -0.06, 95% CI: [-0.11, -0.01], P =0.020) (Table S18). In the mediation analysis, the indirect effect of *Bifidobacteriales* order on urate level *via* DHA was (β: -0.004, 95% CI: [-0.003, -0.01]) and the proportion of DHA mediation was 7.6% (P =0.016). Consistent with DHA, PUFAs level also had a causal effect on both gout (β: -0.40, 95% CI: [-0.62, -0.18], P <0.001) and urate level (β: -0.21, 95% CI: [-0.29, -0.12], P <0.001), while the *Bifidobacteriales* order *and Bifidobacteriaceae* family had a significant causal effect on serum PUFAs level (both β: 0.04, 95% CI: [0.01, 0.08], P =0.010) (Table S19). However, the indirect effect of the *Bifidobacteriales* order on urate level *via* PUFAs was not statistically significant (β: -0.0097, 95% CI: [-0.020, 0.034], P =0.667).

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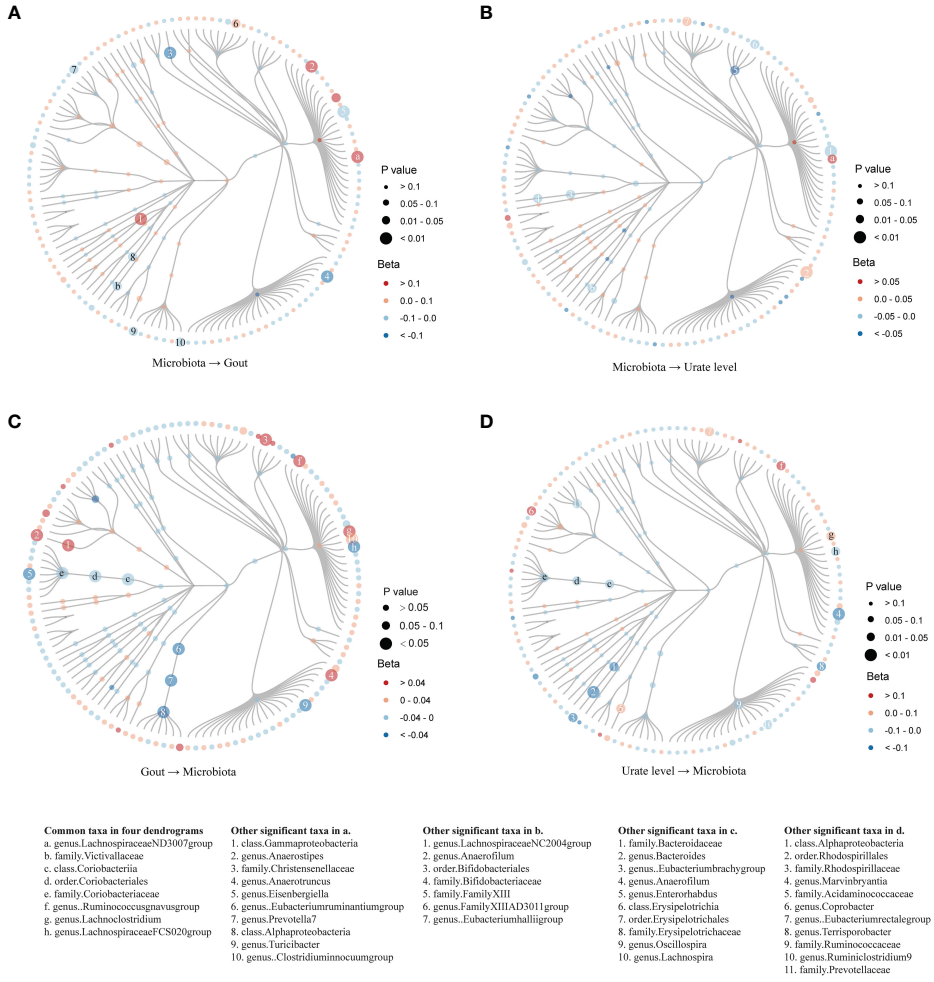

FIGURE 2
Dendrograms showing the taxonomy structure of the association between gut microbiota and host gout/urate level. Four dendrograms compared with the MR results of inferior and superior taxa according to the taxonomy structure. We included 203 taxa in the figures and excluded eight taxa named unknown. Taxa with significant MR results were listed at the bottom of the figure: the first column showed eight common taxa in both gout and urate level; the other four columns showed significant taxa in the respective figures. **(A)**. MR results from microbiota to gout; **(B)**. MR results from microbiota to urate level; **(C)**. MR results from gout to microbiota; **(D)**. MR results from urate level to microbiota.

We then estimated the causal effect of the *Bifidobacteriales* order and *Bifidobacteriaceae* family on urate level under a stricter significance threshold of IVs (P <1×10^{-8}). The Wald ratio showed the only remaining rs182549 (P =5.9×10^{-20}) had a significant causal effect on DHA level (β: 0.22, 95% CI: [0.15, 0.30], P <0.001). We then accessed the colocalization evidence to the 5000 bp region encompassing the SNP rs182549 at *MCM6* locus using the summary statistics from *Bifidobacteriales* order and *Bifidobacteriaceae* family, DHA, and urate level. The colocalization posterior probability (H4) of COLOC is high with 0.999 in both DHA and urate, which indicated the *Bifidobacteriales* order and *Bifidobacteriaceae* family shared a specific common variant rs182549 with both DHA and urate. We then searched the PhenoScanner database for rs182549 and mapped this variant in the *MCM6* gene combined with *LCT* in the pQTL analysis.

4 Discussion

In the present study, we found that 20 genetically predicted taxa significantly affected urate metabolism, and 29 taxa were affected by urate metabolism, followed by supportive genetic correlation from LDSC. Two taxa had a common causal effect on both gout and urate, whereas the *Victivallaceae* family was replicable in the independent GUGC cohort. Six taxa were commonly affected by both gout and urate, whereas the *Ruminococcus gnavus group* genus was replicable in the GUGC. DHA may mediate the protective effect of *Bifidobacteriales* order and *Bifidobacteriaceae* family on the host urate level. *Bifidobacteriales* order and *Bifidobacteriaceae* family shared a common causal variant rs182549 associated with *MCM6/LCT* with both DHA and urate level. To our knowledge, this is the first study to comprehensively examine the genetic association

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TABLE 2 Replicated significant taxa of bidirectional MR supported the causal effect between gut microbiota and gout/urate level.

Taxonomy	Gout				Urate level			
	Population	Methods	Effect size	P value	Population	Methods	Effect size	P value
Direction: microbiota → gout/urate level								
Victivallaceae Family	Primary: Gout from GBMI	IVW	-0.04 (-0.08, -0.01)	0.033	Primary: Urate from CKDGen	IVW	-0.02 (-0.04, -0.01)	0.025
		WM	-0.02 (-0.08, 0.04)	0.492		WM	-0.03 (-0.06, 0.00)	0.057
		MRPRESSO	-0.04 (-0.08, -0.01)	0.040		MRPRESSO	-0.02 (-0.04, -0.01)	0.030
		Con-mix	-0.01 (-0.17, 0.04)	0.697		Con-mix	-0.03 (-0.05, 0.00)	0.052
		Steiger test	True	<0.001		Steiger test	True	<0.001
	Replication: Gout from GUGC	IVW	0.07 (-0.15, 0.29)	0.548	Replication: Urate from GUGC	IVW	-0.05 (-0.09, -0.01)	0.025
		WM	0.09 (-0.19, 0.38)	0.518		WM	-0.05 (-0.10, 0.00)	0.053
		MRPRESSO	0.07 (-0.15, 0.00)	0.567		MRPRESSO	-0.03 (-0.06, 0.00)	0.090
		Con-mix	0.22 (-0.48, 0.62)	0.177		Con-mix	-0.04 (-0.10, -0.01)	0.051
		Steiger test	True	<0.001		Steiger test	True	<0.001
	Meta-analysis		**-0.02 (-0.04, -0.01)**	**0.047**	**Meta-analysis**		**-0.01 (-0.02, -0.01)**	**0.003**
Direction: gout/urate level → microbiota								
Ruminococcus gnavus group Genus	Primary: Gout from GBMI	IVW	0.09 (0.02, 0.15)	0.012	Primary: Urate from CKDGen	IVW	0.12 (0.01, 0.23)	0.028
		WM	0.07 (-0.02, 0.17)	0.133		WM	0.24 (0.07, 0.41)	0.005
		MRPRESSO	0.09 (0.02, 0.15)	0.016		MRPRESSO	0.08 (0.02, 0.15)	0.016
		Con-mix	0.09 (0.02, 0.18)	0.023		Con-mix	0.12 (0.03, 0.30)	0.021
		Steiger test	True	<0.001		Steiger test	True	<0.001
	Replication: Gout from GUGC	IVW	0.09 (0.02, 0.16)	0.009	Replication: Urate from GUGC	IVW	0.16 (0.05, 0.28)	0.004
		WM	Insufficient SNPs	/		WM	0.15 (0.01, 0.30)	0.039
		MRPRESSO	Insufficient SNPs	/		MRPRESSO	0.16 (0.05, 0.27)	0.010
		Con-mix	0.09 (0.03, 0.18)	0.021		Con-mix	0.20 (0.08, 0.30)	0.005
		Steiger test	True	<0.001		Steiger test	True	<0.001
	Meta-analysis		**0.04 (0.02, 0.06)**	**<0.001**	**Meta-analysis**		**0.06 (0.03, 0.09)**	**<0.001**

The MR Steiger test was used to ensure the right causal direction (not confounded) from microbiota to gout/urate or gout/urate to microbiota. The meta-analysis integrated the effect size of IVW methods based on a random-effect model. The bold form showed the results of meta-analysis.
IVW, inversed-variance weighted; WM, weighted median; MRPRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier; Con-mix, contamination mixture; GBMI, Global Biobank Meta-analysis Initiative; GUGC, Global Urate Genetics Consortium.

between gut microbiota and urate metabolism. Our findings implicated the critical role of gut microorganisms in host-microbiota crosstalk of urate metabolism disorders, underlying the importance of modulating host-microbe balance in the prevention and treatment of hyperuricemia diseases.

Gut microbiota plays an essential role in the production, catabolism, metabolism, and excretion of host uric acid. It could either convert purines to uric acid by secreting active enzymes or accelerate uric acid degradation by synthesizing urate-metabolizing enzymes (37). However, most studies focusing on the interaction of

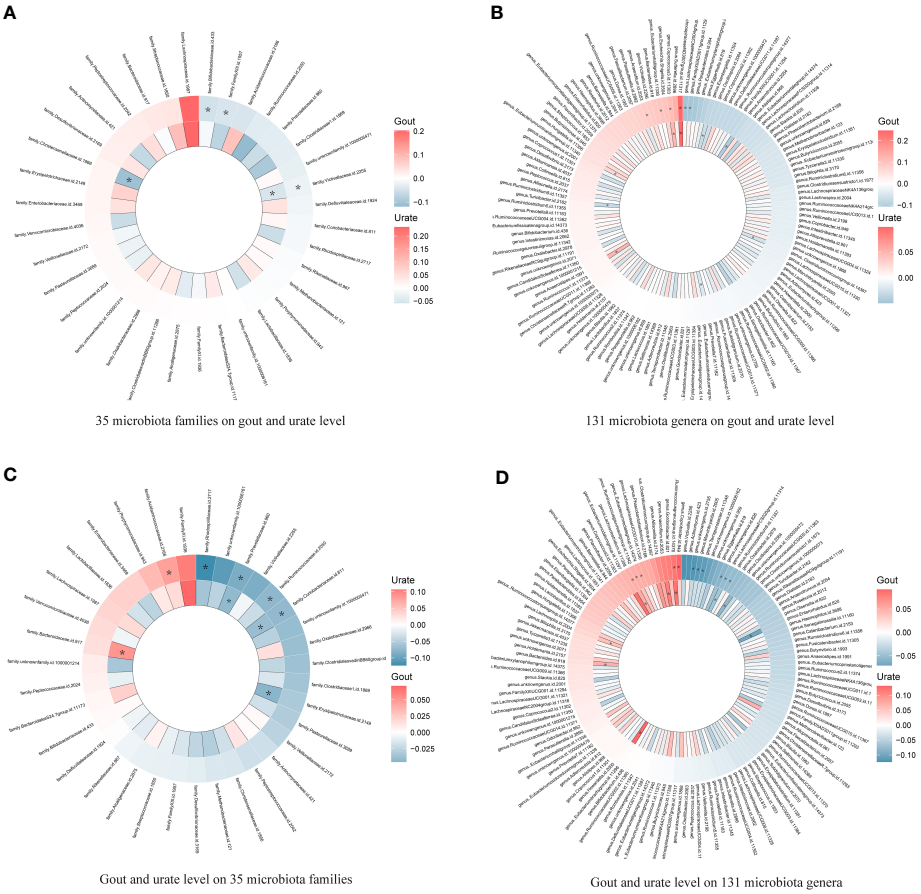

FIGURE 3
Heatmap showing bidirectional causality of 35 families and 131 genera. Four heatmaps comparing the results of gout with the urate level of each taxon in 35 families and 131 genera. The outer ring represents urate level and the inner ring represents gout. **(A)**. 35 microbiota families on gout and urate level; **(B)**. 131 microbiota genera on gout and urate level; **(C)**. Gout and urate level on 35 microbiota families; **(D)**. Gout and urate level on 131 microbiota genera. * means significant results.

gut microbiota and uric acid metabolism were cross-sectional studies, which could hardly determine the chronological order of the change in uric acid level and microbiota abundance (7–10). For example, elevated serum urate level could be related to the change in abundance of both purine-decomposition and purine-synthesis bacteria (5). Whether this change in abundance is pathogenic or compensatory for hyperuricemia remains unclear. The present study used bidirectional MR and MR Steiger direction tests to ensure the right causal direction between microbiota and urate metabolism (38). We found that the *Victivallaceae* family and *Lachnospiraceae ND3007 group* genus had a causal effect on both gout and urate level. The former taxon has not been well investigated, while the latter taxon belonged to an important disease-inducing family, the *Lachnospiraceae* family (39). Our MR results indicated five genera in this family had causal effects on gout or urate metabolism. However, the negative results of other taxa in the *Lachnospiraceae* family were not insignificant. Due to the controversial role of different inferior genera, function-dependent cluster analysis or taxon-to-taxon ratio on urate metabolism is warranted for further investigation (39).

In turn, gout and hyperuricemia could also trigger variations in gut microbiota abundance and metabolism. Consistent with observatory studies, the abundance of the *Coriobacteriaceae* family, *Prevotellaceae* family, *Lachnoclostridium* genus, and *Bacteroides* genus changed in patients with gout during treatment (9, 40–42). Therefore, similar to biomarkers of specific diseases, microbial dysbiosis and metabolic disorders serve as a profound reference for future studies in uric metabolism. Patients' fecal microbiome could be considered a pre-diagnostic target of gout and hyperuricemia (43).

Our results highlighted the different profiles of significant taxa in gout and urate level. Hyperuricemia and gout share common characteristics but also have differences. While a large proportion of individuals with hyperuricemia have never had a gout flare, some patients with gout can have a normuricemia status (44, 45). Hyperuricemia is generally considered to be the pathophysiologic basis of gout flares, and uric acid has dual effects *in vivo* with antioxidant properties as well as being an inflammatory promoter, which places it in a delicate position in balancing metabolisms (46). On the other hand, gout is a multifactorial metabolic disease, and its

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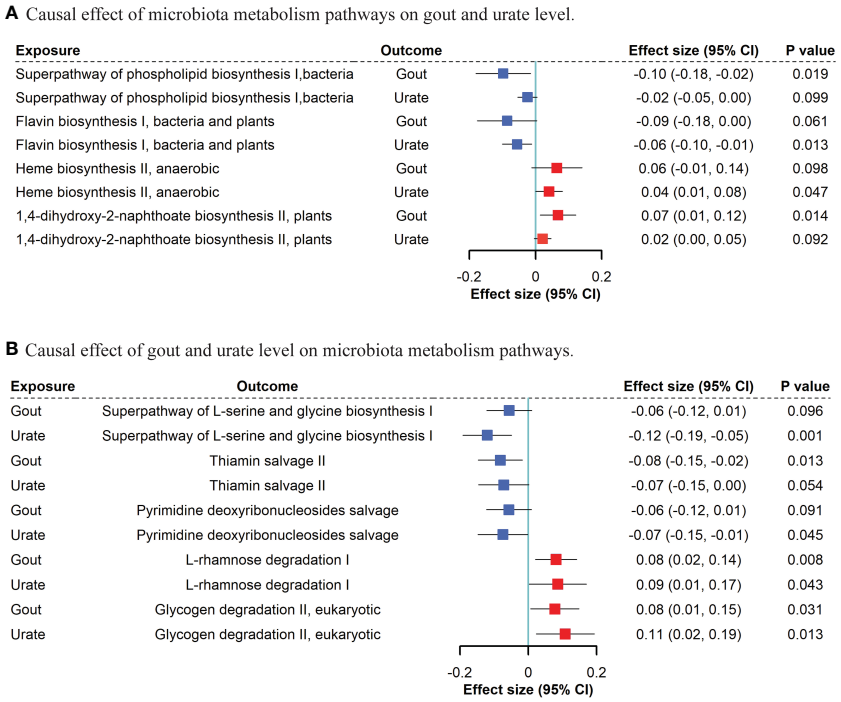

FIGURE 4
Bidirectional MR suggestive causal association between microbiota metabolism pathways and gout/urate level. **(A)**. Causal effect of microbiota metabolism pathways on gout and urate level. **(B)**. Causal effect of gout and urate level on microbiota metabolism pathways. We included "suggestive" significant pathways in Figure 4 (P <0.05 in gout or urate level and P <0.1 with the same direction in the other phenotype).

pathogenesis should not rely solely on hyperuricemia or monosodium urate crystals (46). Thus, the spectrum of significant taxa, combined with the mediation effect of DHA of hyperuricemia was quite different from that of gout; this might explain the difference between gout and urate metabolism in the MR analysis.

Bifidobacteria is a well-known probiotic to treat hyperuricemia, which is consistent with our MR (47). Studies indicated that *Bifidobacteria* supplement could elevate DHA levels in animal experiments and DHA supplement could improve hyperuricemia in humans (12, 48). We found that both the *Bifidobacteriales* order and *Bifidobacteriaceae* family had a protective effect on urate metabolism partially through DHA level, whereas rs182549 in *MCM6* was the most predominant variant. *MCM6* could be activated by both DHA supplements in the human body and hyperuricemia status in mice (49, 50). The complex *MCM6/LCT* variation also participated in several diseases' progress such as obesity, lactose intolerance, and irritable bowel syndrome (51, 52). This suggested that rs182549, which is related to *Bifidobacteria*, might play a critical role in the interplay of gut microbiota, unsaturated fatty acid, and urate metabolism. Furthermore, *MCM6/LCT* might be a potential therapeutic target for treating hyperuricemia.

Although gut microbiota was previously thought to be influenced by host health status, the GWAS significant variants representing the microbiota phenotype were relatively not largely confounded (19). Also, we assessed the genetic effect of microbiota in both gout and urate level. These two statistics were highly correlated but were derived from two independent populations. This not only avoided sample overlap but also replicated MR results in a related phenotype.

This study has several limitations. Firstly, a relatively non-significant heritability of the gut microbiota of the MiBioGen and DMP GWAS might impair the detective power of MR. However, owing to the sufficient sample size of publicly available data from the largest GWAS such as MiBioGen, DMP, and GBMI, the weak instrument bias could be somewhat complemented. Thus, we had adequate power to detect significant lifelong causality. Secondly, due to the difference between gout and urate, it is predictable that some bacteria related to one phenotype could not be replicable in the other phenotype. Therefore, some significant taxa in gout might not be associated with the urate level but might be associated more with systematic inflammation and intestinal transportation (53, 54). This could partly explain the different significant taxa in the two phenotypes.

In conclusion, this is the first study comprehensively assessing the association between gut microbiota and urate metabolism based on genetic methods. Our findings underlined the critical role of gut microorganisms in host hyperuricemia pathogenesis and progress. Taking advantage of host-microbiota crosstalk in urate metabolism could not only indicate directions of clinical prediction and the monitoring of gout but also draw a future blueprint for a cutting-edge therapeutic method based on fecal bacteria transplantation.

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

MX, TH, YB and GN conceived and designed the study. TH, QW, HD and YH conducted the analysis and finished writing the paper. XZ, JZ, TW, ML, HL, SW, ZZ, YG and YX offered guidance and methods for the analysis and data selection. YC, JL, WW, GN and YB reviewed the article and offered clinical advice. MX, YB and GN took responsibility for the contents of the article. All authors contributed to the article and approved the submitted version.

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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.2023.1148591/full#supplementary-material

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EDITED BY
Sergey Ponomarev,
Russian Academy of Sciences (RAS), Russia

REVIEWED BY
Peng Xie,
Huaiyin Normal University, China
Min Jin,
Ningbo University, China
Houguo Xu,
Chinese Academy of Fishery Sciences (CAFS), China

*CORRESPONDENCE
Shuyan Miao
msytougao@126.com

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Effects of dietary tryptophan on the antioxidant capacity and immune response associated with TOR and TLRs/MyD88/NF-κB signaling pathways in northern snakehead, *Channa argus* (Cantor, 1842)

Xin Zhang, Anran Wang, Enhui Chang, Bei Han, Jie Xu, Yu Fu, Xiaojing Dong and Shuyan Miao*

Aquaculture Nutrition and Feed Laboratory, College of Animal Science and Technology, Yangzhou University, Yangzhou, China

Introduction: Dietary tryptophan (Trp) has been shown to influence fish feed intake, growth, immunity and inflammatory responses. The purpose of this study was to investigate the effect and mechanism of Trp on immune system of juvenile northern snakehead (*Channa argus* Cantor, 1842).

Methods: A total of 540 fish (10.21 ± 0.11 g) were fed six experimental diets containing graded levels of Trp at 1.9, 3.0, 3.9, 4.8, 5.9 and 6.8 g/kg diet for 70 days, respectively.

Results and Discussion: The results showed that supplementation of 1.9-4.8 g/kg Trp in diets had no effect on the hepatosomatic index (HSI) and renal index (RI), while dietary 3.9 and 4.8 g/kg Trp significantly increased spleen index (SI) of fish. Dietary 3.9, 4.8, 5.9 and 6.8 g/kg Trp enhanced the total hemocyte count (THC), the activities of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD). Malondinaldehyde (MDA) levels in the blood were significantly decreased by consuming 3.9 and 4.8 g/kg Trp. Fish fed with 3.0 and 3.9 g/kg Trp diets up-regulated interleukin 6 (*il-6*) and interleukin 8 (*il-8*) mRNA levels. The expression of tumor necrosis factor α (*tnf-α*) was highest in fish fed with 3.0 g/kg Trp diet, and the expression of interleukin 1β (*il-1β*) was highest in fish fed with 3.9 g/kg Trp diet. Dietary 4.8, 5.9 and 6.8 g/kg Trp significantly decreased *il-6* and *tnf-α* mRNA levels in the intestine. Moreover, Trp supplementation was also beneficial to the mRNA expression of interleukin 22 (*il-22*). Additionally, the mRNA expression levels of target of rapamycin (*tor*), toll-like receptor-2 (*tlr2*), toll-like receptor-4 (*tlr4*), toll-like receptor-5 (*tlr5*) and myeloid differentiation primary response 88 (*myd88*) of intestine were significantly up-regulated in fish fed 1.9, 3.0 and 3.9 g/kg Trp diets, and down-

regulated in fish fed 4.8, 5.9 and 6.8 g/kg Trp diets. Dietary 4.8 and 5.9 g/kg Trp significantly increased the expression of inhibitor of nuclear factor kappa B kinase beta subunit (*ikkβ*) and decreased the expression of inhibitor of kappa B (*iκbα*), but inhibited nuclear transcription factor kappa B (*nf-κb*) mRNA level. Collectively, these results indicated that dietary 4.8 g/kg Trp could improve antioxidant capacity and alleviate intestinal inflammation associated with TOR and TLRs/MyD88/NF-κB signaling pathways.

KEYWORDS

tryptophan, antioxidant capacity, TOR signaling pathway, TLRs/MyD88/NF-κB signaling pathway, northern snakehead *Channa argus*

1 Introduction

Amino acids play a significant role in regulating growth, immunity and intestinal health of animals (1, 2). As the lowest concentration of essential amino acids in most common protein sources (3), dietary tryptophan (Trp) has been found to influence the feed intake, growth, immunity and inflammatory responses of fish (4–12), while the regulatory mechanisms need to be further studied.

When fish are subjected to oxidative stress, the content of reactive oxygen species (ROS) in fish increases (13, 14), which can attack macromolecules such as proteins and nucleic acids in the organism, leading to oxidative damage (15). In addition, the lipid peroxidation of ROS with polyunsaturated fatty acids (PUFA) in the cell membrane, and the end products of peroxidation, such as malondialdehyde (MDA), have toxic effects on cells (16, 17). Total antioxidant capacity (T-AOC), superoxide dismutase (SOD) and catalase (CAT) are important components of the antioxidant defense system in fish, which can remove excess ROS in the body, and maintain normal physiological and life activities (16, 18). Supplementation of amino acids in the diet has improved antioxidant capacity and reduced oxidative stress in several fish (19–21). Study has been demonstrated that dietary Trp improved the activity of plasma antioxidant enzymes in juvenile blunt snout bream (*Megalobrama amblycephala*) (22). Dietary Trp has been reported to prevent the increase of MDA content in grass carp (*Ctenopharyngodon idella*) (23). However, the effect of dietary Trp on the enzymatic and nonenzymatic antioxidant capacity of northern snakehead, *Channa argus* (Cantor, 1842) remains to be investigated.

Intestine performs multiple functions, including digestion and absorption of nutrients, recognition of external factors, and signal transduction related to innate and adaptive immunity (24). As is well-known, intestinal cytokines are closely related to intestinal health (25). According to the research, the production of pro-inflammatory cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), play a significant role in the development of intestinal inflammation (26). Accumulating evidence indicates that amino acids have powerful regulatory roles in cell signaling and mRNA translation (27, 28). Trp was reported to exert beneficial regulatory function in mucosal growth or maintenance, as well as alleviation of intestinal inflammation by 5-hydroxytryptophan (5-HT) (29). Target of rapamycin (TOR) and nuclear factor-kappa B (NF-κB) signaling pathways have been considered to have momentous functions in cell proliferation, differentiation, growth, and metabolism (30, 31). In addition, a large number of studies have shown that various amino acids can regulate intestinal inflammation through the TOR or NF-κB signaling pathway (32–34). Glutamine was found to attenuate intestinal inflammation dependent on its function *via* the mechanistic target of rapamycin (mTOR) and NF-κB signaling pathways (35, 36). Studies have shown that when the TLRs/MyD88/NF-κB signal pathway is inhibited, the inflammatory response in *Oreochromis niloticus* decreases (37). However, there are few reports on the effects and mechanisms of dietary Trp on immunoregulation of *Channa argus*, which need to be further investigated.

C. argus is one of the main economic species in China, due to its rich edible and medicinal value (38). The production of *C. argus* in 2021 was about 548,500 tons (39). To our knowledge, limited information about the nutritional immunity of *C. argus* is available (40, 41). In our previous research, based on the second-degree polynomial regression analysis of specific growth rate and feed efficiency against dietary Trp levels, the optimum dietary Trp requirements for *C. argus* were respectively estimated to be 4.6 and 4.5 g/kg (42). Therefore, in the present study, we investigated the effects of different dietary Trp levels on the antioxidant capacity and intestinal health of *C. argus*, and discussed its possible mechanisms of immune regulation by analyzing the expressions of related genes of the signaling pathways.

2 Materials and methods

2.1 Experimental diets

The formulation and proximate composition of the basal diet are shown in Table 1. Fish meal, poultry by-product meal and mixed L-amino acids were chosen as the main protein sources, fish

TABLE 1 Formulation and proximate analysis of the basal diet (g/kg diet).

Ingredient	Content
Fish meal (70.2% crude protein)	105.00
Poultry by-product meal (66.5% crude protein)	200.00
Wheat meal (12.0% crude protein)	150.00
Gelatin (97.5% crude protein)	40.00
Yeast powder (58.2% crude protein)	30.00
Wheat bran (18.6% crude protein)	110.00
Fish oil	25.00
Soybean oil	30.00
Vitamin mixture [a]	15.00
Mineral mixture [b]	15.00
Amino acid mixture [c]	150.00
Calcium dihydrogen phosphate	10.00
Choline chloride	10.00
Cellulose	99.00
Dimethyl-β-propiothetin	1.00
Composition (% dry weight basis)	
Moisture	7.00
Crude protein	48.56
Crude lipid	10.38
Crude ash	10.04
Carbohydrate[1]	24.02
Gross energy[2], kJ/g	19.72

a.Vitamin premix (mg/kg diet): vitamin B_1, 25 mg; vitamin B_2, 45 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; vitamin K, 10 mg; vitamin A, 32 mg; vitamin C, 2000 mg; vitamin D_3, 5 mg; vitamin E, 240 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 60 mg; calcium pantothenate, 60 mg; micocrystalline cellulose 12273 mg.
b Mineral premix (mg/kg diet): inositol, 800 mg; $MgSO_4{\cdot}H_2O$, 1200 mg; $CuSO_4{\cdot}5H_2O$, 10 mg; $FeSO_4{\cdot}H_2O$, 80 mg; $ZnSO_4{\cdot}H_2O$, 50 mg; $MnSO_4{\cdot}H_2O$, 45 mg; $CoCl_2{\cdot}6H_2O$, 50 mg; $Ca(IO_3)_2$, 60 mg; Na_2SeO_3, 20 mg; zeolite powder, 12685 mg.
c Amino acid mixture (g/kg diet): arginine, 9.98 g; histidine, 3.37 g; isoleucine, 9.91 g; leucine, 18.60 g; lysine, 23.52 g; methionine, 6.27 g; cysteine, 10.27 g; phenylalanine, 7.57 g; threonine, 10.79 g; valine, 8.48 g; aspartic acid, 28 g; glycine, 14 g.
1 Carbohydrate (%) = 100 - (% crude protein + % crude lipid + % moisture + % ash).
2 Calculated based on 17.2 kJ g^{-1} carbohydrate; 23.6 kJ g^{-1} protein and 39.5 kJ g^{-1} lipid according to the method described in a previous study (43).

oil and soybean oil were chosen as the main lipid sources. 1.0, 2.0, 3.0, 4.0 and 5.0 g/kg diet Trp (Sinopharm Chemical Reagent Co., Ltd, SHH, CHN) were added into the basal diet to produce five experimental diets, respectively. Trp supplement is balanced with glutamate, accounting for 1% of the diet. Prior to the addition of oil and water, all raw ingredients were ground through a 246-micron sieve, then were fully mixed and extruded into particles with diameter of 2.0 × 3.0 mm. Finally, all diets were dried at 40°C and stored at -20°C until use.

Amino acid contents of diets were analyzed with automatic amino acid analyzer (L-8900, Hitachi, Japan). The amino acid profile of the basal diet is presented in Table 2. The final Trp content in six diets are 1.9, 3.0, 3.9, 4.8, 5.9 and 6.8 g/kg, respectively.

TABLE 2 The amino acid profile of the basal diet (% dry weight).

Amino acid	Amino acid composition of basal diet
Essential amino acids (EAAs)	
Lysine	4.29
Methionine	0.99
Arginine	2.89
Phenylalanine	2.18
Histidine	1.01
Isoleucine	2.04
Leucine	3.93
Threonine	2.21
Valine	2.10
Tryptophan	0.19
Non-essential amino acids (NEAAs)	
Aspartic acid	5.06
Glutamic acid	4.65
Serine	1.22
Glycine	4.15
Alanine	2.01
Tyrosine	0.87
Cysteine	1.10

2.2 Fish and culturing conditions

Healthy *C. argus* were purchased from a commercial hatchery in Guangdong, China. Before the trial, fish were acclimated to experimental conditions in the greenhouse in Yangzhou University with a water-recirculating system, and fed with the basal diet for two weeks. Then, 540 fish with initial weight of 10.21 ± 0.11 g were randomly assigned to 18 cages (1 m × 1 m × 80 cm) with 30 fish in each cage, and three cages of fish were randomly provided for each experimental diet. During the feeding trial, all fish were fed at 8:00 and 17:00 daily at apparent satiation level. Feed consumption and fish mortality were recorded every day. The water dissolved oxygen was 6.2-6.6 mg/L and the temperature was 25.5-28.5°C. All experimental protocols were approved by the Animal Care Advisory Committee of Yangzhou University.

2.3 Sample collection and analysis

After 70 days feeding trial, all *C. argus* were fasted for 24 h, then anaesthetized by MS-222 solution (250 mg/L, Sigma). Liver, head kidney and spleen samples were collected from 10 fish in each cage and weighed to calculate the hepatosomatic index (HSI), renal index (RI) and spleen index (SI). Blood samples were collected in heparin sodium-anticoagulant tubes from 10 fish in each cage and divided into two parts, one part was prepared for the determination of

malondinaldehyde (MDA) value and the antioxidant enzymes activity, including superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (T-AOC), another part was prepared for the determination of total hemocyte count (THC). The whole intestine samples were collected from 5 fish in each cage for determination of the relative mRNA levels of genes, including tumor necrosis factor α (*tnf-α*), interleukin 1β (*il-1β*), interleukin 6 (*il-6*), interleukin 8 (*il-8*), interleukin 22 (*il-22*), target of rapamycin (*tor*), toll-like receptor-2 (*tlr2*), toll-like receptor-4 (*tlr4*), toll-like receptor-5 (*tlr5*), myeloid differentiation factor 88 (*myd88*), inhibitor of nuclear factor kappa B kinase beta subunit (*ikkβ*), inhibitor of kappa B (*iκbα*), nuclear transcription factor kappa B (*nf-κb*).

The proximate composition of the ingredients and diet, including the moisture, crude protein, crude lipid and crude ash were determined using standard procedures of the AOAC (44). The content of amino acids in ingredients and diet was determined by the method of Rajendra (45). THC was measured and calculated according to Sierra et al. (46). MDA value and the antioxidant enzymes activity were measured using commercial kits provided by Jian Cheng Bioengineering Institute, Nanjing, China. The relative mRNA level of genes was analyzed by RT-*q*PCR method according to Miao et al. (47). The cDNA sequences of the relevant genes were queried in the NCBI, and all primers were designed using Primer Premier 6. All specific primers for genes are provided in Table 3. *β-actin* was chosen as the internal reference gene based on the preliminary tests and Norm Finder algorithms (48, 49).

2.4 Calculations and statistical analysis

Hepatosomatic index (HSI), spleen index (SI), and renal index (RI) were calculated as following:

$$HSI\ (\%) = 100 \times \frac{w_h}{w_b}$$

$$SI\ (\%) = 100 \times \frac{w_s}{w_b}$$

$$RI\ (\%) = 100 \times \frac{w_r}{w_b}$$

W_b: fish weight (g); W_h: liver weight (g); W_s: spleen weight (g); W_r: head kidney weight (g)

After homogeneity variance tests by SPSS 18.0 (SPSS Inc., Chicago, IL, USA), the data (means ± S.D.) were subjected to a one-way analysis of variance (ANOVA) by Tukey's multiple comparison test to assess the significant differences among the treatments at $P < 0.05$. In addition, to determine if the effect was linear and/or quadratic, a follow-up trend analysis using orthogonal polynomial contrasts was performed (50).

3 Results

3.1 Organ index

The effects of dietary Trp on organ index and THC of *C. argus* are shown in Figure 1. The HSI and RI in fish fed the 1.9, 3.0, 3.9 and 4.8 g/kg Trp diets were significantly higher than that in fish fed the 5.9 g/kg Trp diet ($P < 0.05$), and there was no significant difference among fish fed the 1.9, 3.0, 3.9 and 4.8 g/kg Trp diets ($P > 0.05$). The SI and HSI of fish increased at first and then decreased as Trp content increased. Fish fed 3.9 and 4.8 g/kg Trp diets had the highest SI. There were significantly negative linear and positive quadratic trends between the dietary Trp levels and the dependent variables including HSI, SI and RI ($P < 0.05$).

TABLE 3 Primers sequence for RT-*q*PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′–3′)
β-actin	CACTGTGCCCATCTACGAG	CCATCTCCTGCTCGAAGTC
tor	GAGCCTCTCTCATCCTCACCAC	GATTCATTCCTTTCTCTTTAGCCA
tlr-2	CTGGACGAATCATCGAATCACCT	AACTTTGGCTTCCTCTTGGCTCT
tlr-4	GGAGGAGACAGAAGGTGTAGATTTG	AGGTTGTGATCTTGGGCTGAGTG
tlr-5	ACCTCTTCCGCTGTTGTTTCG	AGTGAGCCACCTTCCCTACCA
myd88	TGTCCGAGGTGGAAAGAAGTG	TCAAAGTCGCTCTGGCAGTAG
ikkβ	ATCACAGAGCAACCCCTTTT	CCACTGTAGTTAGGGAAGGA
iκbα	AAAATGTTACCGTGCCAGGAC	ATGTATCACCGTCGTCAGTC
nf-κb	CAGCCAAAACCAAGAGGGAT	TCGGCTTCGTAGTAGCCATG
tnf-α	ACAATACCACCCCAGGTCCCA	ACGCAGCATCCTCTCATCCAT
il-1β	ATGACATGCAATGTGAGCAAAAT	TTAACTCGTATGCTGAATGGTGA
il-6	CATGGAGCACTCAAAGAGGATAG	CTGAGGTGGAGGTAGTGTTGTCG
il-8	GAGTCTGAGCAGCCTGGGAGT	CTGTTCGCCGGTTTTCAGTG
il-22	CAGGCTGTGCAGACGGAGGAAGA	GCGTGGTGATGGTCGTGATAGTGAG

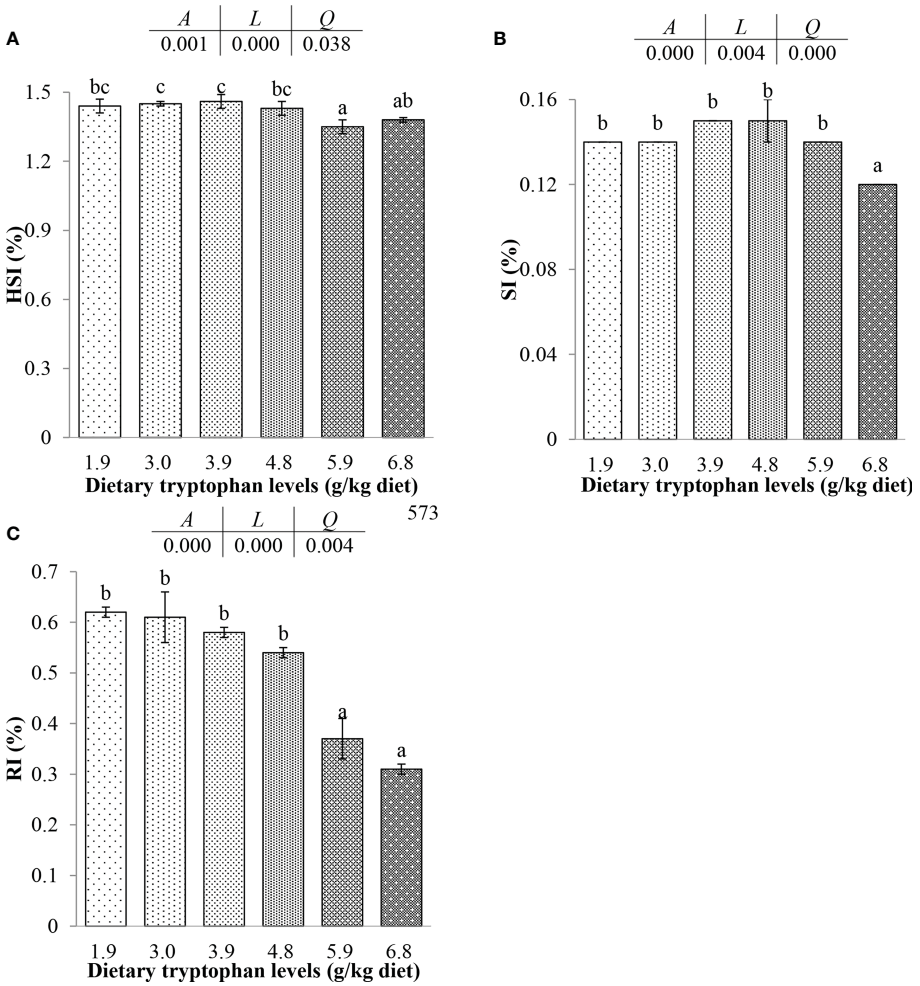

FIGURE 1
Effects of dietary tryptophan with different levels on organ index of *C. argus* (means ± S.D. of three replications). Bars with different letters indicate significantly among the treatments ($P < 0.05$). **(A)** Hepatosomatic index (HSI); **(B)** Spleen index (SI); **(C)** Renal index (RI).

3.2 Total hemocyte count and hematologic antioxidant-related parameters

The effects of dietary Trp level on THC and the hematologic antioxidant-related parameters are shown in Figure 2. The THC levels in *C. argus* increased as Trp levels increased until the dietary Trp level reached 3.9 g/kg, then began to decrease when the dietary Trp level exceeded 4.8 g/kg, and had significantly positive linear and negative quadratic trends with dietary Trp levels ($P < 0.05$).

With the increase of Trp, the activities of SOD, CAT and T-AOC were increased first and then decreased. SOD reached the highest activity in fish fed the diet with 3.9 g/kg Trp, and T-AOC reached the maximum activity in fish fed the 3.9 and 4.8 g/kg Trp diets ($P < 0.05$). However, the highest CAT activity was showed in fish fed the diet with 3.0 g/kg Trp ($P < 0.05$), but there was no significant difference between the fish fed the 1.9 and 3.9 g/kg Trp diets ($P > 0.05$), and the activities of CAT in fish fed the 1.9-3.9 g/kg Trp diets were significantly higher than that in other fish ($P < 0.05$). There were significantly linear and positive quadratic trends between the dietary Trp levels and the dependent variables including SOD, T-AOC and CAT ($P < 0.05$). However, the MDA contents had positive quadratic trend with dietary Trp levels ($P < 0.05$). The MDA contents in fish fed the 3.9 and 4.8 g/kg Trp diets were significantly decreased compared with those in fish fed the 1.9, 3.0 and 6.8 g/kg Trp diets ($P < 0.05$), and there was no significant difference with fish fed the 5.9 g/kg Trp diet ($P > 0.05$).

3.3 Relative mRNA expression of genes related to intestinal inflammatory factors

As shown in Figure 3, dietary Trp level significantly affected the relative expressions of genes related to intestinal inflammatory factors, including interleukins and TNF-α ($P < 0.05$).

The expressions of *tnf-α*, *il-6* and *il-8* increased first and then decreased with dietary 1.9-4.8 g/kg Trp. There were significantly negative linear trends between the dietary Trp levels and the dependent variables including *tnf-α* and *il-6* ($P < 0.05$). For *il-6*

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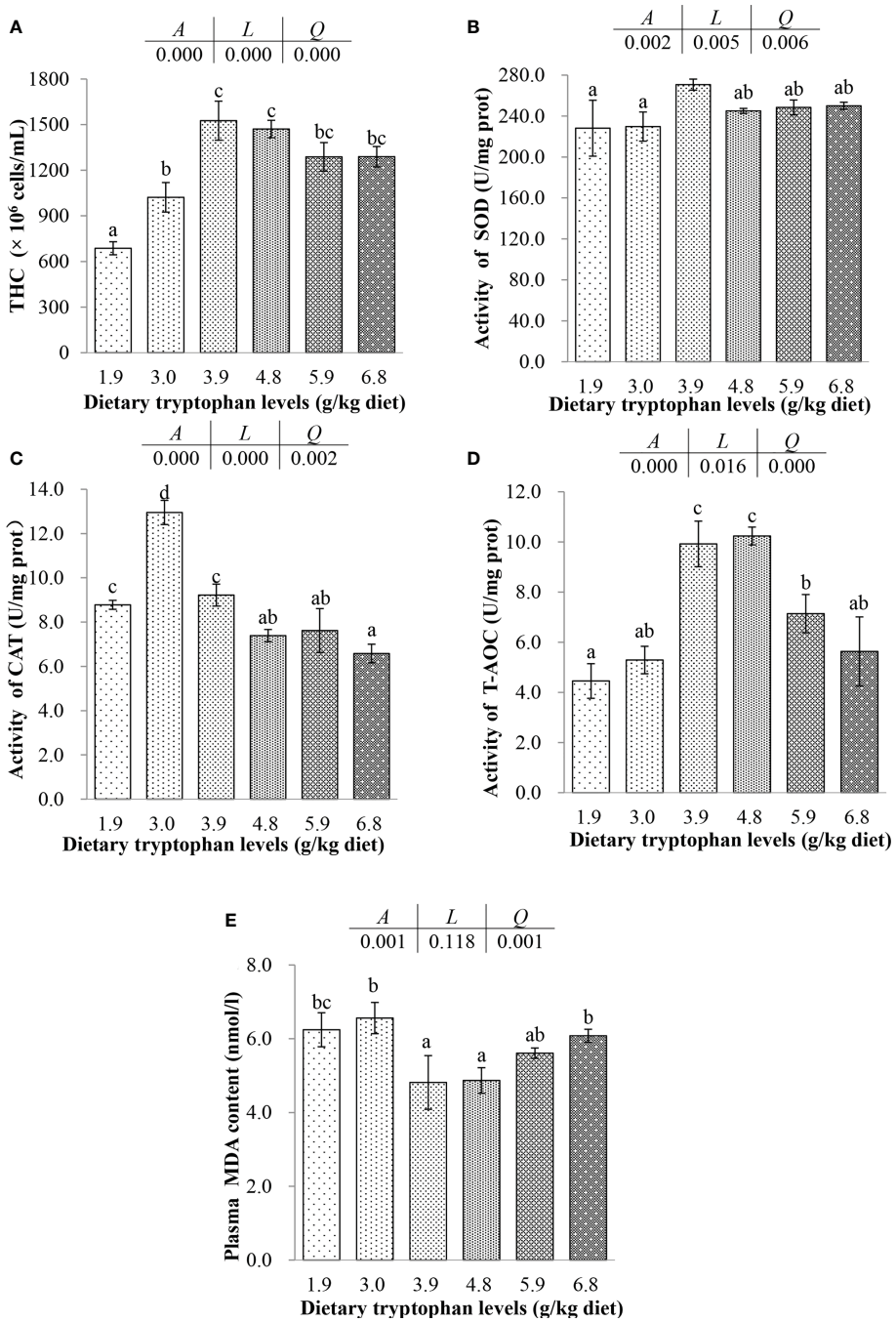

FIGURE 2
Effects of dietary tryptophan different levels on total hemocyte count and hematologic antioxidant parameters of blood in *C argus* (means ± S.D. of three replications). Bars with different letters differ significantly among the treatments ($P < 0.05$). **(A)** Total hemocyte count (THC); **(B)** Superoxide dismutase (SOD); **(C)** Catalase (CAT); **(D)** Total antioxidant capacity (T-AOC); **(E)** Malondialdehyde (MDA).

and *il-8*, the expressions of them in fish fed the 4.8 g/kg Trp diet were significantly lower than those fed with 1.9, 3.0 and 3.9 g/kg Trp diets ($P < 0.05$), and the expressions of them were higher in fish fed the 3.0-3.9 g/kg Trp diets compared with 1.9 g/kg Trp diet group ($P < 0.05$). The expression of *tnf-α* was highest in fish fed 3.0 g/kg Trp diet and significantly decreased in fish fed 3.9 and 4.8 g/kg Trp diets compared to fish fed the basal diet ($P < 0.05$). The expression of *il-1β* in fish fed diets with 3.0, 4.8, 5.9 and 6.8 g/kg Trp showed a decreasing trend compared with the control group, but there was no significant difference except for dietary 6.8 g/kg Trp ($P > 0.05$). There were significantly negative quadratic trends between the dietary Trp levels and the dependent variables including *il-1β*, *il-6* and *il-22* ($P < 0.05$). The higher expression of *il-22* was found in fish fed diets with 3.0-4.8 g/kg Trp ($P < 0.05$), and there was no significant difference between the fish fed diets with 3.0-4.8 g/kg Trp ($P > 0.05$).

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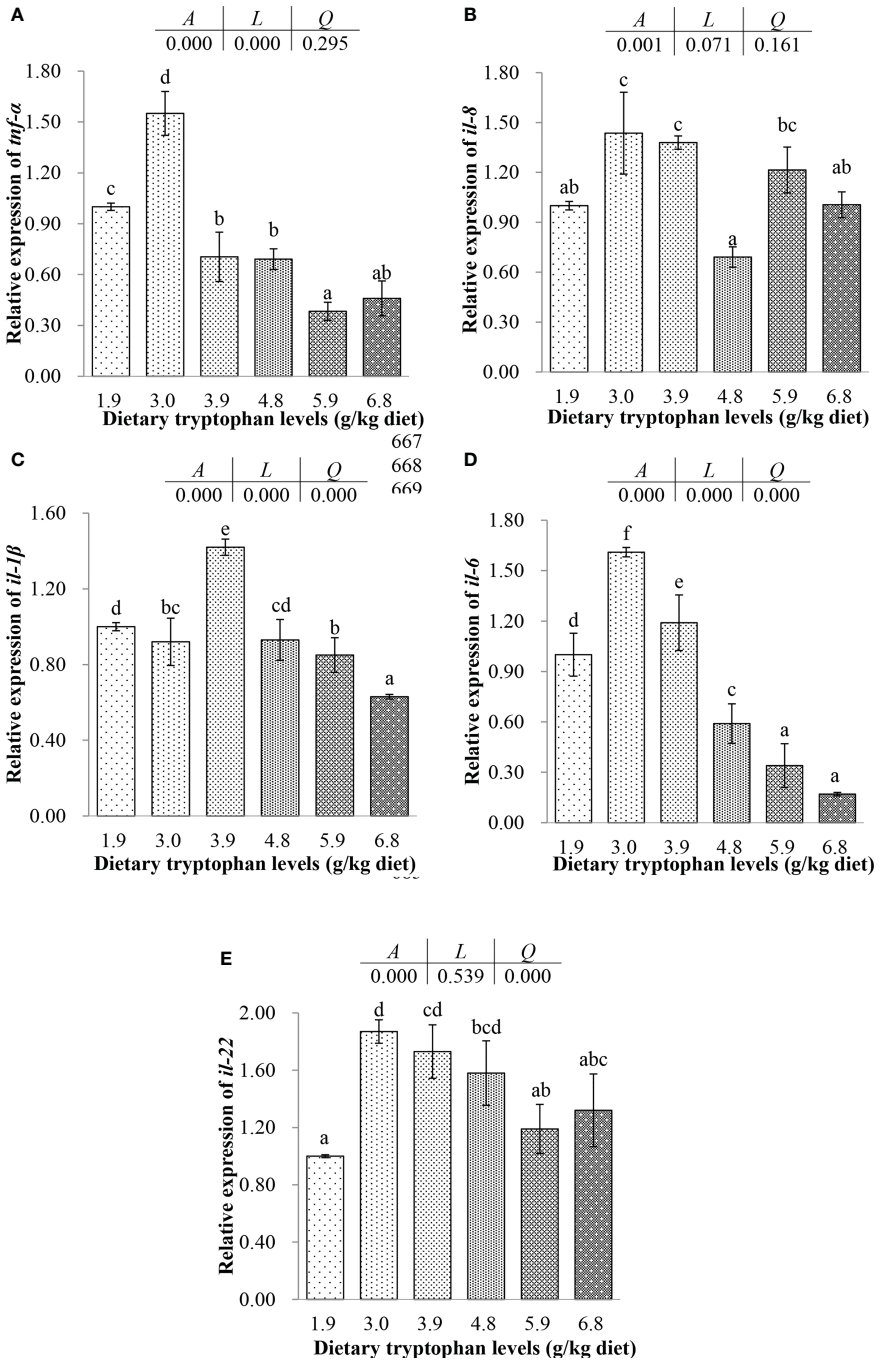

FIGURE 3
Effects of dietary tryptophan with different levels on the relative mRNA expression of genes related to intestinal inflammatory factors of *C argus* (means ± S.D. of three replications). Bars with different letters differ significantly among the treatments ($P < 0.05$). **(A)** Tumor necrosis factor α (*tnf-α*); **(B)** Interleukin 8 (*il-8*); **(C)** Interleukin 1β (*il-1β*); **(D)** Interleukin 6 (*il-6*); **(E)** Interleukin 22 (*il-22*).

3.4 Relative mRNA expression of genes related to the intestinal target of signaling pathways

As shown in Figure 4, there were significantly linear and positive quadratic trends between the dietary Trp levels and the expressions of *tor*, *tlr-2*, *tlr-4*, *tlr-5*, *myd88*, *iκbα* and *ikkβ* ($P < 0.05$).

The expressions of *tor*, *tlr-2*, *tlr-4*, *tlr-5* and *myd88* were all significantly increased with the dietary Trp increasing from 1.9 to 3.9 g/kg, and then decreased ($P < 0.05$). The expressions of *tlr-2*, *tlr-5*, *myd88* and *iκbα* in fish fed the 4.8 g/kg Trp diet were significantly lower than those in fish fed the basal diet ($P < 0.05$). Moreover, the lowest *tor*, *tlr-2*, *tlr-5* and *myd88* gene expressions were found in fish fed the 5.9 and 6.8 g/kg Trp diets ($P < 0.05$). The expression of *ikkβ* increased with increasing dietary Trp up to 4.8 g/kg ($P < 0.05$) and

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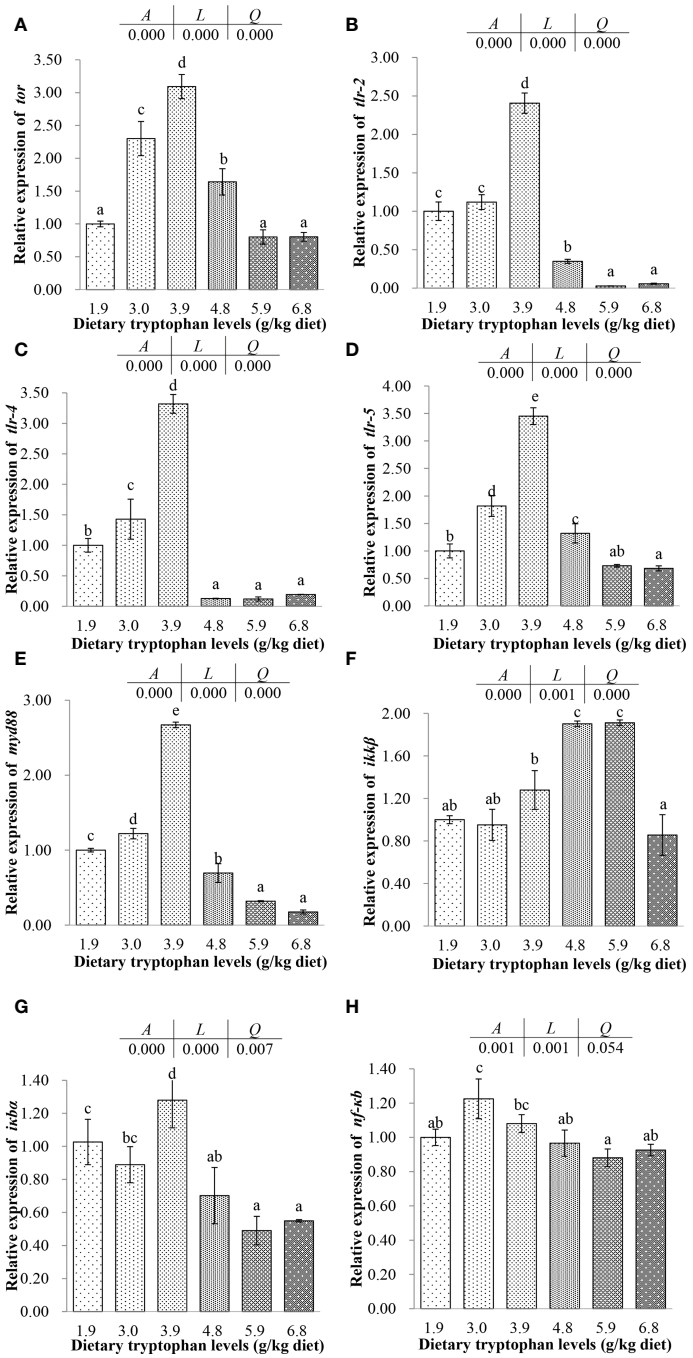

FIGURE 4
Effects of dietary tryptophan with different levels on the relative mRNA expression of genes related to the TOR and NF-κB signaling pathways of *C argus* intestine (means ± S.D. of three replications). Bars with different letters differ significantly among the treatments ($P < 0.05$). **(A)** Target of rapamycin (*tor*); **(B)** Toll-like receptor-2 (*tlr-2*); **(C)** Toll-like receptor-4 (*tlr4*); **(D)** Toll-like receptor-5 (*tlr5*); **(E)** Myeloid differentiation factor88 (*myd88*); **(F)** Inhibitor of nuclear factor kappa B kinase beta subunit (*ikkβ*); **(G)** Inhibitor of kappa B (*ikbα*); **(H)** Nuclear factor-κ-gene binding (*nf-κb*).

decreased with increasing dietary Trp up to 6.8 g/kg ($P < 0.05$). The expression of *ikbα* showed a positive linear except for that in the fish fed diet with the 3.9 g/kg Trp. At the same time, the expressions of *ikbα* were lower in the fish fed with 4.8, 5.9 and 6.8 g/kg Trp diets ($P < 0.05$), compared with the fish fed with basal diet. The expressions of *nf-κb* in the fish fed with 4.8, 5.9 and 6.8 g/kg Trp diets were lower than those in fish fed the basal diet, but there was no significant difference ($P > 0.05$). The expression of *nf-κb* was highest in the fish fed with 3.0 g/kg Trp diet ($P < 0.05$), and had significantly positive linear trend with dietary Trp levels ($P < 0.05$).

4 Discussion

The organ index usually reflects the development of organs and the general nutritional status of animals (51). The liver, spleen and

kidney are the main immune organs of fish, which can reflect the immune state of fish to some extent (52). In the present study, HSI, SI and RI decreased significantly in linear and quadratic curves with dietary Trp increasing, but there was no significant difference in fish fed with 1.9-4.8 g/kg Trp diets. Sharf et al. (53) showed that HSI and viscera somatic index of fingerling *Channa punctatus* decreased with the increase of dietary 0.9-9.1 g/kg Trp. However, studies have shown that dietary supplemented with 0.4-0.6 g/kg Trp could significantly increase SI of ducks (54). Carrillo-Vico et al. (55) also proved that melatonin (the metabolic product of Trp) could positively stimulate the development of spleen. In the present study, although dietary 3.9 or 4.8 g/kg Trp couldn't significantly improve the development of spleen, it has shown a significantly negative quadratic trend, which may be due to the differences in culturing time and breeding subjects. These results provide a hint that the optimum supplementation of Trp in feed was between 3.9 and 4.8 g/kg, which may be beneficial to the development of spleen and had no negative effect on the liver and head-kidney.

The function of blood is closely related to maintaining the stability of various physiological environments in the fish, such as eliminating invading bacteria, phagocytosis of foreign body particles and participating in immune response (56, 57). In this study, the THC in *C. argus* increased at first and then decreased with the increase in dietary Trp level, and there was a significant linear and quadratic relationship. Studies have shown that changes of THC are related to the health status of spleen, liver and other hematopoietic organs (58). This phenomenon was also observed in this study, when inclusion of 3.9-4.8 g/kg Trp in the diets, the SI of *C. argus* showed an upward trend, accompanied by the highest THC. In aquatic animals, antioxidant systems served as the first line of defense against oxidative damage (59). SOD, CAT, T-AOC are commonly used to evaluate the antioxidant capacity and immune response of aquatic animals (11, 32, 59, 60). The present study showed that the activities of SOD and T-AOC in blood were significantly increased when fish fed with 3.9-4.8 g/kg Trp. These results are similar to the findings in the liver of pigs (61), intestine of young grass carp (32). Meanwhile, MDA levels in tissues can be used to estimate lipid peroxidation (62). In this study, the MDA contents in the blood of *C. argus* were significantly decreased with dietary 3.9-4.8 g/kg Trp. Similar results also have been observed in hybrid catfish (*Pelteobagrus vachelli*♀ ×*Leiocassis longirostris*♂) (11). In this study, the activity of CAT in blood was highest in fish feed with 3.0 g/kg Trp diet. However, dietary 4.0 g/kg Trp significantly increased the activity of CAT in juvenile blunt snout bream (22). The reason may be caused by different kinds of fish. These findings suggested that dietary 3.9 and 4.8 g/kg Trp can enhance the antioxidant capacity of *C. argus* by increasing of SOD and T-AOC activities and decreasing the contents of MDA in the blood.

Intestinal cytokines, such as interleukins (ILs) and tumor necrosis factors (TNFs), are important components of the fish mucosal immune system (63). Pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8 can promote the occurrence of inflammatory reactions (64, 65), and anti-inflammatory cytokines such as IL-22 can promote host immune defense against bacterial pathogens (66). In the present study, when dietary Trp reached 4.8 g/kg, the relative expressions of *tnf-α*, *il-1β*, *il-6* and *il-8* in intestine of *C. argus* decreased, whereas the relative expression of *il-22* increased significantly. Trp have been shown to have a similar effect in other study (67). The TOR signaling pathway plays a critical role in the immune system of monocytes (68). And the TOR signaling pathway may improve the innate immune system of fish and human by regulating the transcription of cytokines (69, 70). In the present study, we observed that the expressions of *tor*, *il-1β*, *il-6* and *il-8* were significantly increased when the dietary Trp was up to 3.9 g/kg, but the opposite results were observed when dietary Trp reached 4.8 g/kg. Meanwhile, recent studies have shown that dietary Trp may up-regulate anti-inflammatory factors and down-regulate pro-inflammatory factors of fish partly by regulating the transcription of TOR (5, 22). These results demonstrated that the optimum dietary Trp level could alleviate intestinal inflammation partly by down-regulating the expressions of *tnf-α*, *il-1β*, *il-6* and *il-8*, and up-regulating the expression of *il-22* in fish intestine *via* regulating the expression of *tor*. However, the underlying mechanism by which dietary Trp attenuates inflammatory responses through the TOR signaling pathway remains to be further studied.

Furthermore, the NF-κB translocates to the nucleus and upregulates the expression of genes linked with inflammation, cell survival, proliferation, invasion, and angiogenesis (71), and mediated the proinflammatory action of TOR (70). The increased expression of IKK complex (including IKKα, IKKβ and IKKγ) promotes the phosphorylation and degradation of IκBα, which in turn activates NF-κB, which suppresses the relative expression of anti-inflammatory cytokines and up-regulates the relative expression of pro-inflammatory cytokines (72). In the present study, the relative expression of *ikkβ* increased and the expression of *iκbα* decreased significantly with dietary Trp up to 5.9 g/kg, but there was no difference in *nf-κb*. These immune responses may be caused by multiple pathways regulating NF-κB (73), such as TLRs/MyD88/NF-κB signaling pathway. TLRs (including TLR2, TLR4 and TLR5) are transmembrane proteins that can recognize a variety of related molecules (such as lipopolysaccharide, sodium urate crystal, viral double-stranded RNA, etc.) and cause inflammatory immune response. TLRs can activate the MyD88-dependent pathways, thus activating NF-κB and resulting in the release of inflammatory mediators and cytokines (74, 75). In the present study, the relative expressions of *tlr2* and *tlr4* showed a certain positive correlation with that of *myd88* when dietary Trp 1.9-4.8 g/kg. At the same time, dietary 4.8 g/kg Trp inhibited the relative expression of *nf-κb*, which is consistent with the relative expressions of *tnf-α*, *il-1β*, *il-6* and *il-8*. Li et al. (52) also observed similar results in the kidney of juvenile blunt snout bream fed with 2.8 or 4.0 g/kg Trp. These results implied that the optimum dietary Trp may regulate inflammatory cytokines through the TLRs/MyD88/NF-κB signaling pathway.

In conclusion, the present study provided evidence that dietary 3.9-4.8 g/kg Trp could improve total hemocyte count, antioxidant

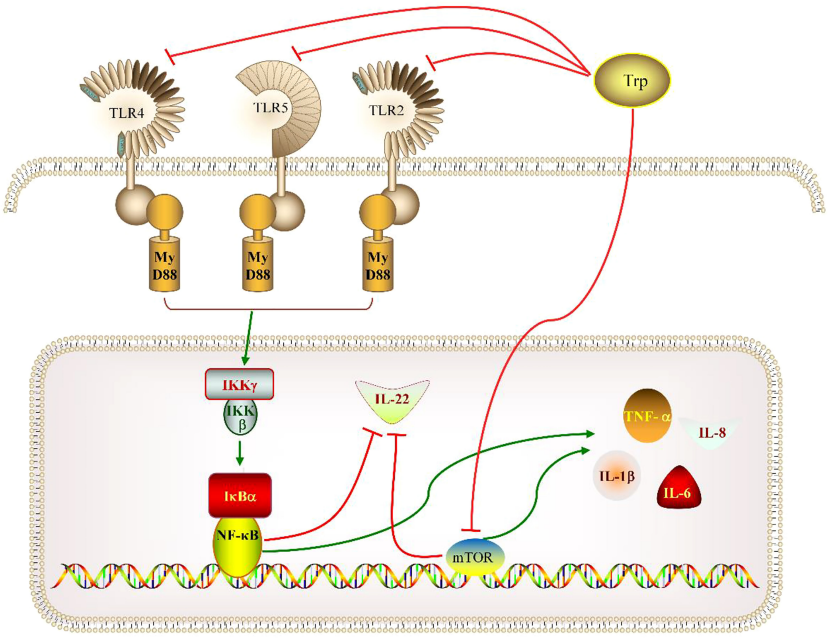

FIGURE 5
Possible mechanisms of the mechanisms of optimal dietary tryptophan promoted intestinal health *via* TOR and TLRs/MyD88/NF-κB signaling pathways.

enzyme activity in blood, and decrease the content of MDA in blood, but have no effect on the development of liver, spleen and head-kidney. Furthermore, dietary 4.8 g/kg Trp could alleviate intestinal inflammation partly by down-regulating the expression of *tnf-α*, *il-1β*, *il-6* and *il-8* and up-regulating the expression of *il-22* in fish intestine *via* the TOR and TLRs/MyD88/NF-κB signaling pathways (Figure 5).

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Care Advisory Committee of Yangzhou University.

Author contributions

XZ: data curation and writing-original draft. AW: writing-reviewing and editing. EC: software. BH: investigation and formal analysis. JX and YF: formal analysis. XD: writing-reviewing and editing. SM: conceptualization, methodology and funding acquisition. All authors contributed to the article and approved the submitted version.

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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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EDITED BY
Isabelle Mack,
University of Tübingen, Germany

REVIEWED BY
Imad Al Kassaa,
Fonterra (New Zealand), New Zealand
Zhaocheng Ma,
Huazhong Agricultural University, China

*CORRESPONDENCE
Meng-Liang Tian
secondat@sicau.edu.cn

†These authors have contributed equally to this work

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Polysaccharide from aerial part of *Chuanminshen violaceum* alleviates oxidative stress and inflammatory response in aging mice through modulating intestinal microbiota

Yuan-Feng Zou[1,2†], Xiao-Ping JiZe[1†], Cen-Yu Li[1], Chao-Wen Zhang[1], Yu-Ping Fu[1], Zhong-Qiong Yin[1], Yang-Ping Li[3], Xu Song[1], Li-Xia Li[1], Xing-Hong Zhao[1], Bin Feng[4], Chao Huang[2], Gang Ye[1], Hua-Qiao Tang[1], Ning-Yuan Li[1], Ji Chen[5], Xing-Fu Chen[5] and Meng-Liang Tian[5*]

[1]Natural Medicine Research Center, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China, [2]Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China, [3]State Key Laboratory of Crop Gene Exploration and Utilization in Southwest China, Chengdu, China College of Agronomy, Sichuan Agricultural University, Chengdu, China, [4]Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, China, [5]College of Agronomy, Sichuan Agricultural University, Chengdu, China

Aging is a biological process of progressive deterioration of physiological functions, which poses a serious threat to individual health and a heavy burden on public health systems. As population aging continues, research into anti-aging drugs that prolong life and improve health is of particular importance. In this study, the polysaccharide from stems and leaves of *Chuanminshen violaceum* was obtained with water extraction and alcohol precipitation, and then separated and purified with DEAE anion exchange chromatography and gel filtration to obtain CVP-AP-I. We gavaged natural aging mice with CVP-AP-I and performed serum biochemical analysis, histological staining, quantitative real-time PCR (qRT-PCR) and ELISA kit assays to analyze inflammation and oxidative stress-related gene and protein expression in tissues, and 16SrRNA to analyze intestinal flora. We found that CVP-AP-I significantly improved oxidative stress and inflammatory responses of the intestine and liver, restored the intestinal immune barrier, and balanced the dysbiosis of intestinal flora. In addition, we revealed the potential mechanism behind CVP-AP-I to improve intestinal and liver function by regulating intestinal flora balance and repairing the intestinal immune barrier to regulate the intestinal-liver axis. Our results indicated that *C. violaceum* polysaccharides possessed favorable antioxidant, anti-inflammatory and potentially anti-aging effects *in vivo*.

KEYWORDS

Chuanminshen violaceum, polysaccharide, antioxidation, antiinflammatory, gut-liver axis, gut microbiota

1 Introduction

Aging is a process of deterioration of the body's tissues and organs over time, with a decline in the function of all systems, eventually leading to cellular and even individual death. With age-related diseases such as Alzheimer's disease, Parkinson's disease, cardiovascular disease as well as cancer increasing exponentially worldwide in recent years (1–3), there is a constant desire to slow or even reverse aging. Aging is a multifaceted biological process resulted from the combined effect of many factors, and the most widely accepted theory of aging is the free radical theory of aging (4). The free radical theory suggests that increased oxidative stress is an important driver of aging. Under normal physiological conditions, redox levels remain in balance. As we age, the body produces more free radicals (mainly ROS), but the reduced activity of enzymes involved in scavenging free radicals leads to oxidative stress damage (5, 6) and chronic inflammation (7, 8), thus exhibiting the characteristics of aging. One of the main features of aging is the decline in intestinal digestive functions, manifested by disturbances in the intestinal flora, atrophy of the intestinal mucosa and destruction of intestinal immune function (9, 10). This intestinal dysfunction allows microorganisms (or derived metabolites) and toxins (LPS) to enter the circulation and reach the liver, causing or worsening a range of liver diseases (11, 12). Similarly, aging-induced decreases in the metabolic and detoxification functions of the liver affect the intestinal tract through bile acids (13, 14). This bidirectional relationship between the gut and liver *via* the portal vein, known as the gut-liver axis, was first proposed in 1988 and has attracted widespread attention (15). Accumulated studies show that liver and intestinal diseases can be treated through the gut-liver axis theory (16, 17), which shows that the gut-liver axis balance is so important for a healthy body. The gut microbes, which are part of this two-way communication between the gut and liver, are essential for maintaining the homeostasis of the gut-liver axis (18, 19). In recent years, there has been an increasing amount of research into the use of microbiota-targeted dietary and probiotic interventions and novel therapeutic applications such as faecal microbiota transplantation in the prevention and treatment of ageing-related diseases (20). Therefore, screening and identifying potential therapeutic molecules that balance the gut microflora against aging is of great significance.

Chuanminshen violaceum Sheh et Shan (CVSS), mainly distributed in Sichuan and Hubei provinces, traditionally used as a medicine from the roots, it is both a bulk Chinese herb and a premium nutritional health product, mostly used as a fitness tonic and as an edible ingredient in folklore for hundreds of years (21). Natural polysaccharides have attracted great interest due to their wide range of sources, low toxicity, few side effects and diverse biological activities (22). Pharmacological studies have shown that polysaccharides are the main active ingredients of CVSS and have various activities such as antioxidant (23), immunomodulatory (24) and antiviral (25). In the industrial production of traditional Chinese medicine, non-medicinal parts, such as stems and leaves of traditional root-based medicines, are generally discarded, resulting in a waste of resources and environmental pollution. These plant parts may be suitable plant resources for the production of phytochemicals (26). Numerous studies have shown that non-medicinal parts of traditional Chinese herbs have the same biological activity as their medicinal counterparts (27, 28). Therefore, in this study, we aimed to evaluate whether CVSS polysaccharide can alleviate oxidative stress injury and inflammatory response in the gut and liver of naturally aging mice, and to investigate the potential function of CVSS polysaccharide in the gut-liver axis and their correlation with gut microbiota to provide evidence for the further development and utilization of CVSS.

2 Materials and methods

2.1 Materials

The stems and leaves of *Chuanminshen violaceum* were collected from Bazhong City, Sichuan Province, in April 2022 and identified by Dr Yuan-Feng Zou, College of Veterinary Medicine, Sichuan Agricultural University. The fresh stem leaves are treated in an oven at 105°C for 15 minutes to destroy and blunt the oxidase activity in the leaves by high temperature, inhibit the enzymatic oxidation of tea polyphenols etc. in the fresh leaves and evaporate some of the water in the fresh leaves, then dried at 55°C to constant weight and sheared into flakes then dried at 55°C to constant weight and sheared into pieces

2.2 Isolation, chemical composition, monosaccharide composition and average-molecular weight of CVSS polysaccharides

The extraction of polysaccharides was carried out based on the previous method (29). Briefly, the dried stems and leaves of *Chuanminshen violaceum* (100g) were pre-treated by 90% ethanol (v/v) to remove impurities until colorless, then the dried residues were treated with boiling water under the following conditions: 100° C, 2 h, solid–liquid ratio 1:20, 2 times. The extract was extracted by filtration, alcohol precipitation, dialysis (cut off 3500 Da) and loaded to a lyophilizer (LGJ-10G, Beijing Sihuan Qihang technology Co., Ltd., Beijing, China) to obtain CVSS crude polysaccharide (CVP) powder. For further purification, CVP (400 mg) was dissolved in distilled water (20 mL), centrifuged, filtrated (0.45 μm) and applied into an anion-exchange column packed with DEAE- Sepharose Fast Flow (50 mm × 40 cm; Beijing Rui Da Heng Hui Science Technology Development Co., Ltd, Beijing, China). The neutral and acidic fractions were eluted with 2 L distilled water followed by 0–1.5 mol/L NaCl solution at a flow rate of 2 mL/min, respectively. The elution profiles of acidic fractions were monitored by the phenol–sulfuric acid method (30), and the acid polysaccharide fraction was collected according to the elution profiles, followed by dialysis (cut off 3500 Da) to remove NaCl, concentrating and freeze-drying, named as CVP-AP. Finally, CVP-AP was further purified by gel filtration (Sepharose 6FF, 2.5 cm ×

100 cm, Beijing Rui Da Heng Hui Science Technology Development Co. Ltd) to obtain a purified fraction (31), named CVP-AP-1, because it was more purified than CVP-AP, CVP-AP-I was collected and lyophilized for subsequent studies.

The total carbohydrate content of CVP-AP-I was determined by the phenol sulfuric acid method (30), the total amounts of phenolic compounds and proteins of CVP-AP-I were determined by Folin-Ciocalteu (32) and Bio-Rad protein assay (33). Referring to previous research methods (34), the monosaccharide composition of CVP-AP-I was determined by HPLC (Agilent Technologies, 1260 Infinity II, USA). Briefly, CVP-AP-I was dissolved in TFA (trifluoroacetic acid) and then hydrolyzed at 100°C for 8 h. After repeated evaporation and concentration with methanol to remove excess TFA, the sample was then derivatized with NaOH and PMP (1-phenyl-3-methyl-5-pyrazolone) -methanol at 70°C for 100 min, and finally the pH was adjusted to neutral and extracted with chloroform to obtain the sample derivative. the standard monosaccharide mix including mannose (Man), ribose (Rib), Rhamnose (Rha), glucuronic acid (GlcA), galacturonic acid (GalA), glucose (Glc), galacturonic (Gal), xylose (Xyl) and arabinose (Ara) were derivatized at the same manner. All derivatives were chromatographed on HPLC equipped with C18 column (250 nm ×4.6 mm, 5 μm), with following settings: mobile phase: 0.05 mol/L phosphate buffer (NaH2PO4–Na2HPO4, pH6.8)-acetonitrile (82:18, V/V); flow rate: 1.0 mL/min; column temperature 30°C; detection wavelength of DAD was 250 nm; injection volume: 20 μL.

The molecular weight (Mw) of CVP-AP-I was determined by gel permeation chromatography method (Waters 515 liquid chromatography pump, Waters 2410 differential detector (Refractive Index), Waters Ultrahyrdogel Linear gel column (300 × 7.8 mm) (34, 35). The Mw control dextran (2.5–5348 Da) and CVP-AP-I were dissolved in the mobile phase. Sample solutions were injected after the system was stable, and the resulting chromatogram was recorded. The weight-average molecular weight (Mw), number-average molecular weight (Mn) and their distribution equivalents were obtained by the standard curve. The chromatographic conditions are as follows: the mobile phase was 0.2 mol/L NaNO3 solution, pH = 6.0; flow rate was 0.6 mL/min; the column temperature: 40°C; the injection volume was 20 μL.

2.3 Animal care and experimental design

All animal studies were conducted in accordance with the Animal Care and Use Committee guidelines of Sichuan Agricultural University. Thirty male specific-pathogen-free C57BL/6 mice (48 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing China), and housed in a pathogen free environment and free access to standard chow and water for 7 days, with an automatically controlled 12 h light/dark cycle, the temperature was 25 ± 2°C and the humidity was 60%. After the acclimatization period, as shown in Figure 1, all mice were randomly divided into three groups (10 mice in each group), and received a daily gavage of solution (same volume of saline), 10 mg/kg polysaccharide, or 20 mg/kg polysaccharide group for 14 consecutive days. Food intake was recorded daily and body weight was measured every 2 days. At the end of the experiment, all mice were fasted overnight, blood was collected from the eyeballs the next day. Finally, they were euthanized and dissected quickly, then the main organs of liver, intestine tissue, and cecum contents were collected rapidly. All tissue samples were divided into two parts, one was frozen in liquid nitrogen, and then stored at -80 °C for gene expression analysis and biochemical analysis, the other one was fixed in 4% paraformaldehyde for subsequent histopathological examination.

2.3.1 Blood biochemical test

The biochemical indexes of serum, including alanine aminotransferase (ALT), aspartic aminotransferase (AST), triglyceride (TG) and total cholesterol (TC), were detected by automatic hematology analyzer (Shenzhen Icubio Biomedical Technology Co., Ltd.).

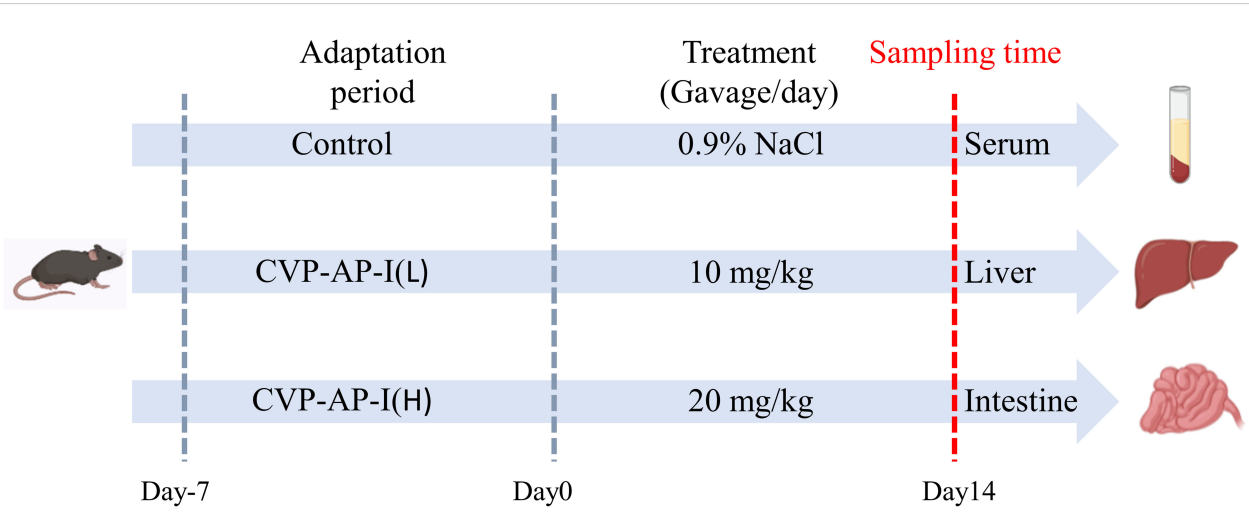

FIGURE 1
Schematic overview of experimental design.

2.3.2 Histological staining

The duodenum, jejunum, ileum and liver tissue fixed in 4% paraformaldehyde solution were cut into appropriate size. The tissues were embedded in paraffin and cut into 5um sections, then stained with hematoxylin and eosin according to the manufacturer's instructions (H&E, G1260, Solarbio, Beijing, China). Brightfield images at 200 × magnification were captured on Nikon Eclipse Ti microscope (Melville, NY, USA).

2.3.3 Quantitative realtime PCR

The intestine (duodenum, jejunum, ileum) and liver tissues were ground under liquid nitrogen. The total RNA was extracted with TRIzol reagent (Biomed, Beijing, China) from the tissue powder, then the RNA (5 μg/μL) was reverse transcribed into cDNA using M-MLV 4 First-Strand cDNA Synthesis Kit (Biomed, RA101-12, China) and further applied to qRT-PCR in a step Oneplus System (Biosystems, Foster City, CA, USA) using determine double-stranded DNA. The procedure conditions were one cycle of 30s at 95°C for pre-denaturation, 40 cycles of 95°C (10 s) and optimum annealing temperature (30s) for denaturation and annealing/extension, respectively. The gene expression levels were calculated according to the regulative relevant quantification method followed by the ΔΔCT method (34). The relative gene expression was normalized to internal control as β-Actin. The primer sequences (Tsingke Biotechnology Co., Ltd, China) for genes expression detection in this study are listed in Table 1.

2.3.4 Determination of oxidative stress indicators, inflammatory factors, LPS and sIgA

The concentrations of inflammatory factors (IL-6, IL-1β, TNF-α) and LPS in serum, liver and jejunum, and sIgA in jejunum were determined by ELISA kits according to the manufacturer's instructions (MlBio, Shanghai, China). The levels of SOD (superoxide dismutase), CAT (catalase), GPX (glutathione peroxidase) and MDA (malondialdehyde) in serum, jejunum and liver tissues, as well as ROS (reactive oxygen species) and T-AOC (total antioxidant capacity) levels in tissues were analyzed with biochemical kits according to the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China). the total protein concentration was assessed according to the BCA assay kit (Beyotime, Shanghai, China).

2.3.5 Gut microbiota analysis

Fresh digesta isolated from the cecum was quickly sent to Novogene Technology Co., Ltd. (Beijing, China), under dry ice conditions for gut microbiota analysis. The DNA was extracted using CTAB/SDS (cetyltrimethylammonium bromide/sodium dodecyl sulfate) Bromide method, and diluted to 1 ng/μL using sterile water. The V4 region of 16S rRNA was amplified by PCR using specific primers (515F: CCTAYGGGRBGCASCAG; 806R: GGACTACNNGGGTATCTAAT), followed by mixing and purification of PCR products, and library construction using TruSeq® DNA PCR-Free Sample Preparation Kit. The libraries were quantified by Qubit and Q-PCR, and then sequenced using NovaSeq 6000.

TABLE 1 primer sequences for qRT-PCR.

Gene	Primer sequence 5′ to 3′	PubMed no.	bp
β-actin	F: TCACGGTTGGCCTTAGGGTTC R: CGCTCGTTGCCAATAGTG	NM_001259638.1	71
IL-1β	F: CCTGTGTTTTCCTCCTTGCCT R: AGTGCGGGCTATGACCAATTC	NM_008361.4	158
TNF-α	F:CTCTTCTCATTCCTGCTCGT R: ACCCCGAAGTTCAGTAGACA	NM_012675.3	62
IL-6	F: AAATATGAGACTGGGGATGTC R: TCAGTCCCAAGAAGGCAAC	NM_001314054	90
CAT	F: ACCAGATACTCCAAGGCAAA R: TAAAATTTCACTGCAAACCCC	NM_009804.2	137
SOD1	F: GAACCATCCACTTCGAGCAG R: ATCACACGATCTTCAATGGAC	NM_011434.2	265
GPX	F: TGCTTGCCTCCTAAATGCTG R: CCCAGAATGACCAAGCCAA	NM_001329860.1	81
Nrf2	F: AACCTCCCTGTTGATGACTTC R: CTGTCGTTTTCTCCCTTTTCTC	NM_001399226.1	76
Muc2	F: TCATCAACCTTCACTACCCCA R: TTTTGCACACTAACCCAAC	NM_023566.4	247
ZO-1	F: TCGATCAAATCATTACGACCCT R: GCTCTCAAAACTTCTTCGGTCAA	NM_001352638.1	55
Occludin	F: TTGAAAGTCCACCTCCTTACAGA R: CCGGATAAAAAGAGTACGCTGG	NM_001360536.1	129

2.4 Statistical analysis

Data represent the mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). One-way ANOVA with LSD *post-hoc* test was performed by using SPSS 27.0 software. The p-value of 0.05 or less was considered with statistically significance.

3 Results

3.1 Isolation, chemical composition, monosaccharide composition and average-molecular weight of CVSS polysaccharide

Crude polysaccharide (CVP) was obtained from the stems and leaves of CVSS by water extraction and alcohol precipitation, with a yield of 5.06%. There are no previous reports related to polysaccharides from the stems and leaves of CVSS, but the extraction rates of crude polysaccharides from CVSS leaves were reported to be in the range of 4.73%-5.41% (36), which were similar to our extraction rates. CVP was separated by DEAE anion exchange chromatography to obtain an acidic component CVP-AP (Figure 2A), but the neutral component isolation was less than 1%, so it was not included in the follow-up study. CVP-AP was further purified by gel filtration to obtain CVP-AP-I with a yield of 81.93% (Figure 2B). The monosaccharide composition of CVP-AP-I was determined by HPLC and the results (Figure 2C) showed that the monosaccharides present in CVP-AP-I were mainly galacturonic acid (GalA), galactose (Gal), arabinose (Ara), rhamnose (Rha), and a small amount of mannose (Man). The total carbohydrate content of the CVP-AP-I was 89.91%, the total protein was 4.51%, and the total polyphenol content was 0.98% (Figure 2C). Compared with the reported polysaccharides from the roots and leaves of CVSS, the monosaccharide types and molar ratios of CVP-AP-I were different (36, 37). The weight-average molecular weight (Mw) of CVP-AP-I was 118.2 kDa, as determined by gel permeation chromatography, and a single symmetric peak was observed, as shown in Figure 2D.

3.2 CVP-AP-I protects naturally aging mice from oxidative stress and inflammation

As we all know, aging is the inevitable process of life. It is characterized by progressive irreversible degenerative changes in the structure and function of the organs with age, under the influence of multiple factors, accompanied by oxidative stress and inflammatory reactions (7, 8, 38, 39). Therefore, drugs that reduce oxidative damage are the first choice for anti-aging. A mouse's life cycle is typically 18 ~ 24 months, with aging-related biomarkers slowly appearing in mice from the 10th month onwards. The mice used in the experiments were nearly 12 months of age, when most of the biomarkers had already started to show changes. Therefore, we used naturally aging mice as a model to study the roles of the CVP-AP-I in the natural aging process of the organism. After two weeks of polysaccharide intragastric administration, the body weight of mice in the polysaccharide group was greater than that in the control group (Figure 3A). Finally, there was a significant increase in body weight in the 20mg/kg polysaccharide group, and no difference in food intake was observed between the three groups. In addition, in the aging process, the activities of liver function

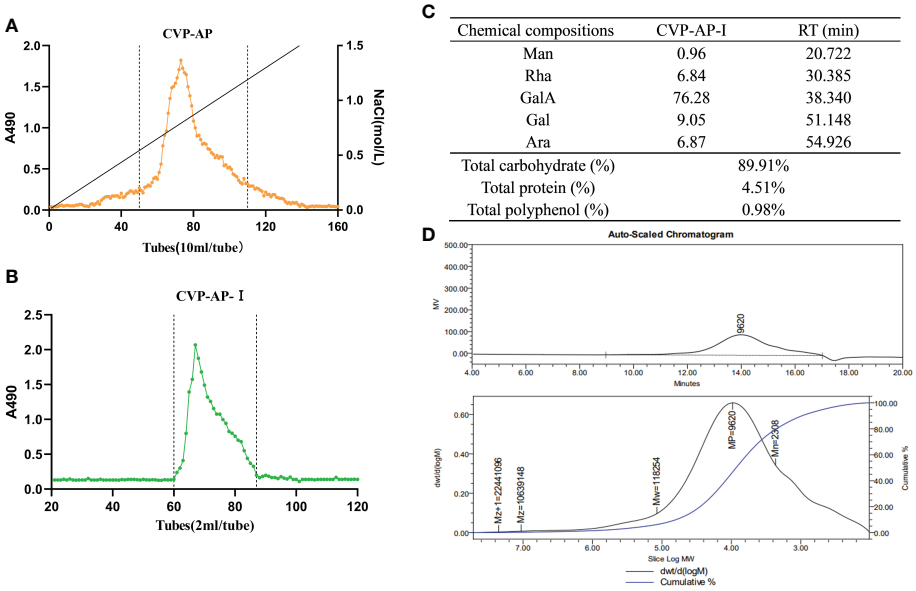

FIGURE 2
Isolation, chemical composition, monosaccharide composition and average-molecular weight of CVSS polysaccharides. **(A)** The elution curve of CVP-AP on DEAE anion exchange chromatography. A single component is obtained. **(B)** CVP-AP-I elution profile was obtained after gel filtration purification. **(C)** The chemical compositions of CVP-AP-I including monosaccharide composition (mol %), total carbohydrate (%), protein content (%), polyphenol content (%). **(D)** The molecular weight determination of CVSS polysaccharide by gel permeation chromatography.

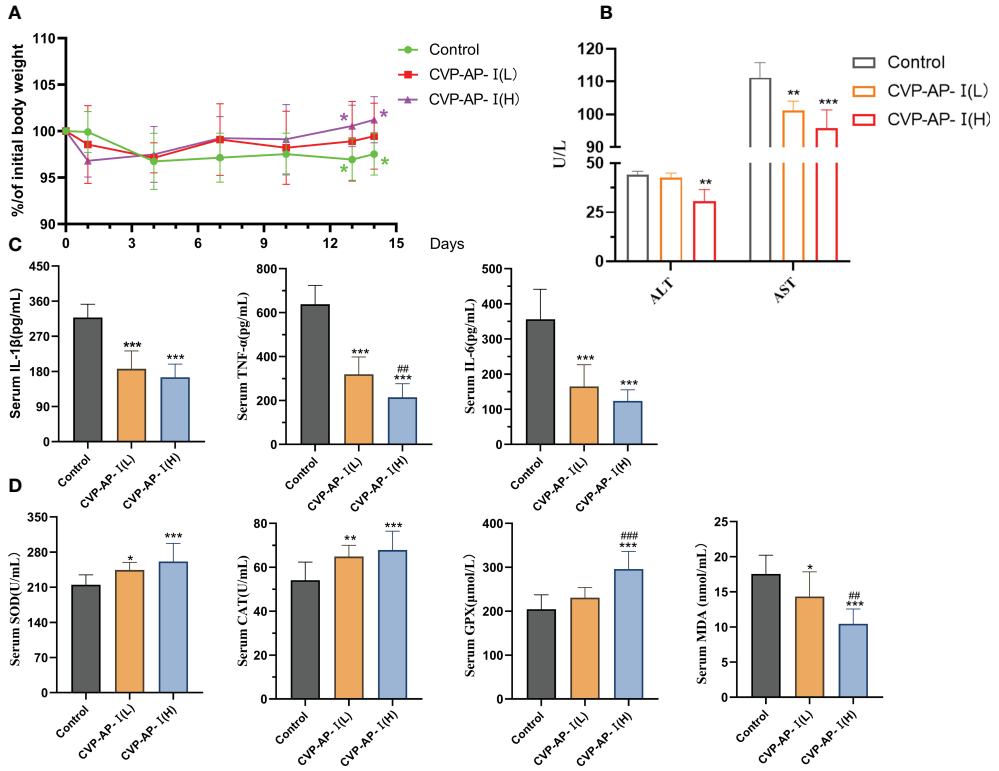

FIGURE 3
Effects of CVP-AP-I on body weight and serum levels of ALT, AST, pro-inflammatory factors (IL-6, IL-1β, TNF-α), antioxidant enzymes (SOD, CAT, GPX) and MDA in aging mice. **(A)** Changes in body weight of mice (Relative to initial body weight; n=10); **(B)** Quantification shows the serum levels of ALT and AST in the mice of different groups (n=4); **(C)** Quantification shows the inflammatory factor IL-1, TNF-α, IL-6 level in the serum from different groups mice(n=6). **(D)** Quantification shows the activity of the antioxidant enzymes SOD, CAT and GPX and the level of MDA in the serum of the mice in the different groups (n=6). All data are represented as means ± SD.*p<0.05, **p<0.01, and ***p<0.001 as compared with Control Group. ##p<0.01 and ###p<0.001 as compared with CVP-AP-I (L) group.

indicators ALT and AST will continue to rise with the decline of liver function (40). We quantified the activities of ALT and AST in serum, and found that the activities of ALT and AST significantly decreased in polysaccharide group, especially in CVP-AP-I(H) group (Figure 3B). In order to further illustrate the effect of CVSS polysaccharides on aging mice, the serum indicators of inflammation and oxidative stress were quantitatively analyzed. Compared with the aging group, the levels of inflammatory factors IL-1β, TNF-α and IL-6 (Figure 3C) and MDA in serum (Figure 3D), significantly decreased in the polysaccharide treatment group, while the activities of antioxidant enzymes SOD, CAT and GPX in serum significantly increased (Figure 3D). Previous studies on anti-aging drugs mainly focused on their antioxidant activity, such as astragalus polysaccharide inhibiting D-galactose-induced oxidative stress in mice by upregulating antioxidant factors (41). Inflammation induced by oxidative stress is also another characteristic change of aging. CVSS polysaccharide not only up-regulated serum levels of antioxidant factors but also down-regulated levels of inflammatory factors. All these data indicated that different doses of CVSS polysaccharide interventions in naturally aging mice exhibited great antioxidant activity and anti-inflammatory activity, and may had some ameliorative effects on liver function.

3.3 CVP-AP-I attenuates oxidative stress and inflammation in the gut and liver of naturally aging mice

Systemic chronic inflammatory responses and oxidative stress are the main features of aging, with a decline in intestinal digestive function, which in turn affects the function of other organ tissues (42). Although, CVP-AP-I could significantly reduce the levels of serum inflammatory factors and liver function indicators ALT and AST, and significantly increase the activity of serum antioxidant enzymes. However, polysaccharides as macromolecules may mainly relay on its indirect effect to liver and the intestine, as reported by our group and other researchers (43, 44). Therefore, we further evaluated the effect of CVP-AP-I on antioxidant and anti-inflammatory capacity of gut and liver in naturally aging mice. We observed a decrease trend in the expression of pro-inflammatory genes (IL-1β, TNF-α and IL-6) and an increase trend in the gene expression of antioxidant enzyme (SOD, CAT and GPX) in the liver, duodenum, jejunum and ileum of aging mice in the polysaccharide-treated groups (Figures 4A, B). Nrf2, an important transcription factor that widely exists in various organs of the body, is considered to be a key transcription factor in regulating cells against xenobiotics and oxidative damage (45, 46);

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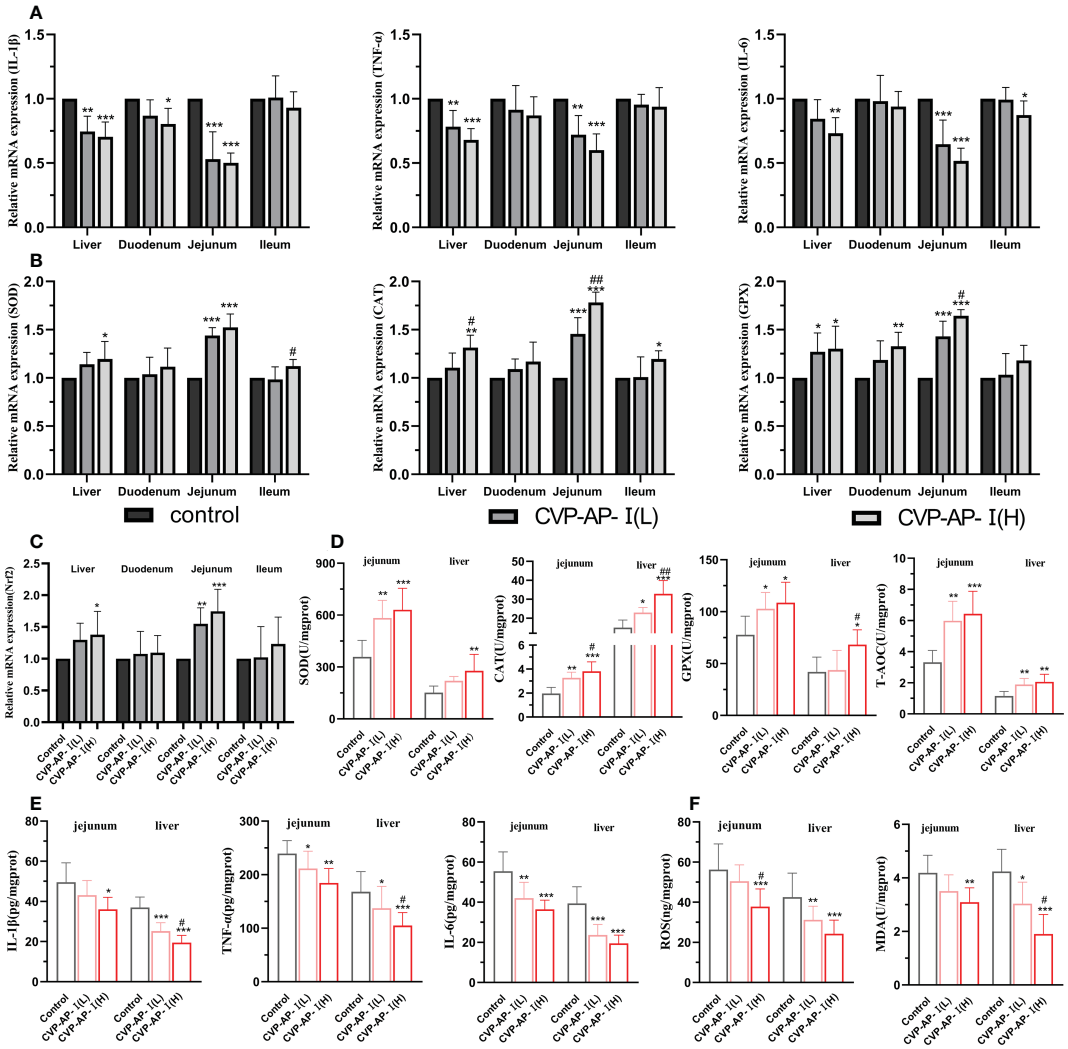

FIGURE 4
Effects of CVP-AP-I on antioxidant and anti-inflammatory capacity of intestine and liver in naturally aging mice. **(A)** qRT-PCR results show the relative expression levels of inflammatory factors IL-1β, TNF-α and IL-6 genes in liver and intestinal tissues (duodenum, jejunum and ileum) of different groups of mice (n=5); **(B)** qRT-PCR results show the relative expression levels of the antioxidant enzymes SOD, CAT and GPX genes in liver and intestinal tissues (duodenum, jejunum and ileum) of different groups of mice (n=5); **(C)** qRT-PCR results show the relative expression levels of transcription factor Nrf2 gene in liver and intestinal tissues (duodenum, jejunum and ileum) of different groups of mice (n=5); **(D)** Quantitative results show the protein expression levels of antioxidant enzymes SOD, CAT, GPX and T-AOC in jejunum and liver of mice in different groups (n=6); **(E)** Quantitative results show the protein expression levels of inflammatory factors IL-1β, TNF and IL-6 in jejunum and liver of mice in different groups (n=6); **(F)** Quantitative results show the protein expression of ROS and MDA in jejunum and liver of mice in each group (n=6); All data are represented as means ± SD. *p<0.05, **p<0.01, and ***p<0.001 as compared with Control Group. #p<0.05 and ##p<0.01 as compared with CVP-AP-I (L) group.

and on the other hand, scholars have found that Nrf2 also plays a key role in inflammatory repair (47, 48). Polysaccharides have been shown to reduce oxidative stress and inflammatory responses by activating Nrf2 (49, 50). We found that the relative expression of the Nrf2 gene was significantly higher in the jejunum and liver of the CVSS polysaccharide group (Figure 4C), and that the increased expression of antioxidant enzymes and reduced pro-inflammatory factors could be associated with the increase in Nrf2. Although there was a tendency for the polysaccharide-treated groups to alleviate aging-induced oxidative stress and inflammation in the duodenum and ileum, the effect was not significant, so we speculated that the site of utilization of CVSS polysaccharides in the small intestine was primarily in the jejunum. To further illustrate the anti-inflammatory and antioxidant effects of CVP-AP-I, we then quantified the protein levels of inflammatory factors and antioxidant enzymes in jejunum and liver. As shown in (Figures 4D, E), at the protein level, compared with the control group, CVP-AP-I significantly increased the activities of antioxidant enzymes (SOD, CAT, GPX) and T-AOC in jejunum and liver, and significantly decreased proinflammatory factors (IL-1 β, TNF- α, IL-6) levels, which was consistent with the qRT-PCR results. Finally, we quantified ROS and MDA protein levels in the jejunum and liver. Specific increases of ROS level have been demonstrated as potentially critical for induction and maintenance of cell senescence process (51, 52). And the MDA content is an important parameter reflecting the body's potential

antioxidant capacity, which can amplify the effect of reactive oxygen species and can reflect the rate and intensity of lipid peroxidation in the body, as well as indirectly reflecting the degree of tissue peroxidative damage (53). Additionally, we found that ROS and MDA were significantly lower in the jejunum and liver of the polysaccharide-treated group compared to the control group, and showed a polysaccharide concentration dependence (Figure 4F). These data collectively show the benefits of the CVSS polysaccharide in improving oxidative stress and inflammatory responses in the gut-liver axis of aging mice.

3.4 CVP-AP-I ameliorates liver and intestinal tissue defects and repairs the intestinal immune barrier of naturally aging mice

Our experimental results show that CVP-AP-I not only alleviates intestinal inflammation and oxidative stress, but also has a similar effect on the liver. While the target organ of polysaccharides is the intestine, the intestinal microbiota, intestinal immune response and intestinal permeability are also related to the function of polysaccharides (54). The intestinal tract has been considered an important target organ mediating the body's lifespan extension due to its immune and nutritional intake functions. The decline of digestive and absorption functions in the elderly is caused by histological degeneration of the gastrointestinal tract (55), so we speculate that the protective effects of CVP-AP-I on the gut and liver are mediated through the gut-liver axis. To confirm this speculation at the histopathological level, we first performed H.E staining of the liver and intestine. Subsequently, we found that the livers of aging control mice were structurally disorganized and showed aging features, with inflammatory necrosis of hepatocytes, multinucleated cells and mild steatosis. In contrast, the hepatocytes of the two groups supplemented with CVSS polysaccharide, especially the high-dose group, were neatly arranged in a cord-like arrangement with clear hepatic sinusoidal structures (Figure 5A). In many previous studies, triglyceride (TG) and cholesterol (TC) metabolism disorders and accumulation have been reported to be closely related to aging (56, 57). For example, increased ROS during aging has been reported to play an important role in the accumulation of cholesterol in the liver by increasing cholesterol and glucose uptake to increase cholesterol synthesis (58). Given the previous improvement in hepatocyte steatosis by CVP-AP-I, we subsequently quantified TG and TC in serum, and found that the CVP-AP-I(H) group similarly reduced serum levels of TG and TC (Figure 5E), which was consistent with the results of hepatic H.E staining. In the intestinal tissues, we noted significant histopathological structural defects in the duodenum, jejunum and ileum of the control mice, including fewer and wide villus and reduced number and depth of crypt. Unsurprisingly, we found that the histopathological structure of the intestine (duodenum, jejunum and ileum) of mice supplemented with polysaccharides was more normal, especially in the jejunum of the CVP-AP-I(H) group (Figures 5B–D, F–H).

The small intestinal epithelium is composed of a single layer of cells, associated with each other by tight junctions, and a mucus layer that protects the intestinal epithelial cells surface (59). In physiological situations, it carries out the exchange and transfer of nutrients and acts as a physical barrier, together with intestinal microorganisms and the host immune system, to maintain intestinal homeostasis (60). Therefore, tight junctions mediated by the integral membrane proteins, peripheral membrane proteins and mucoprotein are essential for the function of the intestinal epithelial barrier, and their defects lead to impaired intestinal permeability and possible translocation of toxic compounds or pathogenic bacteria within the lumen, causing further local or systemic inflammation and many human diseases (61, 62). Intestinal barrier function decreases with age (63, 64), with qRT-PCR results we found that the gene expression of integral membrane protein (Occludin), peripheral membrane protein (ZO-1), and mucoprotein (Muc2) were all elevated in the small intestine of mice in the polysaccharide consumption group (Figure 5I), indicating that CVP-AP-I had an ameliorative effect on intestinal permeability in aging mice. Consistent with this, we detected that CVP-AP-I decreased LPS content in serum and liver (Figure 5J), and promoted the production of sIgA (Figure 5K), a basic and essential enteroendocrine immune antibody. These results suggest that CVSS polysaccharides can improve intestinal and hepatic structural defects and promote the development of the intestinal mucosal immune system.

3.5 CVP-AP-I regulates gut–liver axis through modulating gut microbiota

Intestinal microbial-host interactions are critical in the development and maintenance of body immunity (65). During aging, oxidative stress affects the living environment of intestinal microbes and disrupts the structure and function of intestinal flora, leading to the dysregulation of intestinal flora, and further exacerbates oxidative damage and lipid metabolism disorders (66), thereby aggravating the morbidity and mortality of various chronic diseases. Accumulated studies show that anti-aging can be achieved by regulating the structure and composition of intestinal microbiota (67, 68). As CVSS polysaccharides are not directly available to the organism as macromolecules, we evaluated whether the gut flora was its target and assessed the composition of the gut flora by sequencing the 16S rRNA V4 region of the gut bacteria. The previous data showed that the high-dose polysaccharide administration group had the best anti-aging effect, so the intestinal flora analysis was conducted only for the control group and the high-dose group (named CVP group). As shown in Figure 6A, we first calculated the sample Rarefaction Curves, where the gradual flattening of the curves indicated a reasonable amount of sequencing data and indirectly reflected differences in the abundance of species in different groups respectively (69). Venn diagram analysis revealed 3825 and 1709 OTUs each in the control and CVP groups, with their respective unique OTUs numbers being 2538 and 422, indicating differences in the structure of the gut microflora between the two groups (Figure 6B). Then, we calculated the Chao1 and Shannon indices (70) to assess the richness and diversity of the gut microbiota, and found that they both decreased in the CVP group and Chao1 significantly decreased (Figures 6C, D). To determine the differences in gut microbiota structure between the different groups, Unifrac

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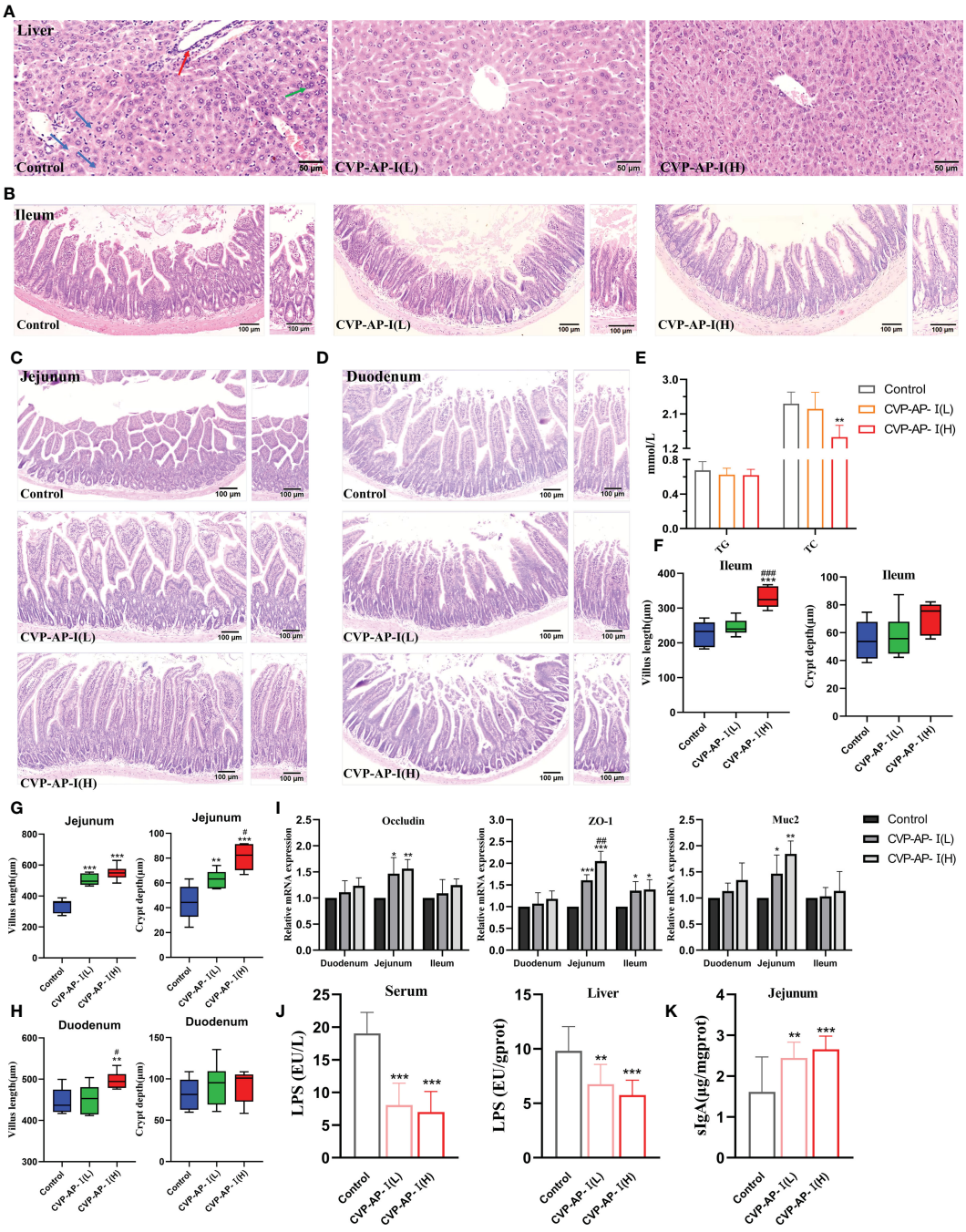

FIGURE 5
Effects of CVP-AP-I on liver and intestinal histology and intestinal immune barrier in naturally aging mice. **(A)** Representative images of H.E staining show that CVP-AP-I improves age-induced liver lesions. ("→" Inflammatory cell necrosis of the liver; "→" Multinucleated hepatocytes; "→" Mild steatosis of hepatocytes); **(B-D)** Representative images of H.E staining show that CVP-AP-I reverses aging-induced villus and crypt defects in the ileum, jejunum and duodenum; **(E)** Quantification shows the levels of TG and TC in the serum of different groups of mice(n=4); **(F-H)** Quantitative show of CVP reversal of aging-induced intestinal villus and crypt defects in the ileum, jejunum and duodenum. Error bars indicate Mix to Max (n = 6); **(I)** qRT-PCR results show the expressions of Occludin, ZO-1 and Muc2 in duodenum, jejunum and ileum of mice from different groups (n=4); **(J)** Quantification shows LPS levels in the serum and liver of different groups of mice (n=4); **(K)** Quantification shows sIgA levels in the jejunum of different groups of mice (n=6). All data are expressed as mean ± SD. *p<0.05, **p<0.01, and ***p<0.001 as compared with Control Group. #p<0.05, ##p<0.01, and ###p<0.001 as compared with CVP-AP-I(L) group.

distance-based Principal Co-ordinate analysis (PCOA) (71), Bray-Curtis distance-based metric-free multidimensional calibration method analysis (NMDS) (72) and UPGMA cluster analysis (73) were performed. We found that the CVP group was completely separated from the control group in both the PCoA and NMDS analyses (Figures 6E, F), and the community composition of the samples was more similar. Similarly, in the UPGMA cluster tree analysis, we found that samples from the polysaccharide group

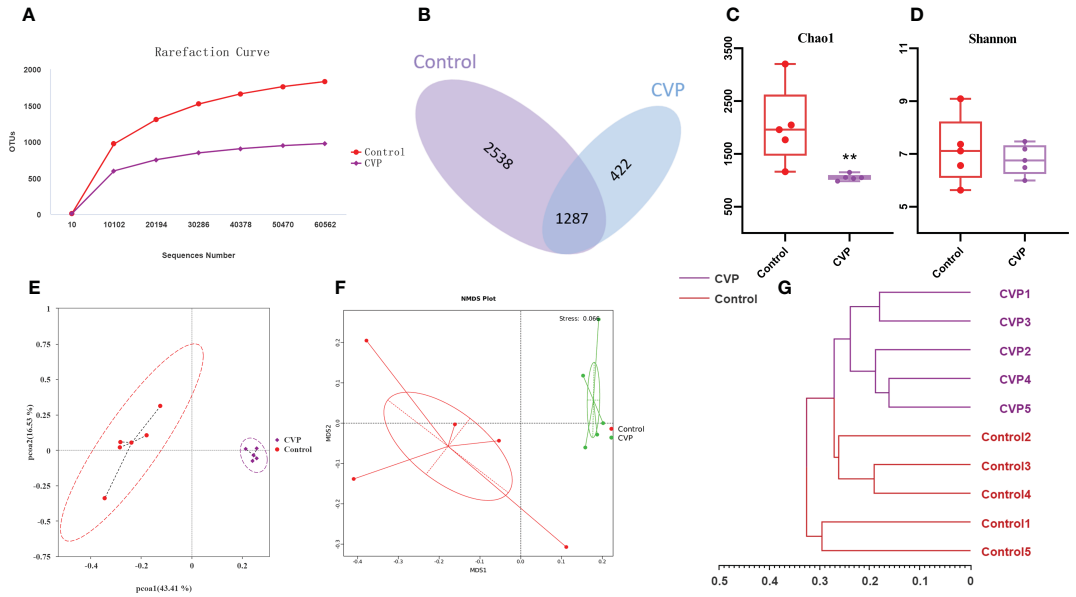

FIGURE 6
CVSS polysaccharides modulate the composition and structure of gut microflora. **(A)** OUT Rarefaction Curves of gut microbiota in different groups; **(B)** Venn diagram showing the unique and shared OTUs from different groups; **(C)** Bacterial community richness measured by Chao1 index in different groups; **(D)** Bacterial community diversity measured by Shannon index in different group; **(E)** Unweighted Unifrac Principal Coordinate Analysis by bacterial microbiota; **(F)** NMDS ordination based on Bray-Curtis similarities of bacterial communities; **(G)** UPGMA clustering tree based on unweighted Unifrac distances. "*" stands for the comparation with Control; **$p<0.01$ by one-way ANOVA.

tended to cluster together, while samples from the control group were scattered or clustered (Figure 6G).

Furthermore, we studied the taxonomic distribution of abundant bacteria at three levels: phylum, genus and species. At the phylum level (Figure 7A), the control group was mainly composed of *Bacteroidota* (33.80%), *Firmicutes* (32.07%), *Proteobacteria* (18.45%), *unidentified_Bacteria* (3.58%) and *Actinobacteria* (2.86%), while the CVP group consisted of *Bacteroidota* (50.25%), *Firmicutes* (36.64%), *Proteobacteria* (4.69%), *unidentified_Bacteria* (2.11%), and *Actinobacteriota* (1.33%). Studies have shown that phylum horizontal *actinomycetes*, *Proteobacteria* and *Verrucomicrobia* increased significantly and *Firmicutes* decreased during aging (74, 75). However, CVSS polysaccharide could reverse this phenomenon, and the abundance of *Bacteroidetes*($p<0.01$) and *Firmicutes*($p>0.05$) in our polysaccharide group increased, while the abundance of *Proteobacteria* ($p<0.01$) and *actinomycetes* ($p>0.05$) decreased. We then calculated the abundance ratio of *Firmicutes/Bacteroidetes* (Figure 7D), which tended to increase with age and has been suggested as a possible marker of senescence (76). The abundance ratio of *Firmicutes/Bacteroidetes* significantly decreased in CVP group ($p=0.023$). The analysis of the relative abundance of microbes at the genus level is shown in Figure 7B. *Bacteroides* (7.63%) was the most enriched genera in Control group, followed by *Staphylococcus* (3.96%), *Acinetobacter* (3.52%), *Lactobacillus* (1.97%), *Parasutterella* (1.90%), *Lactobacillus* (1.97%) and *Lachnospiraceae_NK4A136_group* (1.53%). *Dubosiella* (6.29%) and *Bacteroides* (6.27%) were the most abundant genera in the CVP group, followed by *Candidatus_Bacilloplasma* (5.38%), *Lachnospiraceae_NK4A136_group* (2.84%), *Lactobacillus* (2.75%) and *Parasutterella* (2.42%). At the species level,we observed higher relative abundance of *Lactobacillus_reuteri* and *Faecalibaculum_rodentium* and lower relative abundance of *Acinetobacter_lwoffii*, *Cercis_gigantea*, and *Corynebacterium_glutamicum* in the CVP group compared to the control group (Figure 7C). In addition to *Dubosiella* and *Candidatus_Bacilloplasma*, which increased significantly in the CVP group at the genus level, there were also some increases in the abundance of the genera *Lactobacillus* and *Parasutterella*. Of these, *Lactobacillus* was widely used as one of the most common probiotics (77). Our results showed that *Lactobacillus_reuteri* was the most abundant at the species level in the genus *Lactobacillus*, which protected the micro-ecological balance of the gut by producing reuterin, short-chain fatty acids (SCFA), and lactic acid (78). In addition, *Parasutterella* has been shown to be involved in the maintenance of bile acid homeostasis and cholesterol metabolism (79). All these data indicate that CVSS polysaccharide can regulate the composition and structure of intestinal microorganisms.

To identify key phylotypes of the gut microbiota associated with CVSS polysaccharide function, we used linear discriminant analysis (LDA) to assess cecum microbes in the control and CVP groups. Figure 7E shows species with significant differences, indicated by LDA scores > 4.0, reflecting the degree of impact of species that differed significantly between the two groups. At the genus level, the CVP group significantly increased the abundance of *Dubosiella* and *Candidatus_Bacilloplasma* and significantly decreased the abundance of *Staphylococcus* and *Acinetobacter*. *Dubosiella* is isolated and cultured from mouse intestinal contents (80) but there are few studies on its efficacy. It has been reported that *Dubosiella* significantly improves the state of dysglycemia and lipid metabolism in mice and prolongs the life span of nematodes. Surprisingly, in the treatment of high-fat diet-

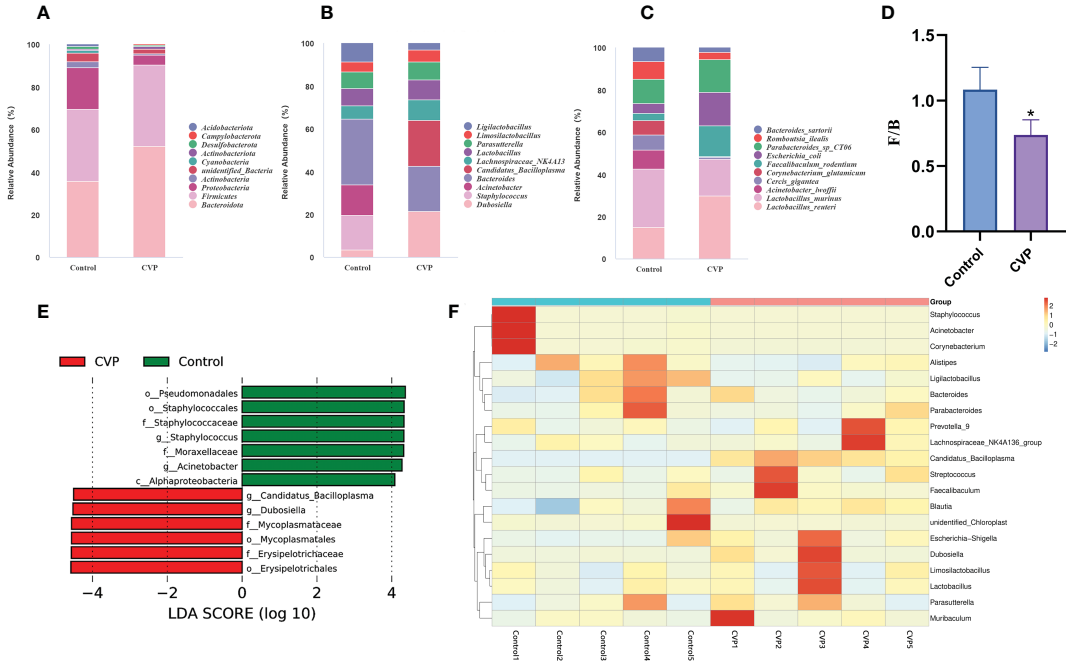

FIGURE 7
Comparative analysis of the effects of CVSS polysaccharide supplementation on the gut microflora. **(A-C)** Relative abundance of species in the top 10 of the intestinal flora at the phylum, genus and species level; **(D)** Quantification shows the abundance ratio of Firmicutes/Bacteroidetes at the phylum level, n=5, "*" stands for the comparation with Control; *p<0.05 by one-way ANOVA; **(E)** The histogram of the distribution of LDA values shows the species with significant differences in abundance in different groups; **(F)** Heatmap depicting the relative abundance of 20 bacterial species significantly enriched in different samples at the genus level.

induced obesity (81), alcoholic fatty liver (82), and inflammatory bowel disease (83) with polysaccharides, the abundance of *Dubosiella* was also increased, in the same way as our results, and in some cases predominantly. To further analyse the differences in species abundance, a heat map was generated using the top twenty bacteria in abundance at the genus level for species composition analysis. We found that 19 genera changed in the CVP group compared to the control group, with a trend towards enrichment in 11 genera and a decrease in abundance in 8 genera (Figure 7F). Among the genera with increased abundance, *Dubosiella* (80, 84), *Candidatus_Bacilloplasma* (85), *Lachnospiraceae_NK4A136_group* (86), *Faecalibaculum* (87), *Limosilactobacillus* (88, 89), *Lactobacillus* (77) and *Parasutterella* (79) were associated with intestinal homeostasis. Some of the genera with reduced abundance were associated with intestinal inflammation and oxidative stress, such as *Staphylococcus* (90, 91), *Acinetobacter* (92, 93), *Corynebacterium* (94, 95). Therefore, CVSS polysaccharide may regulate the homeostasis of the intestinal flora by increasing the abundance of potentially beneficial bacteria such as *Dubosiella* and *Candidatus_Bacilloplasma*, as well as common beneficial bacteria such as *Lactobacillus*, *Lactobacillus_reuteri* and *Parasutterella*, and decreasing the abundance of harmful bacteria such as *Staphylococcus* and *Fusobacterium*, thereby protecting the normal function of the intestine.

Finally, we performed a heatmap correlation analysis using the top ten abundances of intestinal microorganisms at the genus level with pro-inflammatory factors, antioxidant enzymes, MDA, LPS and sIgA in serum (Figure 8A), jejunum (Figure 8B) and liver (Figure 8C). We found that *Dubosiella*, *Candidatus_Bacilloplasma*, *Lachnospiraceae_NK4A136_group*, *Lactobacillus*, *Parasutterella*, and *Limosilactobacillus* were negatively correlated with the levels of pro-inflammatory factors (IL-6, IL-1β, TNF-α), LPS (serum), and MDA in serum, jejunum, and liver, and positively correlated with the levels of antioxidant enzymes (CAT, SOD, GPX) and sIgA (jejunum). *Staphylococcus*, *Acinetobacter*, and *Ligilactobacillus* were positively correlated with the levels of pro-inflammatory factors (IL-6, IL-1β, TNF-α), LPS (serum), and MDA in serum, jejunum, and liver, and negatively correlated with the levels of antioxidant enzymes (CAT, SOD, GPX) and sIgA (jejunum). Seven of the top ten genera in abundance were significantly correlated with inflammatory, oxidative, and other factors, which further verified the above speculated that intestinal flora alleviated oxidative stress and inflammatory response and delayed aging through the gut-liver axis. As shown in Figure 9, during the aging process, under the action of ROS, the intestine and liver were damaged by oxidative stress, the intestinal barrier was damaged, the intestinal flora was disturbed, and inflammation was triggered. LPS produced by Gram-negative bacteria and metabolites produced by other microorganisms, and inflammatory factors entered the liver through the intestinal epithelial layer through the portal vein, aggravating the oxidative stress damage in the liver. By promoting the expression of transcription factor Nrf2, CVSS polysaccharide enhanced antioxidant capacity, reduced inflammatory response, repaired intestinal immune damage and regulated dysbiosis, thereby improving oxidative stress damage and inflammatory response in the intestine and liver of naturally aging mice through the intestine-liver axis. The protective effects on the

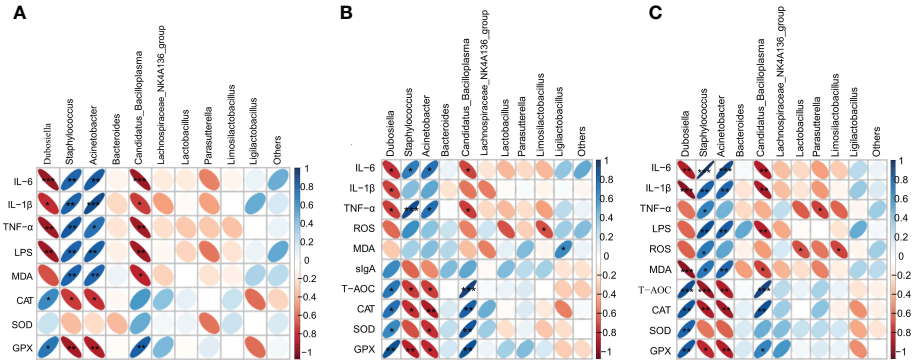

FIGURE 8
Heatmap showing the correlation of gut microbial genera with pro-inflammatory factors (IL-6, IL-1β, TNF-α), antioxidant enzymes (CAT, SOD, GPX), MDA, LPS, sIgA; **(A)** indicates correlation with serum; **(B)** indicates correlation with jejunum; **(C)** indicates correlation with liver. The colour and shape of the ellipse correlates with the strength of the correlation, with darker and flatter ellipses being more strongly correlated (*p<0.05, **p<0.01, ***p<0.001).

intestine and liver exhibited by the polysaccharide from the stems and leaves of *Chuanminshen violaceum* indicated that the polysaccharides from the stems and leaves had the same good pharmacological activity as the polysaccharides from the medicinal parts of the root, suggesting a predictable application *in vivo* against oxidative stress-related intestinal and liver diseases. In order to further develop and utilize the non-medicinal parts of the ground, more in-depth research is needed.

4 Conclusion

In this study, we used DEAE anion-exchange chromatography and gel filtration to obtain a homogeneous acidic polysaccharide, CVP-AP-I, from the stems and leaves of *Chuanminshen violaceum*. The results of chemical composition studies showed that CVP-AP-I is mainly composed of GalA, Gal, Ara, and Rha, and its average molecular weight is 118.2kDa. Subsequently, different doses of CVP-AP-I were orally administered to naturally aging mice, and benefits in terms of oxidative stress and inflammation in the intestine and liver were reported. CVP-AP-I not only improved intestinal function by down-regulating ROS levels and pro-inflammatory factor expression, up-regulating antioxidant enzymes and total antioxidant capacity, repairing structural damage to intestinal tissues and modulating intestinal flora structure, but also has the same effect in liver tissue. These results suggest that CVSS Polysaccharide is an effective modulator and contributes to the homeostasis of the intestinal microbiota and its associated intestinal functions. With improved intestinal flora and intestinal immune barrier, LPS-producing Gram-negative bacteria such as *Acinetobacter* decrease, *Lactobacillus_reuteri*, *Parasutterella*, and possibly the potentially beneficial bacterium *Dubosiella* increase. CVSS polysaccharide reduces serum and hepatic LPS levels, thereby improving oxidative stress and inflammatory responses in the gut and liver through the gut-liver axis.

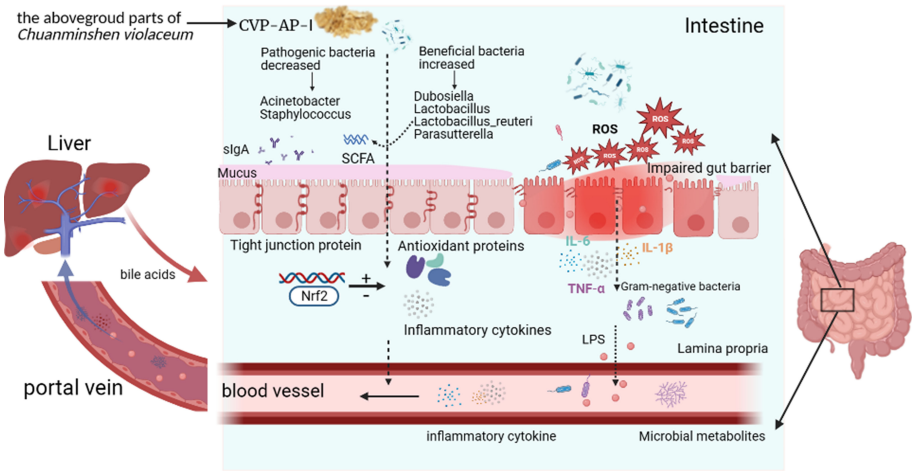

FIGURE 9
Summary diagram of the pharmacological effects of CVP-AP-I.

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: NCBI Bioproject, PRJNA936077.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee guidelines of Sichuan Agricultural University.

Author contributions

Y-FZ and X-PJ designed the study, C-YL, X-PJ, Y-PF, C-WZ and N-YL finished the experiment; X-PJ drafted the manuscript. L-XL, Z-QY and XS were mainly responsible for the determination of characterization of polysaccharides. X-HZ, H-QT, JC, Y-PL and X-FC detected the bioactivity of polysaccharides. BF and GY performed the graphics and helped to improve the research. Y-FZ, M-LT guided the experiments and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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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EDITED BY
Isabelle Mack,
University of Tübingen, Germany

REVIEWED BY
Mario Delfino,
University of Naples Federico II, Italy
Anasuya Roychowdhury,
Indian Institute of Technology Bhubaneswar, India

*CORRESPONDENCE
Maryam Zare
✉ M.Zare@Khalums.ac.ir
Saeid Doaei
✉ sdoaee@yahoo.com

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Potential of macronutrients and probiotics to boost immunity in patients with SARS-COV-2: a narrative review

Afrouz Mardi[1], Aziz Kamran[2], Farhad Pourfarzi[3], Maryam Zare[4]*, Azadeh Hajipour[5], Saeid Doaei[6]*, Negin Abediasl[3] and Daniel Hackett[7]

[1]Department of Public Health, School of Health, Ardabil University of Medical Science, Ardabil, Iran, [2]School of Medicine and Allied Medical Sciences, Ardabil University of Medical Sciences, Ardabil, Iran, [3]Digestive Disease Research Center, Ardabil University of Medical Sciences, Ardabil, Iran, [4]Department of Nutrition, Khalkhal University of Medical Sciences, Khalkhal, Iran, [5]School of Health, Qazvin University of Medical Sciences, Qazvin, Iran, [6]Department of Community Nutrition, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [7]Physical Activity, Lifestyle, Ageing and Wellbeing Faculty Research Group, School of Health Sciences, Faculty of Medicine and Health, The University of Sydney, Lidcombe, NSW, Australia

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) may cause inflammation and increased cytokine secretion. Dietary factors may play an important role in enhancing the immune responses against infectious diseases such as SARS-COV-2. This narrative review aims to determine the effectiveness of macronutrients and probiotics to improve immunity in SARS-COV-2 patients. Dietary proteins may boost pulmonary function in SARS-COV-2 patients through inhibitory effects on the Angiotensin-converting enzyme (ACE) and reduce Angiotensin (ANG-II). Moreover, omega-3 fatty acids may improve oxygenation, acidosis, and renal function. Dietary fiber may also produce anti-inflammatory effects by reducing the level of high-sensitivity C-Reactive Protein (hs-CRP), Interleukin (IL-6), and Tumor necrosis factor (TNF-α). In addition, some evidence indicates that probiotics significantly improve oxygen saturation which may enhance survival rate. In conclusion, the consumption of a healthy diet including adequate macronutrients and probiotic intake may decrease inflammation and oxidative stress. Following this dietary practice is likely to strengthen the immune system and have beneficial effects against SARS-COV-2.

KEYWORDS

protein, lipid, carbohydrate, probiotic, immune system, SARS-CoV-2

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV-2) is a viral infectious disease that in the previous 2 years has caused a worldwide pandemic (1). In December 2019 (Wuhan, China) SARS-COV-2 was first reported and characterized as a pandemic in March 2020 by the World Health Organization (WHO) (2). Millions of people worldwide have been infected with SARS-COV-2, while over six million people have died from this disease (3). The symptoms of COVID-19 vary from asymptomatic to mild upper respiratory tract symptoms

and can progress to pneumonia. Patients with severe pneumonia rapidly deteriorate over a short period and require advanced medical support (4–6). SARS-COV-2 colonizes the respiratory tract but can also invade the gastrointestinal tract, neurological system, kidneys, and other organs (7, 8). SARS-COV-2 utilizes the host cell membrane receptor ACE2 to enter the virus into lymphocytes, monocytes, pulmonary alveoli, and esophageal epithelial cells (9). The cytokine storm can be induced by increases in the production of pro-inflammatory cytokines, such as IL-6, IL-1, and TNF-α, in which a hyperactive immune response is experienced. The effects of this response include vessel and lung alveoli damage, and systemic organ failure, such as acute respiratory syndrome (ARDS), which has been associated with high rates of mortality (10).

Immunity to viruses can be influenced by several factors such as genetics (11), vaccination history (12), illness, medications, sex (13), stage of life (e.g., pregnancy, infancy, and old age) (14), and diet (15). Specifically, an adequate intake of some dietary components is essential for developing, supplying, and expressing immune responses (16). A balanced diet of macronutrients (i.e., carbohydrates, protein, and fats) has anti-inflammatory effects and can affect different stages of the immune response. Consequently, an unbalanced diet of macronutrients can affect innate and adaptive immunity, hence making people more susceptible to infections and severe clinical conditions (17).

Recent studies reported that critically ill patients with SARS-COV-2 are at high risk for malnutrition (18). In this context, a recent study emphasized the supportive role of dietary supplementation in SARS-COV-2 patients (19). Therefore, following a well-balanced diet in combination with certain dietary supplements may strengthen and optimise the function of the immune system (20). Particular types of supplements shown to have antiviral activities against common respiratory viruses are probiotics. These dietary supplements can regulate cytokine secretion and affect the immune response (20, 21). At present there is limited evidence on macronutrients and probiotics intake in patients with SARS-COV-2. The aim of this narrative review was to examine the effects of macronutrients and probiotics on the immune system in patients with SARS-COV-2.

Protein

Proteins are essential to life because they facilitate biochemical reactions and enzyme production. They also act as cellular signals in the form of hormones and cytokines (22). An adequate protein intake is necessary for antibody production in the body. An adult requires 0.8 g of protein per kg of body weight to meet the recommended dietary allowance (RDA) (23). Key amino acids for stimulating the immune system include arginine and glutamine (24). Arginine serves as the vital substrate for synthesizing nitric oxide by macrophages and endows them with pro-inflammatory and microbicidal properties (25). As for glutamine, this amino acid is used as an energy source for the immune cells similar to glucose (26).

The immunomodulatory role of protein in SARS-COV-2

To defeat the barrier and bind the host cells in the alveoli, SARS-COV-2 utilizes ACE2 (membrane-bound enzyme localized on type II pneumocystis) and cellular serine protease transmembrane protease, serine 2 (TMPRSS2) to prime and replicate in infected organisms (27). ACE2 is expressed in endothelial cells including the heart, kidneys, and intestines (28). When the SARS-COV-2 has entered ACE2-positive cells, there is a down-regulation of the enzyme (29). ACE2 usually binds angiotensin II, a molecule recently certified as a critical player in the conventional renin-angiotensin system (RAS) which promotes inflammation, oxidative stress, and apoptosis (23, 30). The pro-inflammatory angiotensin II is converted into the anti-inflammatory angiotensin 1–7 by binding the enzyme to angiotensin receptors, which exerts a pro-inflammatory effect (23). Therefore, higher production of angiotensin 1–7 can protect the endothelial barriers of the lungs, kidneys, heart, and intestines supporting patients with viral respiratory tract infections from harmful inflammation (31). Dietary peptides with inhibitory effects on the angiotensin-converting-I enzyme (ACE-I) can significantly reduce angiotensin (ANG-II). Dietary-derived peptides can contribute to the downstream ANG-II function. The lactoferrin-released tetra-peptide RPYL (62% of concentration 300 μmol/l) has been found to have inhibitory effects on ANG-II vasoconstriction (32). Additionally, lysine has been found to down-regulate the level of ANG-II (33).

Asymptomatic and mild SARS-COV-2 patients treated with lactoferrin (a milk-derived 80-kDa glycoprotein) had faster clinical recovery compared to untreated patients (34). Therefore, lactoferrin (Lf) may be a safe treatment combined with other therapies for COVID-19 patients. A retrospective study focused on the antiviral activity of Lf found that SARS-CoV-2 RNA was negativized faster in Lf treated versus untreated patients (35).

Evidence from previous review studies indicates that a diet that positively impacts immune function contains adequate amounts of protein, particularly glutamine, arginine, and branched-chain amino acids (BCAAs) (36, 37). Dietary intake of anserine, carnosine, and 4-hydroxyproline has been shown to improve the body's immunological defenses against bacteria, fungi, parasites, and viruses (38). Soy protein enhances cellular immunity through a reduction in TNF-α concentrations (39). However, primary sources of amino acids include fish, meat, and poultry. Therefore ensuring that the intake of peptides is optimised may improve respiratory function, symptoms, and fatigue in SARS-COV-2 patients (40). Support for this recommendation is shown by impaired immunity with protein-deficient diets, which would increase the risk of COVID-19 infection (26) (Figure 1).

Patients with SARS-COV-2 receiving enteral nutrition for >7 days where consistent levels of adequate protein and energy are received have a lower death rate compared to patients with suboptimal protein intake (32). Additionally, patients who died of SARS-COV-2 in the hospital were found to have a lower protein and energy intake compared to patients that survived (41). It could be postulated that the intake of protein and energy in critical status patients with SARS-COV-2 hospitalized in ICU are suboptimal. Therefore, it would be prudent that patients with SARS-COV-2 consumed higher protein and energy intake in the early acute phase increasing their chances of survival and lowering the risk of in-hospital mortality.

Lipid

Dietary intake of fatty acids positively influences the immune response. The two essential fatty acids include omega-3 and omega-6

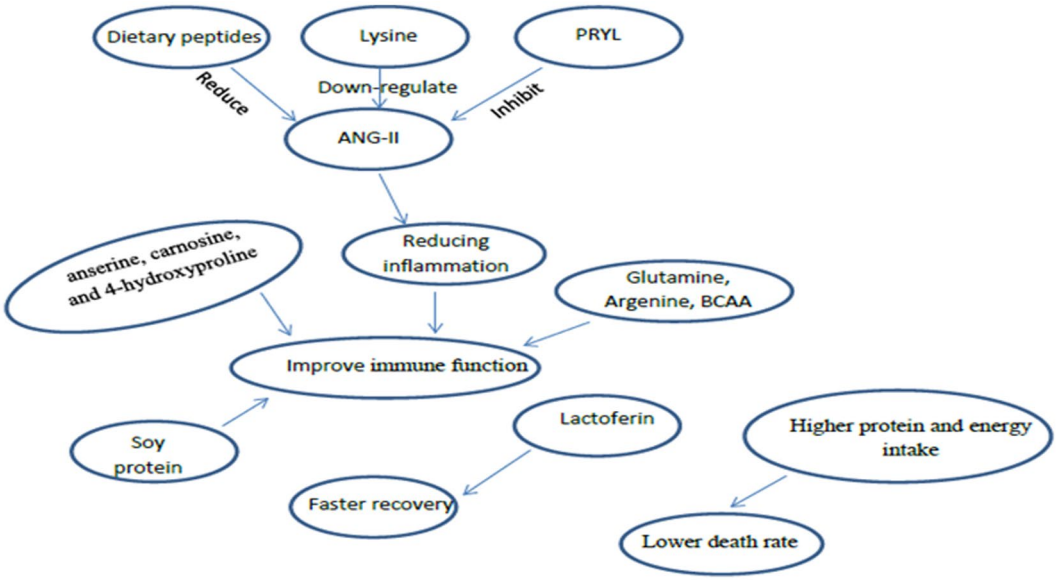

FIGURE 1
The immunomodulatory role of protein in SARS-COV-2.

fatty acids. Additionally, long-chain polyunsaturated fatty acids (PUFA) need to be obtained from foods in the diet since they cannot be synthesized by the human body (42). The omega-3 fatty acids essentially comprise of α-linolenic acid (ALA) from plant sources and docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from fish and seafood sources (43). Anti-inflammatory responses in the body have been shown with the consumption of omega-3 fatty acids (44). In contrast, omega-6 fats mainly provide energy to the body, but the population should ideally receive more omega-3 fats (45). The ratio of omega-6 and omega-3 fatty acids in diets should be between 1:1–4:1.

The immunomodulatory role of lipids in SARS-COV-2

EPA and arachidonic acid (ARA) compete with the lipoxygenase and cyclooxygenase enzymes to synthesize eicosanoids [prostaglandins (PG), thromboxane (TXA), leukotrienes (LT), hydroxylated fatty acids, and lipoxins] and lipid mediators. ARA is a precursor for pro-inflammatory eicosanoids that are both immunosuppressive and thrombotic (46). EPA converts eicosanoids to antithrombotics. EPA-derived LTB5 has a less chemotactic effect on neutrophils than ARA-derived LTB4. Therefore, EPA has a lower pro-inflammatory response than ARA. LTB4 is a critical pro-inflammatory mediator in cell proliferation and immune response (16).

Omega-3 fatty acids alter the phospholipid bilayer of the cell membrane, thus preventing viral entry. EPA gets incorporated into the plasma membrane and affects the clumping of toll-like receptors. This results in inhibiting signals that activate NF-kB, producing fewer pro-inflammatory mediators, and finally, reducing complications of SARS-COV-2 infection (33, 38). Another mechanism by which Omega-3 fatty acids reduce inflammation levels is *via* the inhibition of leukocyte chemotaxis, expression of adhesion molecules, and interaction between leukocytes and endothelial (26, 40). They also have antiviral effects through the inhibition of influenza virus replication. Furthermore, the use of omega-3 fatty acids is shown to improve oxygenation in SARS-COV-2 patients (47). Consistent with this finding is evidence that omega-3 fatty acids *via* parenteral and enteral means could improve oxygenation in SARS-COV-2 patients, although currently, the evidence is insufficient (48).

Supplementing with omega-3 fatty acids might be beneficial in managing inflammation-mediated clinical symptoms in SARS-COV-2 patients (49). Omega-3 fatty acids are precursors of specialized pro-resolution mediators (SPMs) that present long-lasting analgesic and anti-inflammatory effects (50). As such, omega-3 fatty acids are used for the clinical management of patients with SARS-COV-2. Resolvins, protecting, and maresins of omega-3 fatty acids can inactivate polymorphonuclear leukocytes and stimulate the movement of non-inflammation leukocytes, thereby eliminating programmed cell death (32). Omega-3 supplementation was found to have promising effects on acidosis and renal function, possibly improving clinical outcomes in SARS-COV-2 patients (51). a systematic review and meta-analysis of randomized controlled trials on the effect of Omega-3 fatty acids supplementation in patients with SARS-COV-2 have reported that Omega-3 supplementations have been associated with alleviating the inflammatory response by the CRP level reduction (52).

A study found that omega-3 fatty acids from marine food were correlated with lower SARS-COV-2 mortality rates. Omega-3 fatty acids could reduce SARS-COV-2 medical complications by limiting the entry of the virus into human cells *via* a mechanism that involves fatty acids binding to viral spike proteins (53). Hathaway et al. proposed that omega-3 fatty acids supplementation might inhibit

entry of the virus through changes to the lipid composition in a cell's bilipid membrane. Moreover, the omega-3 fatty acids role is critical in mediating inflammatory processes and producing a modulation effect on innate and acquired immune responses (54) (Figure 2).

Caution has been warranted with the use of omega-3 fatty acids in SARS-COV-2 patients. This is due to evidence of a counter-intuitive increase in oxidative stress and inflammation because of a higher susceptibility to damage to cell membranes (55). However, the correlation between omega-3 fatty acids and recovery in SARS-COV-2 patients is still controversial.

Carbohydrates

Carbohydrates are mostly found in plants and used to support parts (fiber) of the plant, for plant growth, while the remaining part is for food storage (i.e., starch or sugar). Rich food sources of carbohydrates include bread, grains, vegetables, fruits, and legumes (56, 57). Carbohydrates intake should comprise 45 to 65% of total daily calories (58). Furthermore, the recommended daily fiber intake for males and females is 25 g and 38 g, respectively. A recent review study reported that the consumption of dietary fiber has anti-inflammatory effects by producing short-chain fatty acids, mucosa protection, and lower glycemic index (36). Diets with a high glycemic index produce inflammatory cytokines, TNF-α, IL-6, and C-reactive protein. Dietary fibers reduce inflammation, hs-CRP, IL-6, and TNF-α. Production of short-chain fatty acids occurs through the fermentation of carbohydrates in the gut and causes anti-inflammatory effects with up-regulated IL-10 production in monocytes (36, 57) (Figure 3). The effect of dietary fiber and prebiotic oligosaccharides is greater and typically promotes the growth of lactobacilli and bifidobacteria which benefits immunity (57). There is no evidence suggesting that carbohydrates influence SARS-COV-2 prevention and improvement (59).

Probiotics

The human body hosts many bacteria and organisms that colonize the skin, mouth, and gut. When there is a community of organisms within a particular location is referred to as the microbiota. The colon is the site of the most significant number and variety of bacterial species (60, 61). The usual diet strongly affects the intestinal microbiota. Probiotics including lactobacillus and bifidobacterium are live microorganisms with long-term effects as part of a typical diet through fermented foods. These foods include traditionally cultured dairy products, some fermented products (dairy and non-dairy), or supplements for their beneficial effects on gut microorganisms (62). Furthermore, both aging and health significantly affect the microbiota composition. An abnormal intestine microbiota, called dysbiosis, is commonly seen in obesity and adults with various human diseases (63). Probiotics have been investigated to prevent and treat various diseases (64). It has been suggested that probiotics are beneficial for human health through their effects on strengthening the immune system (40).

The immunomodulatory role of probiotics in SARS-COV-2

Beneficial effects from probiotics are likely the result of immune regulation, and controlling proinflammatory and anti-inflammatory cytokines (65). Studies have reported that microbiota improves resistance to viruses or pathogenic attacks of respiratory mucosa (66, 67). The results of the network and meta-analysis study showed that probiotics have therapeutic effects through many procedures such as: limiting virus entry *via* ACE2 receptor, mitigating the adverse effects of dysregulated RAS system, improving the systemic immune response, mediating Toll-like receptors (TLRs) innate immune response, improving cardiovascular complications, and reducing

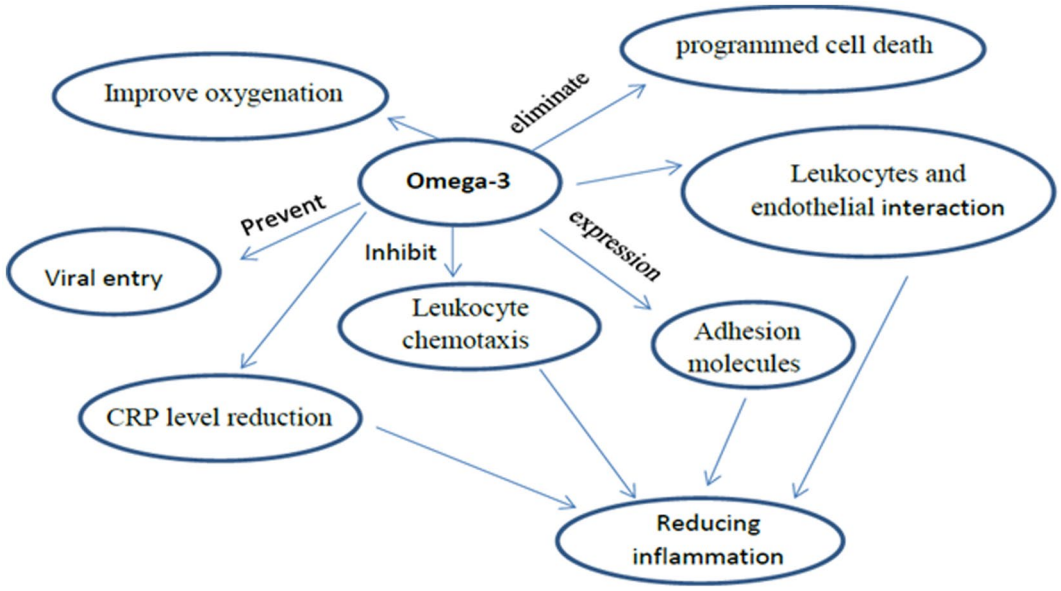

FIGURE 2
The immunomodulatory role of lipid in SARS-COV-2.

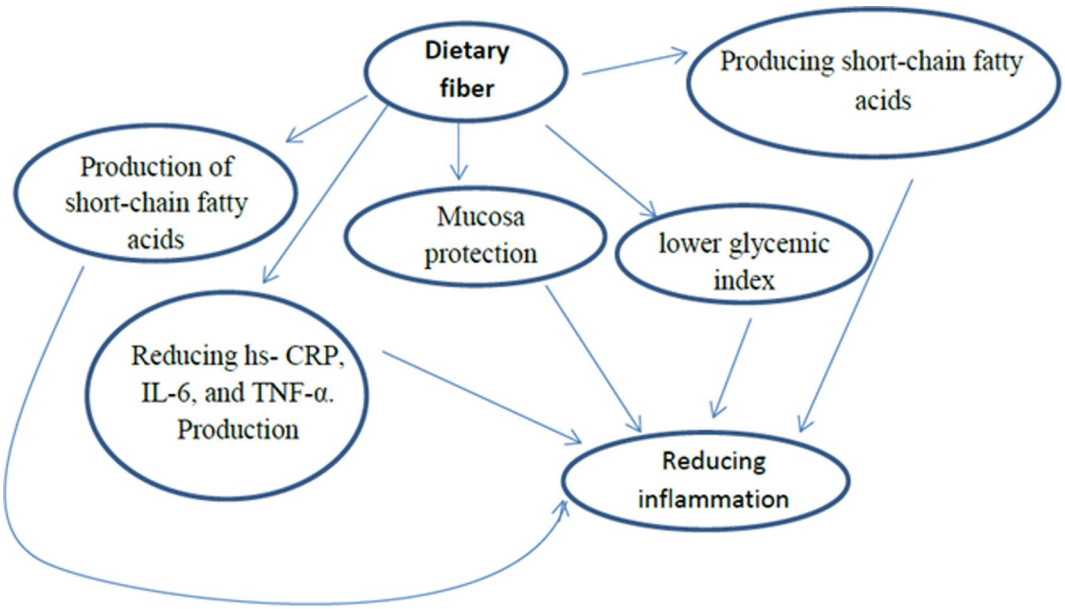

FIGURE 3
The immunomodulatory role of carbohydrates in SARS-COV-2.

nitric oxide (NO) production, hypertension, and oxidative stress (68). Furthermore, another meta-analysis reported a positive correlation between probiotics intake and a reduction in COVID symptoms, especially cough, headaches, and diarrhea (69). Some specific mechanisms that explain the health benefits of probiotics include the activation of the immune reaction by interleukins and natural killer cells. Additionally, probiotics with macrophages lead to the formation of IL-12 which activates the generation of interferon-γ, considered a principal antiviral cytokine (70).

Probiotics enhance mucosal protection *via* increasing IgA production and the differentiation of $CD8^+$ T cells into cytotoxic T-cells as well as $CD4^+$ T cells into Th1 and Th2 cells (71). Different trials have demonstrated that probiotics exert positive effects on the gut and lungs through increases of regulatory T cells, improving anti-viral defense, and reducing pro-inflammatory cytokines during systemic and respiratory infections. Immunomodulatory benefits are crucial for SARS-CoV-2 patients or people at risk of contracting SARS-CoV-2 (72). This effect is likely the result of the gut-lung axis, which can form immune responses and disrupt respiratory diseases (73). The probiotics also affect the junction's integrity and maintenance of the respiratory and gastrointestinal tract epithelium enterocytes, hence limiting the risk of SARS-CoV-2 entry (74). Furthermore, several probiotics especially lactic acid bacteria (LAB) produce peptides with an ACE inhibitory effect, which binds to the site of SARS-CoV-2.

A cross-sectional study on COVID-19 patients, influenza patients, and healthy controls (HC) examining gut microbiota reported that COVID-19 patients had lower gut microbiota diversity compared to HC, which was related to respiratory viral infectious diseases. Moreover, disease-specific shifts in microbiota composition were found between COVID-19 patients and HC (75). The studies on SARS-CoV-2 found significantly decreased gut bacterial diversity compared to HC, while it was increased in numerous bacterial species, such as *Collinsella aerofaciens*, *Morganella morganii*, and *Streptococcus infantis* (76, 77).

Several randomized controlled trials are underway investigating the effect of probiotic strains on SARS-CoV-2 severity, which may directly affect patients (78, 79). A study comparing probiotic-enriched formulas with placebo formula in adult symptomatic Covid19 outpatients reported that the probiotic-enriched formulas reduced symptom duration, viral load, and lung infiltrates while increasing SARS-CoV2-specific IgM and IgG in Covid19 outpatients (80). In a randomized trial, adults with symptomatic SARS-CoV-2 outpatients' consumed probiotic formula (strains Lactiplantibacillus Plantarum KABP022, KABP023, and KAPB033, plus strain *Pediococcus acidilactici* KABP021, totaling 2×10^9 colony-forming units (CFU)) or placebo for 30 days. The results showed a reduction in symptom duration, viral load, and lung infiltrate while increasing SARS-CoV-2 specific IgM and IgG (81).

A randomized controlled clinical trial was conducted with eighty SARS-CoV-2 patients in stages III. The control group received a hospital diet and medical treatment. The intervention group received a hospital diet, medical treatment, and the NSS (vitamins, minerals, fiber, omega-3, amino acids, B-complex, and probiotics). In the intervention group, a significant increase in survival and a decrease in mortality were observed compared to the control group (82) (Figure 4).

The authors of this study assumed that this probiotic might primarily help the gut-lung axis (GLA) by interfering with the host's immune system. However, with the lack of evidence that probiotics affect fecal microbiota, it seems that this hypothesis cannot be confirmed. More studies are needed to confirm this hypothesis.

Conclusion

The adequacy of globally considered macronutrients and microbiota is always fundamental for health in general and the ability of the immune system to respond adequately, not only to SARS-CoV-2, but to any other aggression by microorganisms. This article

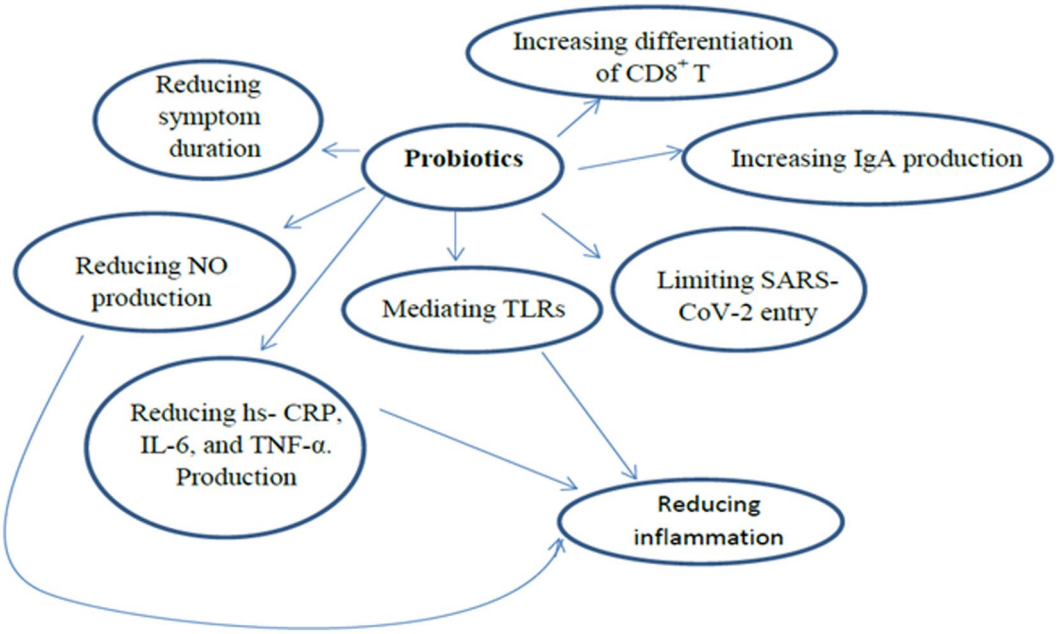

FIGURE 4
The immunomodulatory role of probiotics in SARS-COV-2.

highlights the influential role of macronutrients and probiotics against SARS-CoV-2. Boosted immunity properties of macronutrients have opened a new window for their potential use for SARS-CoV-2 treatment and prognosis. Immunomodulatory agents, including macronutrients and probiotics, are among the current therapies applied in clinical settings for SARS-CoV-2. There is evidence that a diet high in protein and amino acids facilitates antibody production (83). Furthermore, a healthy balanced diet enriched in proteins, dietary fiber, and omega-3 fatty acids is highly recommended during the pandemic. Malnutrition, such as macronutrient deficiencies, is common in vulnerable groups, especially in elderly patients with multiple comorbidities and also in those groups of subjects who voluntarily submit to specific nutritional indications (for example vegan diets, ketogenic diets, vegetarian diets, food exclusion diets, etc…), or in patients affected by particular pathologies (anorexia, celiac disease, bulimia, etc…). As a result of the comorbidities and associated drugs, this may lead to tissues depleted of valuable nutrients, increase urinary excretion, or reduce nutrient absorption (37). These conditions conflict with an upcoming cytokine storm. Currently, no definitive recommendations for macronutrient and micronutrient supplementation in patients with SARS-CoV-2 exist. However, it is advised that SARS-CoV-2 patients be monitored to assess risk factors for nutrient depletion, i.e., inadequate intake and nutrient-depleting drugs. Education would also be imperative concerning depletion symptoms for laboratory monitoring of nutrients.

With numerous research groups currently involved in developing vaccines, and novel therapeutics against SARS-CoV-2, a healthy diet, balanced macronutrient status, and probiotics that reduce inflammation and oxidative stress may be an important strategy for managing SARS-CoV-2. This review highlights the role of macronutrients and probiotics in reducing inflammation, cytokine storm, and strengthening the immune system in SARS-CoV-2 patients (Supplementary Table S1).

Author contributions

MZ, SD, AH, and AK: writing original draft. FP, AM, MZ, and SD: writing—review. NA, and DH: review and editing. All authors contributed to the article and approved the submitted version.

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.2023.1161894/full#supplementary-material

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EDITED BY
Julio Villena,
CONICET Centro de Referencia para Lactobacilos (CERELA), Argentina

REVIEWED BY
Astghik Zaveni Pepoyan,
Armenian National Agrarian University, Armenia
Prem Prashant Chaudhary,
National Institutes of Health (NIH), United States

*CORRESPONDENCE
Isabelle Mack
✉ isabelle.mack@uni-tuebingen.de

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Effects of isolation and confinement on gastrointestinal microbiota—a systematic review

Bea Klos[1], Christina Steinbach[1], Jasmin Ketel[1], Claude Lambert[2,3], John Penders[4,5], Joël Doré[6], Paul Enck[1] and Isabelle Mack[1]*

[1]Department of Psychosomatic Medicine and Psychotherapy, University Hospital Tübingen, Tübingen, Germany, [2]CIRI—Immunology Lab University Hospital, Saint-Étienne, France, [3]LCOMS/ENOSIS Université de Lorraine, Metz, France, [4]Department of Medical Microbiology, Infectious Diseases and Infection Prevention, Maastricht University Medical Center, CAPHRI Care and Public Health Research Institute, Maastricht, Netherlands, [5]Department of Medical Microbiology, Infectious Diseases and Infection Prevention, Maastricht University Medical Center, School of Nutrition and Translational Research in Metabolism, Maastricht, Netherlands, [6]UMR Micalis Institut, INRA, Paris-Saclay University, Jouy-En-Josas, France

Purpose: The gastrointestinal (GI) microbiota is a complex and dynamic ecosystem whose composition and function are influenced by many internal and external factors. Overall, the individual GI microbiota composition appears to be rather stable but can be influenced by extreme shifts in environmental exposures. To date, there is no systematic literature review that examines the effects of extreme environmental conditions, such as strict isolation and confinement, on the GI microbiota.

Methods: We conducted a systematic review to examine the effects of isolated and confined environments on the human GI microbiota. The literature search was conducted according to PRISMA criteria using PubMed, Web of Science and Cochrane Library. Relevant studies were identified based on exposure to isolated and confined environments, generally being also antigen-limited, for a minimum of 28 days and classified according to the microbiota analysis method (cultivation- or molecular based approaches) and the isolation habitat (space, space- or microgravity simulation such as MARS-500 or natural isolation such as Antarctica). Microbial shifts in abundance, alpha diversity and community structure in response to isolation were assessed.

Results: Regardless of the study habitat, inconsistent shifts in abundance of 40 different genera, mainly in the phylum Bacillota (formerly Firmicutes) were reported. Overall, the heterogeneity of studies was high. Reducing heterogeneity was neither possible by differentiating the microbiota analysis methods nor by subgrouping according to the isolation habitat. Alpha diversity evolved non-specifically, whereas the microbial community structure remained dissimilar despite partial convergence. The GI ecosystem returned to baseline levels following exposure, showing resilience irrespective of the experiment length.

Conclusion: An isolated and confined environment has a considerable impact on the GI microbiota composition in terms of diversity and relative abundances of dominant taxa. However, due to a limited number of studies with rather small sample sizes, it is important to approach an in-depth conclusion with caution, and results should be considered as a preliminary trend. The risk of dysbiosis and associated diseases should be considered when planning future projects in extreme environments.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42022357589.

KEYWORDS
isolation, confinement, human, microbiota, gut, gastrointestinal, space, Antarctica

1. Introduction

The indigenous microbiota of the human is known to be closely associated with various physiological functions, with the gastrointestinal (GI) microbiota in particular playing a prominent role in the protection against infection (1, 2), in the digestion of food (3), or in the production of neurotransmitters (4). There is also evidence that intestinal microbes affect energy metabolism (5), intestinal epithelial proliferation (6) and immune response in the host (1). As a consequence, there is increasing evidence of a link between GI dysbiosis and the development of metabolic (obesity), infectious and immune-mediated diseases (allergies, inflammatory bowel diseases) (7).

There are significant interindividual variations in GI microbiota composition, with each individual harboring a unique combination of microbial species (8, 9). The microbiota richness, diversity and community structure are quantified using various ecological measures and indices. Alpha diversity refers to both the number of different species and their distribution (evenness) within a given microbial habitat, and is therefore divided into richness and biodiversity (10). Beta-diversity compares the structure of microbial communities and is determined by the degree of similarity/dissimilarity between communities within different microbial habitats (11).

Generally, the microbiota composition is dependent of environment and food intake in early childhood but is considered to be rather stable in adulthood (12, 13). However, extreme hin diet (14, 15), bariatric surgery (16, 17), the use of medications (especially antibiotics) (18–20) and high hygiene (21) can significantly affect the composition and the function of the indigenous microbiome, and could contribute considerably to a dysbiosis between beneficial and potentially harmful bacteria. Besides environmental factors, genetic background (22, 23) and local immunity (24) also have an important impact on the composition of the microbial community; however, studies suggest that external factors may have a greater impact on dysbiosis than genetic factors (22, 25).

Current literature supports changes in intestinal microbiota due to extreme environmental conditions such as extreme temperatures (26, 27), high altitudes (28–30) or radiation (31–33). Studies in mice have demonstrated that the gut microbiota changes significantly during both real spaceflights and simulated microgravity (34, 35). Additional changes in the microbiota occurred during a real spaceflight that were beyond those observed in ground-controlled animals. This suggests that the space experience has unique features that cause changes in the microbiome (36, 37).

If environmental bacterial load influences the gut microbiota, what happens in case of poor bacterial diversity exposure? The effects of antigen-limited or poor environments on the human gut microbiota have to date received rather little attention. There is evidence that spaceflights can cause dysbiosis in humans, with a reduction in symbiotic microbes and a rise in opportunistic pathogens, affecting both microbial diversity and community structure significantly (25, 37). Several space mission experiments have detected changes in GI bacterial species composition and function (25, 38–40), bacterial gene expression and protein regulation (41) suggesting a potential host-microbial interaction that may contribute to a decline in protein metabolism in the host (35) during spaceflight and after its completion, but results remain conflicting (42). Another long-term spaceflight found neither a reduction in richness nor a change in community structure in the in-flight samples compared to the pre-flight and post-flight samples. The highly variable core microbiome is expected to be present in both spaceflight astronauts and ground-based controls with fecal microbial communities differing significantly between spaceflight astronauts and ground-based controls and remaining distinct over the time (25). Along with the host immune system, the core microbiome is thought to be pivotal in the maintenance of human health even during and after spaceflights (39).

Terrestrial ways to limit antigen exposure include exposure to environmental conditions that are highly challenging for humans, such as in Antarctica, or in specialized facilities known as space simulation units. These units simulate the conditions of spaceflights, including weightlessness, increased radiation, and other factors. The stay of a healthy subject in an environment with altered parameters is accompanied by dysbiotic changes resulting in a decrease in colonization resistance of the intestine and integumentary tissues (43). Within the MARS-500 experiment (44), stool samples were collected and analyzed during a long-term stay (520 days) in a facility simulating spaceflight. Data showed significant changes in the taxonomic composition during the initial stages of the experiment, but the basic composition of the intestinal ecosystem remained unchanged in all 5 individuals without changes in the enterotypes of individual taxonomic groups. After the confinement the taxa tended to reverse to their original state. By clustering the gut microbiota of subjects into two enterotypes, Chen et al. (45) suggested that the composition of the gut microbiota is a crucial factor in the adaptability of individuals to antigen-limited environment exposure, as subjects showed either no significant differences in health indicators before and after confinement or experienced several health problems after confinement, such as increased uric acid, anxiety, and constipation, and lack of sleep. Similarly, another simulation experiment (46) reported mixed results: The data showed increased abundances of the genera *Roseburia*, *Prevotella*, *Lachnospira*, and *Phascolarctobacterium*, while abundances of the genera *Faecalibacterium*, *Parabacteroides*, *Bacteroides*, *Bifidobacterium*, and *Anaerostipes* dropped. However, it remains unclear whether these effects result from exposure to an antigen-limited or antigen-poor environment or from microgravity, which has already been linked to changes in virulence factors, bacterial stress responses and biofilm formation (47).

Although there have been various studies on the changes or stability of microbiota in response to antigen-limited or poor environments, the underlying mechanisms behind these observations are not yet fully understood and remain an active area of scientific inquiry. Effect of exposure is time dependent and long-term experiments on a sufficient number of volunteers are rare. A systematic investigation of the impact of a long-term residence (>28 days) in such an environment on the composition, diversity and stability of the GI microbiota has not yet been conducted. Therefore, we aim to fill this gap in knowledge through

a comprehensive review of the available literature and a systematic search for relevant studies. We ask for two questions:

1. What is the effect of long-term residence (>28 days) in an antigen-limited or poor environment on the relative abundances of key bacterial taxa, and what happens to GI microbiota in terms of richness and biodiversity (alpha diversity) and community structure?
2. How reversible are the isolation-induced effects on the relative abundances, alpha diversity and community structure after the exposure to an antigen-limited or poor environment?

2. Materials and methods

2.1. Literature information sources and search strategy

This review was developed and executed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (48). To identify all relevant studies examining the effect of isolation and confinement on the human gut microbiota the databases PubMed, Web of Science, Cochrane Library (Wiley) and EBM-Reviews (Ovid) Cochrane Library were searched on September 2nd 2022. The protocol of this systematic review is registered on the PROSPERO platform with the registration number CRD42022357589. The full search strategy was conducted in assistance with a specialized librarian and is documented in the supporting information (Supplementary Text S1). It consists of three models: isolation condition, human gut microbiota and exclusion of animals. For the search, a very specific search term was chosen to represent the isolation conditions as best as possible. Broader search terms were also tested, but not all relevant studies were found.

2.2. Eligibility criteria

Inclusion criteria were established based on the five PICOS dimensions, i.e., participants, interventions, comparator, outcome and study design (49).

Participants: Healthy adults, regardless of sex, age, or weight status, who had been under isolation conditions for at least 28 days were included.

Intervention: Isolation in an environment with constant / reduced antigen exposure. Such isolation conditions are found (a) in space missions; (b) in isolation simulations, such as MARS-500, SIRIUS and Lunar Palace-1 or in bed-rest studies; (c) in extreme environmental conditions, such as in Antarctica, Arctic and Siberia. The intake of probiotics was allowed. Studies that focused on the use of antibiotics for preventing infectious and inflammatory diseases in humans were excluded.

Comparator: Studies with or without control groups met eligibility criteria.

Outcome: Assessment of the microbiota of the human GI tract.

Study design: Randomized controlled trials or non-randomized controlled trials with any publication date and written in English, German and Russian. Only original articles were included.

2.3. Study selection and organization

To identify eligible studies, the search results of the databases were combined, and the duplicates were removed. Two authors (BK and CS) independently screened titles and abstracts to identify relevant trials. Full-text articles were evaluated regarding their eligibility (BK, CS), with uncertainties being discussed between the authors (<3% cases). A third author (IM) was involved if the discrepancy persisted.

Throughout the decades, the methods of microbial analysis changed from cultivation and cell counting to molecular-based approaches like next-generation sequencing, micro-arrays or quantitative polymerase chain reaction. Due to the huge diversity between the method procedures and the associated heterogeneity of the outcomes, the results were assessed separately from each other. The studies were classified into two groups according to microbiota analysis method:

Group 1–Cultivation-based approaches for microbiota analysis.

Group 2–Molecular-based approaches for microbiota analysis.

Additionally, subgroups were created to provide a more homogenous summary of findings.

Subgroup 1–Isolation caused by space missions.

Subgroup 2–Isolation caused by spaceflight- or gravity simulators, e.g., MARS-500, bioregenerative life support systems (BLSS) or bed rest-studies.

Subgroup 3–Isolation in a natural, earth-bound habitat, e.g., Antarctica.

Certain experiments may have resulted in multiple publications concerning the GI microbiota. As the outcomes may differ in detail and description, all publications are listed in the tables. However, a summary of these studies is provided in the text and data evaluation sections.

2.4. Data items and statistics

The following information was extracted from each included article for groups 1 and 2: study characteristics, conditions of isolation, methods of GI microbiota analysis and outcomes. Each study's characteristics are reported using the original data and summarized in tabular form. Characteristics across the studies are presented as mean, minimum and maximum for sample size, age, body mass index (BMI) and study length.

Primary outcomes concerning the GI microbiota were alpha diversity (richness and biodiversity), community structure/beta diversity and significant shifts in the abundance of individual microbial taxa (according to the current version of the International Code of Nomenclature of Prokaryotes (50)). Significant differences for alpha diversity, community structure, and taxonomy abundances at both phylum and genus level were summarized for isolation/in-mission and post-isolation/post-mission. We also described conclusions on a pre/post comparison if this was possible. For the microbial abundance outcomes in studies using molecular based methods which were not specifically referred to in text, no effect was presumed and written as unchanged (↔). If data was only presented graphically, the abundance shifts were extracted as best as conceivable. The graphical representation of relative abundance shifts was limited to the phylum level to avoid over- or underreporting of data. Non-isolated control subjects were not considered in further analysis. The data were analyzed separately for groups and subgroups. Finally, data was summarized across the habitats. Secondary outcomes

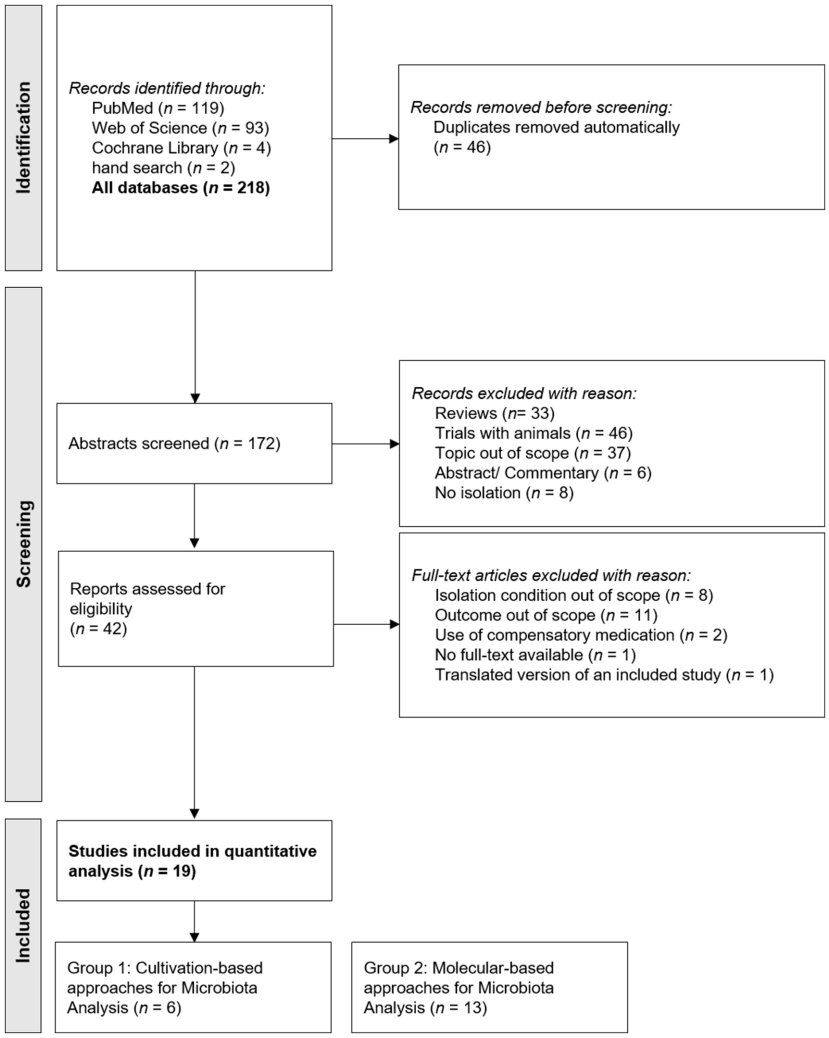

FIGURE 1
PRISMA flow chart for study inclusion.

including anthropometric, clinical, behavioral, psychological changes were also retrieved.

2.5. Risk of bias

For the included studies, a risk of bias assessment was performed using Risk of Bias In Non-randomized Studies of Interventions (ROBINS-I) tool (51). As only non-randomized isolation interventions were included in this systematic review, we chose the Cochrane tool as this tool views each study as an attempt to emulate a hypothetical pragmatic randomized trial and covers seven distinct domains through which bias might be introduced. In the first two domains, issues related to confounding and selection of participants are addressed before the interventions to be compared ("baseline"), while the third domain discusses intervention classification. In the remaining four domains, the following issues are addressed after the start of interventions: biases due to deviations from intended interventions, missing data, measurement of outcomes, and selection of the reported result.

The rating ranged between "Low risk," "Moderate risk," "Serious risk" and "Critical risk" of bias. The authors declare that "Low risk" corresponds to the risk of bias in a high-quality trial, however, due to the limited number of studies found, no study was excluded for risk of bias.

3. Results

The literature search process for identification of eligible studies is shown in Figure 1. Out of 218 identified studies, 19 studies remained for qualitative analysis. Six articles were categorized in group 1 (cultivation-based approaches for microbiota analysis) and 13 articles in group 2 (molecular-based approaches for microbiota analysis).

3.1. Summary of the study characteristics

A detailed overview of the characteristics for the single trials is presented in Table 1 for cultivation methods and Table 2 for molecular-based approaches. The characteristics across the studies are summarized in the text.

The 19 included studies ranged from 1964 to 2021 and data was mainly published by Asian researchers ($n = 15$), otherwise from

TABLE 1 Characteristics of studies using cultivation methods, characteristics are split according to corresponding isolation habitats.

Study ID	Author (year)	Origin	Isolation	Length (days)	Sample size (participants)	Age (years)	Sex, % f	Pre-BMI (kg/m^2)	Sampling time	Cultured microbes	Data derivation	Diet	Additional intake
Subgroup 1: Isolation caused by space missions													
1	Lizko et al. (52)	USSR	Salute 4[1] Sojus 17	30	2	43	0	N.R.	Pr/Du/Po	*Staphylococcus*, *Streptococcus*, *Proteus*, *E. coli*, *Lactobacillus*, Bacteroids, Bifidobacteria, Spore-forming bacteria, Yeast	Text	N.R.	No
			Salute 4[1] Sojus 18	63	2	32, 40							Yes (not further described)
Subgroup 2: Isolation caused by spaceflight- or gravity simulators													
2	Chen et al. (53)	CHN	6 ° HDBR[2]	45	7	26.13 ± 4.05	0	21.7	Pr/Du/Po	N.R.	Text	Controlled, but no special diet	N.R.
3	Cordaro et al. (54)	USA	Oxygen chamber[3]	56	4	27–29	0	23.9	Pr/Du/Po	Coliforms, *Proteus*, Salmonellae, Staphylococci, Enterococci, *Streptococcus salivarius*, Yeasts, Diphtheroids, Bacteroids, Clostridia, Lactobacilli	Graphic	Compressed freeze-dried cubes	N.R.
4	Gall and Riely (55)	USA	Experimental activity facility	45	4	21–34	0	N.R.	Du	Staphylococci, Streptococci, Lactobacilii, *Haemophilus*, Neisseria, Enterobacteriaceae, *Shigella, Salmonella, E. coli*, *Klebsiella*, Yeast, *Proteus, Pseudomonas*	Text	Space-type freeze dehydrated diet (60 foods) and equivalent fresh food	N.R.
5	Rerberg et al. (56)	SIB	BIOS-3[4]	120–180	4	N.R.	N.R.	N.R.	Du	N.R.	Text	N.R.	N.R.
6	Shilov et al. (57)	RUS	Hermetic chamber	365	3	N.R.	N.R.	N.R.	Pr/Du/Po	Bifidobacteria, Lactobacilli, *E. coli,* Streptococci, Clostridia, Staphylococci, Yeast, Protea	Text + graphic	Common mixed diet from natural products	N.R.

"Isolation": Isolated and confined extreme environments. "Length": length of the isolation intervention, no pre/post surveys are considered. The sampling times are either before the start of the intervention ("Pre"), during the isolation itself ("During"), after the isolation ("Post"), or a combination of more than one of these times. "Additional intake": intake of pro/pre/antibiotics during the isolation period.

[1]A Soviet space station. [2]Gravity simulation. [3]Oxygen chamber has an oxygen content of 70%, a helium content of 30%. [4]Experimental project of a closed ecosystem. 6°HDBR, −6° head-down bed rest; CHN, China; Du, During; f, female; N.R., not reported; Po, Post; Pr, Pre; RUS, Russia; SIB, Siberia; USA, United States of America; USSR, Union of Soviet Socialist Republics; BMI, Body Mass Index.

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TABLE 2 Study characteristics of studies using molecular-based approaches, characteristics are split according to corresponding isolation habitats.

Study ID	Author (Year)	Origin	Isolation	Length (days)	Sample Size	Age (years)	Sex, % f.	Pre-BMI (kg/m²)	Sampling point	Analysis method	Microbiota classification	Data derivation	Diet	Additional intake
Subgroup 1: Isolation caused by space missions														
1	Garret-Bakelman et al. (25)	USA	Spaceflight	340	1	50	0	N.R.	Pr/Du/Po	WMGS (Illumina)	N.R.	Text + graphic	Restricted diet	N.R.
2	Liu et al. (40)	CHN	Spaceflight	35	2	N.R.	N.R.	N.R.	Pr/Po	WMGS (Illumina)	N.R.	Text + graphic	Similar with ground life	N.R.
3	Voorhies et al. (37)	RUS/ USA	Spaceflight	180–360	5	N.R.	N.R.	N.R.	Pr/Du/Po	16S rRNA gene (V4 hypervariable region) (Illumina)	OTUs	Text + graphic	N.R.	none
Subgroup 2: Isolation caused by spaceflight- or gravity simulators														
4	Brereton et al. (58)	RUS	Mars-500[1]-unit	520	6	29–40	0	25.5 (23.0–31.3)	Du	16S rRNA gene (V3-V4 hypervariable region) (Illumina)	ESVs	Text + graphic	Tinned foods similar to those used in ISS, by RUS/EUR/KOR/CHN firms, 15.1% protein, 33.4% fat, and 51.2% CH	*Streptococcus thermophilus* via yoghurt
5	Turroni et al. (59)					31.8 (27–38)			Pr/Du/Po		OTUs	Text		N.R.
6	Mardanov et al. (44)			510	5	28–38	N.R.	N.R.	Pr/Du/Po	16S rRNA gene (V3-V5 hypervariable region) (Pyrosequencing)	N.R.	Text + graphic		*Enterococcus faecium*, Eubikor, Vitaflor
7	Hao et al. (60)	CHN	Lunar Palace 1 (BLSS)	79–105	3	27–32	66. 6	21.3 ± 3 (19–24)	Pr/Du/Po	16S rRNA gene (V3-V4 hypervariable region) (Illumina)	OTUs	Text	Predesigned high-plant and high-fibre diet	N.R.
8	Li et al. (46)							N.R.				Graphic		
9	Meng et al. (61)	CHN	Lunar Palace 1 (BLSS)	63	4	26	50	N.R.	Du	WMGS (Illumina)	N.R.	Text	NASA astronauts' dietary standards	N.R.
10	Chen et al. (62)	CHN	BLSS	60	4	23–27	50	18.5–22.9	Pr/Du/Po	WMGS (BGI-SEQ500 platform)	N.R.	Text	↑ CH, ↓ fat	N.R.
11	Dong et al. (63)	CHN	CELSS	180	4	26–36	25	18.6–24.6	Pr/Du	16S rRNA gene (V3-V4 hypervariable region) (Illumina)	OTUs	Text + graphic	in accordance with customs of Chinese population, 3 meals/d, main composition: CH, protein, fat, fiber	N.R.
Subgroup 3: Isolation in a natural, earth-bound habitat														
12	Jin et al. (64)	JPN	Antartica	60	6	37–55	0	N.R.	Pr/Du/Po	16S rDNA (T-RFLP, rtPCR)	OTUs	Text	N.R.	N.R.
13	Zhang et al. (65)	CHN	Sea voyage	30	42	25 ± 4.2	0	22.3 ± 2.7	Du	WMGS (Illumina)	N.R.	Graphic	Controlled, similar diet, Buffet style, min. 2 staple foods/d, 5 entrées/d, 2–3 fruit/d	*Lactobacillus casei Zhang*, *Lactobacillus plantarumP-8*, *Lactobacillus rhamnosus M9*, *Bifidobacterium lactis V9*, *Bifidobacterium lactis M8*
					40									no

"Isolation": Isolated and confined extreme environments. "Length": length of the isolation intervention, no pre/post surveys are considered. The sampling times are either before the start of the intervention ("Pre"), during the isolation itself ("During"), after the isolation ("Post"), or a combination of more than one of these times. "Additional intake": intake of pro−/pre−/antibiotics during the isolation period. Control groups are not shown.

[1]Project to simulate a manned flight to Mars. 16S rDNA, 16S ribosomal Deoxyribonucleic acid; 16S rRNA, 16S ribosomal Ribonucleic acid; BLSS, Bioregenerative life support systems; CELSS, Controlled (or closed) ecological life-support systems; CH, carbohydrates; CHN, China; d, day; f, female; Du, During; JPN, Japan; N.R., not reported; Po, Post; Pr, Pre; rtPCR, reverse transcription polymerase chain reaction; RUS, Russia; T-RFLP, Terminal restriction fragment length polymorphism; USA, United States of America; WMGS, Whole metagenome shotgun sequencing. BMI, Body Mass Index; OTU, Operational taxonomic unit; ESV, exact sequence variants; KOR, Korea; EUR, Europe; NASA, National Aeronautics and Space Administration; ISS, International Space Station.

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American ($n = 3$) or by multiple institutions working together ($n = 1$). Shifts in microbial abundance as a consequence of residency in space ($n = 4$) or long-term confinement in natural, terrestrial habitats ($n = 2$) have been reported. However, the alterations of the human GI microbiota by isolation was mainly studied in spaceflight- and gravity simulation facilities ($n = 13$ via 9 different units). There are several publications on two of these experimental units: In the case of the Mars-500 experiment, 3 publications were identified (44, 52, 53) that differed partly regarding sample size, methodology and microbiota classification. Likewise, in case of the Chinese Lunar Palace 1 experiment, 3 publications (46, 54, 55) were found showing similar differences between each other. We thus examined a total of 142 participants being mainly men (exact number unclear). Most of the studies were conducted exclusively in men (9 studies), in 5 studies both sexes were included while in 5 studies sex was not reported. The median number of subjects involved in an intervention was 4, ranging from 1 to 82, covering the ages between 21 and 50 years (median age 30.4 years). Due to the natural or artificially created extreme conditions, all study subjects were of healthy condition and predominantly of normal weight (median BMI 22.6 kg/m^2). The isolation intervention lasted on average 120 days (covering 30–520 days) and was accompanied by pre- and/or post-intervention measurements in >65% ($n = 13$). In 10 experiments, the diet was very tightly controlled and based on a typical space diet. Furthermore, 4 studies investigated the possibility of maintaining the GI microbiota composition by providing the volunteers with supportive probiotics or prebiotics for either regular or intermittent intake. Although all research groups collected stool samples to study the microbiota composition, no study reported stool frequency and/or consistency.

3.2. Summary of study outcomes

A detailed overview of the in-mission outcomes for the single trials is presented in Table 3 for cultivation-based methods and Table 4 for molecular-based approaches. The outcomes across the studies are in the text and summarized in Figure 2. Further outcomes of pre- and post-mission are provided in the Supplements (Supplementary Table S1, S2).

In the cultivation studies, changes in *Lactobacillus*, *Bifidobacterium*, (potentially pathogenic) *Escherichia*,

TABLE 3 Study outcomes of microbiota analysis by cultivation method in-mission, outcomes are split according to corresponding isolation habitats.

Study ID	Author (year)	Subject	*Lactobacillus* spp.	*Bifidobacterium* spp.	*Escherichia* spp.	Other
Subgroup 1: Isolation caused by space missions						
1	Lizko et al. (52)	Sojus 17/1	↓	N.R.	N.R.	N.R.
		Sojus 17/2	↔	↔	↔	↔
		Sojus 18/1	↑	↑	↑	Spore-forming bacteria ↑ *Proteus* spp. ↔
		Sojus 18/2	↑	↑	↑	Spore-forming bacteria ↑ *Proteus* spp. ↓
Subgroup 2: Isolation caused by spaceflight- or gravity simulators						
2	Chen et al. (53)	∑	↓	↓	N.R.	N.R.
3	Cordaro et al. (54)	∑	↔	N.R.	N.R.	*Bacteroides* spp. ↔ *Enterococci* spp. ↓ Coliforms ↔
4	Gall and Riely (55)	1	↔	N.R.	↓ / ↑	*Klebsiella* spp. ↔ *Citrobacter* spp. ↓ *Shigella boydii* ↓ *Corynebacteria* spp. ↑
		2	↔	N.R.	↔	*Corynebacteria* spp. ↑
		3	↔	N.R.	↔	*Klebsiella* spp. ↑ *Corynebacteria* spp. ↑
		4	↔	N.R.	↑	*Klebsiella* spp. ↑ *Corynebacteria* spp. ↑
5	Rerberg et al. (56)	∑	↓	↓	N.R.	*Bacteroides* spp. ↔ *Clostridium perfringens* ↑
6	Shilov et al. (57)	∑	↓	↓	N.R.	*Clostridium perfringens* ↔
			↓	↓	N.R.	*Clostridium perfringens* ↑
			↔	↔	N.R.	*Clostridium perfringens* ↑

The arrows represent the direction of the microbial abundance shift. ↓: abundance reduction, ↑: abundance increase, ↔ no change in abundance detected. The study subjects can be reported individually, in groups or summarized for all study participants (∑). However, since this table summarizes multiple species within a genus (spp.), it is possible for the direction of shifts to vary among participants. N.R., not reported; spp.: multiple species of a genus.

TABLE 4 Bacterial diversity and microbial abundance shifts in-mission analyzed by molecular-based techniques, outcomes are summarized on phylum level.

Study			Microbial diversity shifts			Relative abundance shifts at phylum level					
Study ID	Author (Year)	Subject	α-D: Richness	α-D: Biodiversity	Community structure	Bacillota	Bacteroidota	Actinomycetota	Pseudomonadota	Verrucomicrobiota	Other
Subgroup 1: Isolation caused by space missions											
1	Garret-Bakelman et al. (25)	∑	↔	↔	Dis.	*Taxa shifts have not been assigned by name.*					
2	Liu et al. (40)	A	↑		Dis.	↑	↓	↑	↑	↑	Fusobacteria ↔ Chlamydiae ↓ Tenericutes ↓ Aquificae ↔
		B	↓		Dis.	↓	↑	↓	↔	↑	Fusobacteria ↔ Chlamydiae ↓ Tenericutes ↔ Aquificae ↓
3	Voorhies et al. (37)	∑	↑	↑	Dis.	↔	↔	↔	↔	↔	↔
Subgroup 2: Isolation caused by spaceflight- or gravity simulators											
4	Brereton et al. (58)	∑	↔	↔	N.R.	↔	↔	↔	↔	↔	↔
5	Turroni et al. (59)	∑	N.R.	N.R.	Sim.	↔	↔	↔	↔	↔	↔
6	Mardanov et al. (44)	A	N.R.	N.R.	N.R.	↔	↔	↔	↔	↔	↔
		B	N.R.	N.R.	N.R.	↔	↔	↔	↔	↔	Fusobacteria ↓
		C	N.R.	N.R.	N.R.	↔	↔	↔	↔	↓	↔
		D	N.R.	N.R.	N.R.	↔	↔	↑	↔	↔	↔
		E	N.R.	N.R.	N.R.	↔	↔	↔	↔	↔	↔
7	Hao et al. (60)	∑	↑		Dis. *	↑	↓	↔	↔	↔	↔
8	Li et al. (46)	∑	N.R.	N.R.	N.R.	↔	↔	↔	↔	↔	↔
9	Meng et al. (61)	∑	N.R.	N.R.	N.R.	↔	↔	↔	↔	↔	↔
10	Chen et al. (62)	∑	N.R.		Dis. *	↔	↔	↔	↔	↔	↔
		f	↓			↔	↔	↔	↔	↔	↔
		m	↑			↔	↔	↔	↔	↔	↔
11	Dong et al. (63)	∑	↓	↓	N.R.	↓	↑	↔	↔	↔	Fusobacteria ↑
Subgroup 3: Isolation in a natural, earth-bound habitat											
12	Jin et al. (64)	∑	N.R.	N.R.	N.R.	↔	↔	↔	↔	↔	↔
13	Zhang et al. (65)	Plac.	↔	↔	Dis.	↔	↔	↔	↔	↔	↔
		Pro.	↔	↔	Sim.	↔	↔	↔	↔	↔	↔

The arrows represent the direction of the microbial abundance shift. ↓: abundance reduction, ↑: abundance increase, ↔ no change in abundance detected, *: Trend to converge. The study subjects can be reported individually, in groups or summarized for all study participants (∑). α-D, alpha-Diversity; Dis, dissimilar; f, female; m, male; N.R., not reported; Plac., Placebo group; Pro., Group taking additional probiotics during the intervention; Sim., similar.

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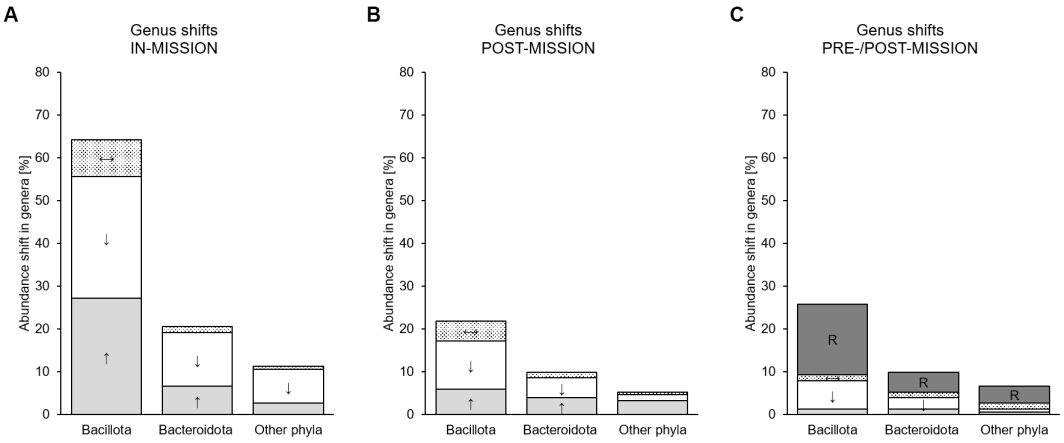

FIGURE 2
Microbial shifts in studies using molecular-based approaches for microbiota analysis [%] summarized in the genera-related phyla assuming that all shifts were reported, but everything else remained unchanged. Shifts that occured during isolation (in-mission, **(A)**, after isolation (post-mission, **(B)** and reported in the before-after comparison (pre/post-mission, **(C)** are presented. Summarized are Bacillota-, Bacteroidota- and other Phyla-related genera. ↓, white bar: Genus/species abundance [%] decreased during intervention; ↑, light grey bar: Genus/species abundance [%] increased during intervention; ↔, dotted bar: Genus/species abundance [%] remained unchanged; R, dark grey bar: Genus/species frequency [%] restored or partly restored to baseline levels.

spore-forming bacteria, *Proteus, Bacteroides*, *Enterococcus*, *Klebsiella, Citrobacter*, *Shigella, Corynebacterium* and, in simulated emergency situations, changes in *Clostridium perfringens* were detected. One study found that the Bacteroides were the most numerous and stable, whereas severe fluctuations occurred in the transit microbiota (mentioned *Staphylococcus* and yeast) (56). Another study also reported alterations, which, however, tended to return normal after the end of the confinement (58). Finally, one study examined the aerobic and anaerobic flora in four young men over a six-week period. As the subjects had been on the space-diet for a longer period of time, differences in both the aerobic and anaerobic flora began to appear. Seven new types of organisms became prevalent, which had not been described before, mostly gas-forming, black slime-producing, and proteolytic organisms. It was assumed that the specialized space-diet (not further reported) was a contributing factor (59). Due to the different cultivation conditions, the cultivated bacterial groups observed were heterogeneous between studies.

The introduction of molecular-based approaches led to a better assessment of the microbiota allowing for a comprehensive description of microbial communities. Out of 13 studies, alpha diversity was reported to be reduced (richness: $n = 3$; biodiversity $n = 3$), to remain stable (richness: $n = 4$; biodiversity $n = 4$) or to increase (richness: $n = 4$; biodiversity $n = 4$). Richness and biodiversity were not reported separately in 3 studies (40, 56, 60). More dissimilarities were reported in microbial community structure ($n = 7$) with a tendency towards convergence observed repeatedly (54, 60). However, in a study that reported the differences between control subjects, the scale of microbiota changes in microbial diversity in the test subject during isolation was relatively small (25).

At the phylum level abundances predominantly remained stable following the confinement; in all but 4 of the 13 included studies ($n = 14$ participants) shifts in abundances were observed.

Changes reported at genus (Table 5) and species level (Supplementary Table S3) were not consistent across studies. Even more, *Bacteroides* (spp.), *Eubacterium* (spp.), *Faecalibacterium* (spp.), *Lactobacillus* (spp.), *Prevotella* (spp.), *Alistipes* (spp.), Blautia (spp.), *Lachnospira*, *Ruminococcus* (spp.), *Bifidobacterium* (spp.) and *Clostridium* (spp.) were reported to be either increasing or decreasing in their relative abundances in-mission. Across all studies, abundance shifts were predominantly associated with the phyla Bacillota (67.5%) and Bacteroidota (20.5%, Figure 2). Regardless of the taxonomic ranking, two studies reported no shifts at all (25, 55). The proportions of increasing and decreasing phyla decrease in Figures 2B,C due to the diminishing number of reporting studies.

No isolation type provided an indication that the intensity of a change is related to the length of the confinement.

3.3. Subgroup analyses

Due to the heterogeneity in outcome comparisons and microbiota analysis techniques used, it was not feasible to summarize the findings clearly in figures. This led to the breakdown into groups and subgroups, as mentioned in the methods section.

3.3.1. Subgroup analysis 1: GI microbiota under isolation conditions in space

One study cultivated and analyzed the samples collected during a space mission lasting 30 and 63 days each with 2 astronauts independent of one another (61). Most of the bacteria analyzed remained stable in the pre/post comparison except for a decline in *Lactobacillus* and a reduction of *Escherichia* and *Proteus* in two individuals.

Three other studies (25, 37, 40) published data from space samples ($n = 8$ astronauts) using molecular-based techniques to analyze microbiota changes.

Beta-diversity has consistently been described as dissimilar in-mission (25, 37, 40), while both richness and evenness demonstrated a rather heterogeneous distribution. In one study, Shannon's alpha diversity and richness significantly increased in space

TABLE 5 Study outcomes for each subgroup at genus level for all studies and all sampling times (pre-intervention, during-intervention, post-intervention) for molecular-based analyses.

Phylum	Genus	Space									Simulation									Natural, earth-bound								
		In-mission			Post-mission			Pre/post			In-mission			Post-mission			Pre/post			In-mission			Post-mission			Pre/post		
Verrucomicrobiota	*Akkermansia* (spp.)		↓[3]						R[3]						↓[5]														
Bacteroidota	*Alistipes* (spp.)	↑[2]				↓[2]		↑[2]		↔[2]	↑[6]	↓[11]				↔[6]		pR[6]			↓[13]								
Bacillota	*Anaerostipes* (spp.)											↓[4,10]																	
Bacteroidota	*Bacteroides* (spp.)	↑[2]	↓[2]		↑[2]	↓[2]		↑[2]	↓[2]		↑[4,5]	↓[6,10,11]	↔[6]	↑[6]	↓[6]	↔[6]		pR[6]			↓[12,13]		↑[12]			R[12]			
Actinomycetota	*Bifidobacterium* (spp.)		↓[2]	↔[2]	↑[2]		↔[2]					↓[10]								↑[12,13]	↓[12]		↑[12]			R[12]			
Bacillota	*Blautia* (spp.)	↑[2]		↔[2]	↑[2]	↓[2]		↑[2]		↔[2]	↑[7]	↓[4]			↓[7]														
Bacteroidota	*Butyricimonas*											↓[11]																	
Bacillota	*Christensenellaceae*										↑[4]																		
Bacillota	*Clostridium* (spp.)	↑[2]		↔[2]		↓[2]			↓[2]			↓[10]								↑[12]	↓[12,13]		↑[12]	↓[12]					
Bacillota	*Coprococcus* (spp.)								↓[3]				↔[5,6]					pR[6]											
Bacillota	*Dialister*											↓[11]	↔[6]		↓[6]	↔[6]		pR[6]											
Bacillota	*Dorea* (spp.)		↓[3]						R[3]				↔[5]																
Pseudomonadota	*Enterobacter* (spp.)											↓[10]																	
Bacillota	*Enterobacteriales*																				↓[12]			↓[12]					
Pseudomonadota	*Escherichia* (spp.)		↓[2]		↑[2]	↓[2]			↓[2]	↔[2]		↓[10]																	
Bacillota	*Eubacterium* (spp.)	↑[2]	↓[2,3]			↓[2]		↑[2]	↔[2]	R[3]																			
Bacillota	*Faecalibacterium* (spp.)	↑[3]	↓[2]	↔[2]		↓[2]			↓[2]	R[3]	↑[7,11]	↓[4,5,10]			↓[7]			pR[6]											
Bacillota	*Flavonifractor*																				↓[13]								
Bacillota	*Fusicatenibacter*	↑[3]							R[3]																				
Bacillota	*Kineothrix*										↑[4]																		
Pseudomonadota	*Klebsiella*																				↓[13]								
Bacillota	*Lachnospira*	↑[3]							R[3]		↑[7]	↓[4,11]			↓[7]														
Bacillota	*Lachnospiraceae*	↑[3]							R[3]		↑[4,10]																		
Bacillota	*Lactobacillus* (spp.)	↑[2]	↓[2]			↓[2]	↔[2]		↓[2]			↓[4]								↑[13]									
Bacillota	*Lactococcus*																				↓[13]								
Fusobacteria	*Leptotrichia*		↓[3]						R[3]																				
Bacillota	*Megamonas*										↑[6]			↑[6]				pR[6]										
Bacillota	*Megasphaera*		↓[3]						R[3]																				
Bacteroidota	*Parabacteroides*											↓[11]																	
Pseudomonadota	*Parasutterella*	↑[3]							R[3]																				
Bacillota	*Phascolarctobacterium*											↓[6]	↔[6]	↑[6]	↓[6]	↔[6]		pR[6]											
Bacteroidota	*Prevotella* (spp.)	↑[2]	↓[3]			↓[2]		R[3]	↓[2]	↔[2]	↑[10,11]	↓[6,7]	↔[6]	↑[6,7]	↓[6]		↑[6]	↓[6]	R		↓[13]								
Bacillota	*Pseudobutyrivibrio*		↓[3]						R[3]		↑[11]																		
Bacillota	*Roseburia* (spp.)	↑[2]				↓[2]			↓[2]			↓[4]									↓[13]								
Bacillota	*Ruminiclostridium*	↑[3]							R[3]																				
Bacillota	*Ruminococcaceae*										↑[4]																		
Bacillota	*Ruminococcus* (spp.)		↓[3]						↓[3]		↑[4]	↓[4,11]																	
Bacillota	*Streptococcus*		↓[3]						R[3]		↑[4]									↑[13]	↓[13]								
Bacillota	*Subdoligranulum*										↑[11]																		
Bacillota	*Veillonella*		↓[3]						R[3]		↓[10]																		

Microbiota abundance fluctuations/continuities identified at genus level in (1) Subgroup 1: Isolation in Space [Study ID 1–3, molecular-based approaches (Tables 2, 4)], (2) Subgroup 2: Isolation in a simulation unit [Study ID 4–11, molecular-based approaches (Tables 2, 4)], and (3) Subgroup 3: Natural earth-bound isolation [Study ID 12–13, molecular-based approaches, (Tables 2, 4)] either (i) through the intervention compared to baseline (in-mission) or (ii) after the isolation period compared to the intervention (post-mission) or (iii) after the isolation compared to baseline (pre−/post-mission).

↓, Relative abundance decreased; ↑, Relative abundance increased; ↔, No significant shifts in relative abundance. R, Restored back to baseline level. pR, partially restored back to baseline. Studies have reported only on genus level or also including some species (spp.).

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and returned to their baseline preflight levels after crew members returned to earth (37). Another study reported that alpha diversity at genus level did not fluctuate significantly, but the fluctuations between each subject were dissimilar (40).

Phyla abundance remained rather stable; only Liu et al. (40) observed changes during and after the isolation as well as significant changes in the pre−/post-comparison. Over a 35-day space mission, two study subjects were isolated and examined. The phyla of the two study subjects conflicted; when a phylum of one subject changed, its equivalent did not change or in the other direction. Bacillota and Bacteroidota displayed a remarkable antagonistic behavior in both subjects (Table 4). Additionally, two studies (37, 40) reported data on shifts at the genus level for $n = 7$ subjects (Table 5; Supplementary Table S3). Most changes in the abundance of genera were related to Bacillota. Concurrent divergent shifts to either higher or lower abundances of the same genera were described, confirming heterogeneity. Almost all shifts were abolished after the spaceflight, indicative of resilience. Further results are very heterogeneous and are therefore not discussed here.

3.3.2. Subgroup analysis 2: GI microbiota under isolation conditions in experimental facilities

Isolations conducted in a controlled artificial environment or similar unnatural unit using cultivation techniques predominantly identified *Lactobacillus*. There was either no change or a drop in *Lactobacillus*. *Bacteroides* was characterized to be one of the most stable microbial groups. *Bifidobacterium* spp. was cultivated less commonly, however, most subjects showed a reduction of the genus across studies. Inconsistent changes were observed in *Escherichia coli* and *Klebsiella* and unique shifts in Coliforms and *Enterococcus*, *Corynebacterium*, *Shigella boydii* and *Citrobacter*. In the course of the experiment, some subjects showed a prominent increase in toxigenic strains of *Clostridium perfringens*, which is suspected of being related to lipid metabolism.

Eight other studies published data from simulation unit experiments using molecular-based approaches to analyze microbiota changes (Table 4). For this subgroup, it is hardly possible to comment on diversity, as it was either rarely reported or, if reported, did not give a conclusive trend. Several shifts occurred during isolation (Supplementary Table S3) in *Bacteroides* (predominantly reducing), *Faecalibacterium* (rather reducing) and *Prevotella* (no directional tendency). *Lachnospira* and *Ruminococcus* also frequently showed significant changes in their abundance during the intervention, but these were very heterogeneous with tendencies towards reduction. One study (44) further reported a follow-up time and compared the pre- and post-measurements. Almost all of the investigated genera showed a return to the baseline proportions or they were at least partly restored to baseline levels.

3.3.3. Subgroup analysis 3: GI microbiota under isolation conditions in natural isolated habitats

There was no data from natural habitats using cultivation methods.

Using molecular-based approaches, two studies provided data from natural isolated habitats. During a period of 2 months, study subjects ($n = 6$) were stationed on a research station in Antarctica for 3 months in the study of Jin et al. (62). The study results were presented in terms of Operational Taxonomic Units (OTUs), showing mainly changes in the abundance of *Bacteroides* and *Bifidobacterium* species, decreasing during the expedition in four subjects and increasing in two subjects. Furthermore, a comparison between pre- and post-residence in Antarctica was possible for OTUs, with predominantly *Bacteroides* spp. showing no significant changes while all *Bifidobacterium* spp. increased, decreased, or remained stable. The study found that the participants had interindividual variability in their fecal microbiota, and cold, stress and changes in food intake were possible factors affecting their microbiota.

The study by Zhang et al. (63) determined the effects of probiotics on sailors ($n = 82$) over the course of a 30-day cruise. Here, it needs to be considered, that this study is different from the other with extreme environments due to the large samples size. Probiotics were administered to some but not all of the sailors in this study. None of the described study models caused an increase or decrease in the abundance of phyla; even with the administration of probiotics to sailors, no differences were found. However, the administration of probiotics indicated that the impact of a long sea voyage on the intestinal microbiota were significant as beta diversity distances were significantly larger in the placebo group than in the probiotic group. The result was confirmed by alpha-diversity, which showed no significant differences among groups, but a sharp decline between probiotic and placebo groups while on mission. Only shifts in abundances at the species level were found, whereby the ratio between declines and increases was rather balanced with tendencies towards more increases.

3.4. Similarities and differences across habitats

Studies in space and simulation units were more similar in both genera and species profiles, while in the natural habitats the shifts were reported to be more diverse (Table 5; Figure 3). However, 8 shifts in genera or genus-specific species were shared by all three habitats. Space and simulation experiments shared shifts in 11 genera or genus-specific species.

There was a high degree of heterogeneity throughout all habitats during the intervention. It has been observed that neither species that exhibited abundance shifts across all three habitats showed the same trend. Only some *Streptococcus* species shifted rather consistently in abundance during the intervention (Supplementary Table S3).

3.5. Further outcomes

Besides GI microbiota responses to extreme environmental conditions, significant reductions in body mass (25, 54, 55) as well as energy intake (64) were found. In line with these results are blood values for metabolism. Li et al. (46) and Hao et al. (54) reported that the participants' health parameters (heart rate, blood pressure and BMI) were within the normal range throughout the study.

Furthermore, significant changes in several physiological parameters were observed, accompanied by an increase in inflammatory parameters (37, 53), changes in urinary metabolites (65), a decrease in mineral bone density and in muscle turnover (65), changes in respiratory minute volume (56), telomere elongation (25) as well as disrupted glucose metabolism (53), affected antibiotic resistance genes (40), genome instability, DNA methylation in immune and oxidative stress-related pathways (25) and finally a reduction in 25-hydroxyvitamin D levels (64). However, none of the participants demonstrated symptoms of diseases associated with considerable changes in the composition of the microbiota (44). After the isolation, 25-hydroxyvitamin D levels (64), but also mean

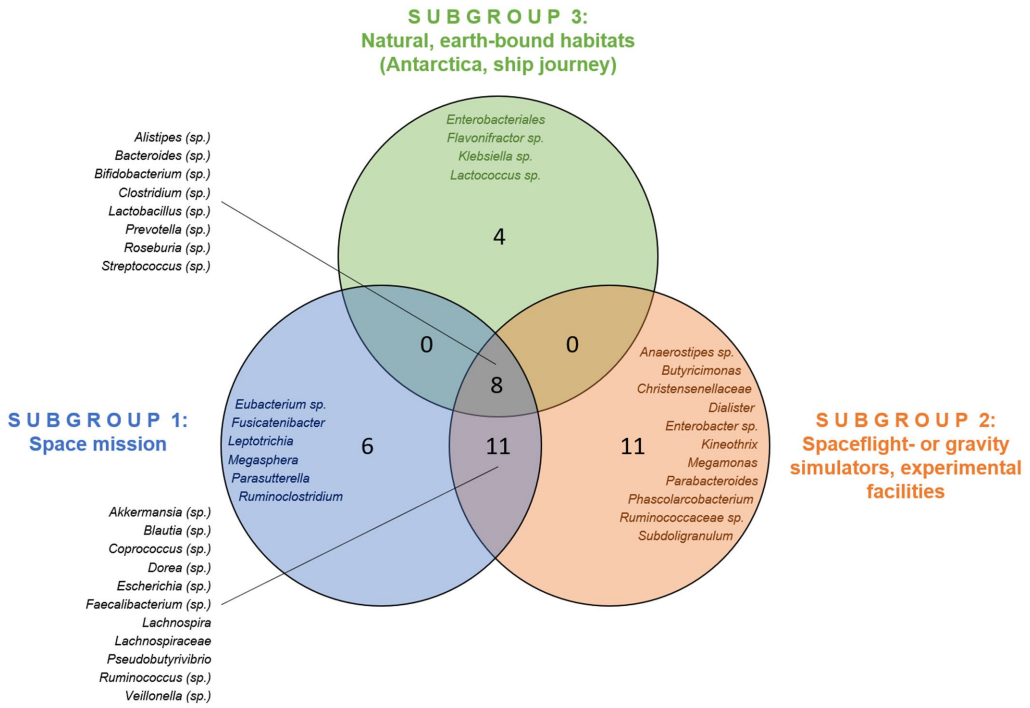

FIGURE 3
Overlap of reported changes in genera across the habitats studied. Species-specific changes (sp.) are also mentioned.

telomere length and global gene expression converged to pre-flight levels, with an increased number of short telomeres observed and the expression of some genes still disturbed (25). However, it remains unclear if these changes are in relation to confinement or in association to microbiota shifts.

In contrast, some psychological parameters were reported to be affected, e.g., an increase in the abundance of *Faecalibacterium* spp. correlating negatively with mood (46). Scores of stress and anxiety seemed to be lower (63), except when in the situation of absence of natural light, where the incidence of anxiety and depression increased (55). After the isolation period of 340 days, a cognitive decline was observed (25).

However, many studies reporting different aspects of human biology and health outcomes due to isolation and confinement have been published elsewhere (42, 66) which is why only the changes that have been reported in the context of our investigation are mentioned here.

3.6. Risk of bias

The risk of bias for the studies included in the review conducted via the ROBINS-I checklist is presented in Table 6 for all studies. Among the 19 non-RCTs, 1 was considered to have critical risk of bias due to confounding bias and 4 were at serious risk of bias. It seems that the older studies have this increased risk. Only one study was included providing a low risk of bias. A moderate risk of bias was commonly attributed to bias in selection of the reported results, which is why the highest number of studies provides a moderate risk (68%).

4. Discussion

This review included 19 articles and examined to what extent the GI microbiota changes during and after a stay in an isolated, antigen-limited or poor environment. Overall, changes in GI microbiota were observed and reported following confinement; however, the type and extent of the reported changes varied between studies. The data do not point to a clear direction, but nevertheless, in line with the assumptions, indicate to some extent trends in changes.

This review affirms that experimental isolation results in a decrease in alpha diversity (richness and biodiversity) and does not lead to increased similarity in the GI microbiota among subjects. A systematic review examining the human GI microbiota during long-term space missions confirms our results suggesting that space travel may lead to microbial dysbiosis and metabolic changes in the human gut, including a drop in alpha diversity (67). Similarly, a recent experimental study investigating the impact of an enhanced spaceflight diet on 16 subjects over a 45-day closed chamber mission found that subjects eating a standard diet demonstrated a decline in Shannon's alpha biodiversity and richness during the mission, but recovered fully by the end of it (68). However, these effects are not evident and consistent across all participants of our review, as an increase in richness and biodiversity has been observed in some test persons, which is suspected in relation to an increased dietary fibre intake (54) or merely due to individual differences (40). The increase of alpha richness and biodiversity is discussed extensively with regard to health protection (37, 54) since a biodiverse ecosystem is more resistant and resilient to perturbations and has a lot more functional redundancy (69). In the studies we know of involving additional administration of probiotics (53, 63), both richness and biodiversity

TABLE 6 ROBINS-I risk of bias for all studies.

Study	Bias due to confounding	Bias in selection of participants into the study	Bias in classification of interventions	Bias due to deviations from intended interventions	Bias due to missing data	Bias in measurement of outcomes	Bias in selection of the reported result	Overall bias
Brereton et al. (53)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Chen et al. (65)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Chen et al. (60)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Cordaro et al. (58)	NI	Low	Low	Low	Moderate	Serious	Moderate	**Serious**
Dong et al. (64)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Gall and Riely (59)	Critical	Low	Low	Low	Low	Moderate	Moderate	**Critical**
Garret-Bakelman et al. (25)	Moderate	Low	Low	Moderate	Low	Low	Moderate	**Moderate**
Hao et al. (54)	Low	Moderate	Low	Low	Low	Low	Moderate	**Moderate**
Jin et al. (62)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Li et al. (46)	Low	Serious	Low	Low	Low	Low	Moderate	**Serious**
Liu et al. (40)	Low	Low	Low	Low	Low	Low	Low	**Low**
Lizko et al. (61)	Low	Low	Low	Low	Serious	Moderate	Moderate	**Serious**
Mardanov et al. (44)	Serious	Low	Serious	Moderate	Low	Moderate	Moderate	**Serious**
Meng et al. (55)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Turroni et al. (52)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
shilov et al. (57)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Rerberg et al. (56)	NI	NI	Low	Low	Low	Low	Moderate	**Moderate**
Voorhies et al. (37)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**
Zhang et al. (63)	Low	Low	Low	Low	Low	Low	Moderate	**Moderate**

A risk of bias is classified as "Low risk" (green coloured), "Moderate risk" (yellow coloured), "Serious risk" (orange coloured), or "Critical risk" of bias (red coloured). Some risks of bias could not be assessed due to lack of information and are therefore marked with "No information (NI)" (grey coloured).

were rather preserved. However, in some studies (40, 54) alpha-diversity is not reported separately for richness and biodiversity, which can limit our understanding of the overall diversity of the studied ecosystem. Alpha-diversity is a measure of diversity within a particular habitat or community. By reporting alpha-diversity as a single value without distinguishing between richness and evenness, researchers may miss important patterns in the distribution of diversity within their study system.

In contrast, hardly any significant changes in community structure were observed. Overall, the community structures remained heterogeneous and dissimilar between individuals, although a convergence of the GI microbiota was reported in several cases. Surprisingly, the effect of convergence was stronger in placebo groups compared to groups supplemented with probiotics (63). This finding is in line with more recent outcomes regarding maintaining GI microbiota eubiosis by probiotics (70). Furthermore, it is speculated that an enhanced spaceflight diet containing increased quantity and variety of fruits, vegetables, fish and other foods rich in flavonoids and omega-3 fatty acids, preserves community structure in the comparative analysis between pre- and in-mission time points (68). To potentially control or better mitigate negative effects better in the future, a diet-based therapy including higher contents of fibre could possibly provide an effective treatment (71, 72). We only know from Hao et al. (54), that a high-fibre diet has been used for the experimental study. The participants' community structure remained dissimilar, however, a convergence was apparent, sup-porting the possible importance of fibre for GI microbiome homeostasis. Previous studies have even highlighted the important effect of diet through the results of their experiments (59). Besides, the administration of probiotic supplements could be considered as another approach to maintain the gut microbiome homeostasis during the stay in isolated environments (70).

Changes in the abundance of a few specific microbiota taxa were reported, supporting our first research question; however, changes reported at the genus and species level were not consistent across studies and at the phylum level abundances remained stable during the mission. Most changes occurred in Bacillota and Bacteroidota, which is consistent with other studies describing microbial changes (14, 15, 17). Both other external and internal parameters influencing the microbiota may induce changes at this phylum level. The reason for this is the high relative abundance of these phyla in the individual core microbiome (8, 9). In more detail, notable changes were reported multiple times for genera *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Faecalibacterium* and *Prevotella*, although there was no clear direction of development for these either. Alterations in intestinal genus levels is often present in case of intestinal microbiota dysbiosis, even more, a dysbiotic abundance of those genera is associated with several diseases (73, 74). Furthermore, the human microorganism ecosystem in an antigen-poor environment, such as a spacecraft, space- or microgravity simulators or Antarctica, has the potential for a loss of the barrier function protecting against pathogens, which, together with a potentially weakened immune system during spaceflight, poses the risk of more severe infection during long-term spaceflight (42). However, none of our included participants demonstrated symptoms of diseases associated with considerable changes in the composition of the microbiota. Thus, it can be assumed that restructuring of the taxonomic composition occurred in their intestinal ecosystems, reflecting their individual responses to the

conditions of the experiment and a new balanced community was formed (44). However, the sample sizes are overall quite low so it is speculative but it can be suspected that the baseline microbiota could play a role, some being more resistant than others to change upon isolation.

A detailed analysis and thus an attempt to compare the results was predominantly possible in the studies using molecular-based approaches. Due to the difficulty in cultivating many types of gut bacteria in laboratory conditions, studies of the GI microbiome have been restricted in the past (75). We have gained a greater understanding of the composition, diversity and roles of the gut microbiome in human health and disease with the development of molecular-based metagenomics (76). A summary of the changes that were carried out via the cultivation method seems even more difficult due to this. However, there appears to be a tendency towards just as unspecific shifts as in the studies that were analyzed using molecular-based approaches. A systematic literature search reported that although still not conclusive, there is a wealth of evidence suggesting that space travel may lead to microbial dysbiosis and metabolic changes in the human gut, including a drop in alpha diversity and changes in gene expression of culturable bacteria (67).

Our second research question aimed to investigate the diversity and abundance of the GI microbiota after experimental exposure. Most of the included studies report a partial recovery after the mission regarding both diversity analysis and microbial abundance within a few weeks, although, studies investigating the recovery were limited. Overall, the data show, that constant environmental factors can partly influence the individual GI microbiota. However, the great interindividual variety remains throughout the experiments which can be attributed to intrinsic factors such as age, genetics and immune system, constantly shaping the GI microbiota persistently. Although resilience of the microbiota following stress such as a course of antibiotics has been commonly observed (77, 78), it is now suspected that harsh or chronic stress such as inflammation could lead to an altered host-microbes relationship associated with a loss of resilience (79). Our analysis would indicate that stress conditions imposed by spaceflight or its simulations does not push the host-microbes system beyond its ecological robustness but rather allows expression of resilience.

4.1. Strengths and weaknesses of the systematic review

Overall, this systematic review has several strengths and limitations. A clear strength is the methodological approach taken according to PRISMA and Cochrane criteria. In order to obtain as broad as possible a knowledge of the current data situation, a very specific search term was used which was superior to broader search terms; however, only 19 articles could be included in the analysis. Despite clear eligibility criteria, the heterogeneity of the studies was high at the methodology (starting from sample preparation and processing) and descriptive levels. To counter this problem, subgroup analyses were performed which reduced heterogeneity to some degree. Despite differences in analysis techniques, habitats, study designs and frameworks for well-conducted studies, all studies were rather highly controlled, which is also reflected in the risk of bias. Here, the ROBINS-I tool for assessing risk of bias in non-randomized studies of interventions, recommended by the Cochrane Handbook, was used.

One of the main issues in the studies reviewed was the extremely low sample sizes, making it challenging to conduct quantitative analyses at the individual study level. Additionally, there were inconsistencies in the study protocols and characteristics, making comparisons between studies difficult. To address these limitations, future studies should aim to increase their sample sizes to enhance statistical power. Furthermore, most of the studies did neither analyze immunological/biochemical parameters in parallel to the microbiota analysis or, the data were published separately and not reported in context. To better understand the effects of long-term isolation on the human GI microbiota, researchers should consider internal and external factors, such as nutrition, genetics, and the immune system. This will enable a clearer differentiation between the effects of isolation and those stemming from other variables. Finally, this systematic review is the first of its kind, providing new insights into the effects of isolation on the human GI microbiota.

5. Conclusion

Overall, our review highlights the complexity of the relationship between the human GI microbiota and its environment. While extreme conditions can affect the composition of the GI microbiota, the internal factors that have shaped the microbiota over time appear to be the primary drivers of its composition and function in response to isolation in antigen-deprived conditions. Maintaining and/or strengthening the host's fitness and immunity through diet, pre- and probiotics, and favorable lifestyle factors may have a positive impact on the human GI microbiota, especially under extreme conditions, and promote GI health and prevent disease.

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

IM and BK: conceptualization. BK and CS: methodology. BK, CS, and JK: formal analysis. BK (80%) and IM (20%): writing–original draft preparation. IM, PE, JP, JD, and CL: writing–review and editing. BK: visualization. IM: supervision. All authors have read and agreed to the published version of the manuscript.

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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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Supplementary material

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