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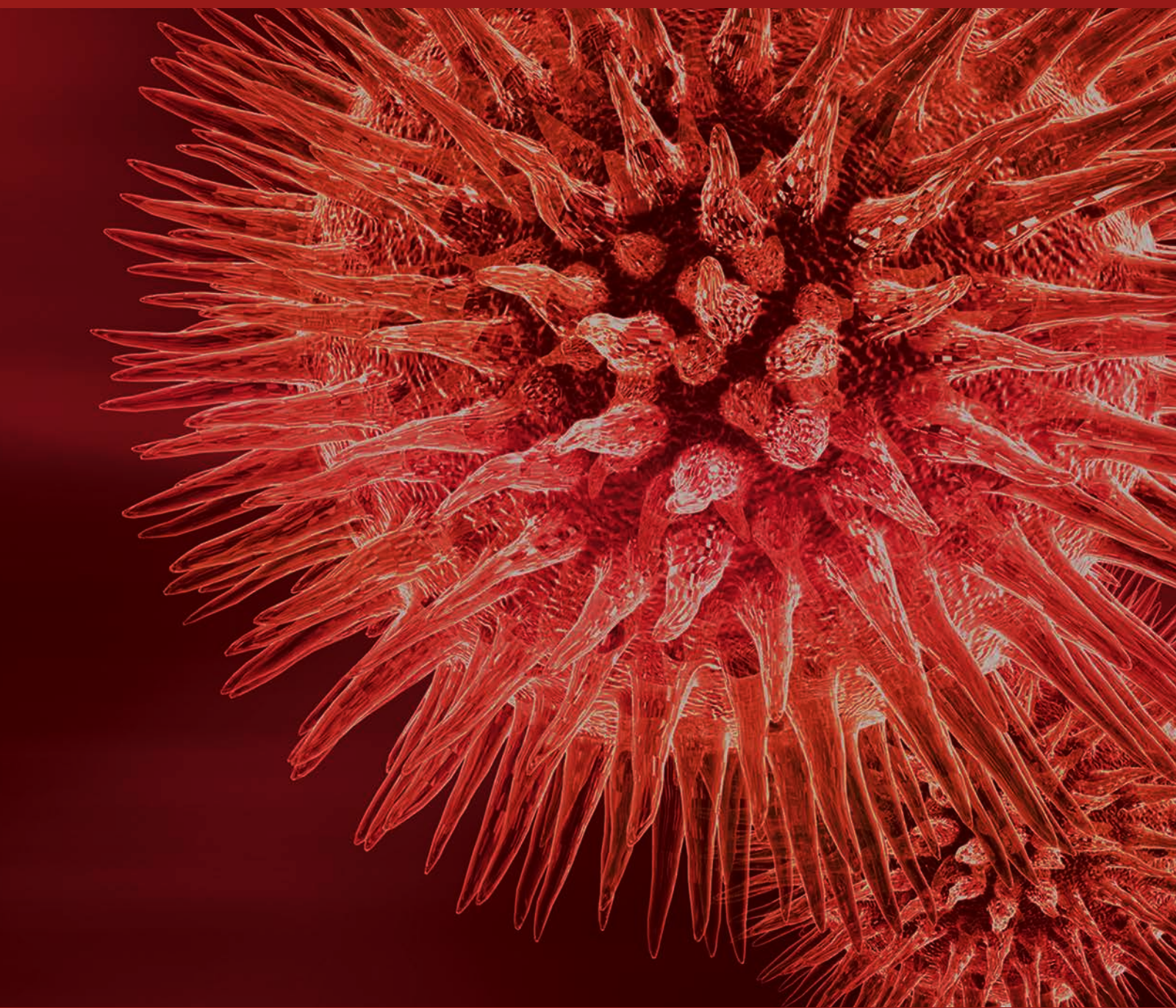
Guest Editors: Wenkai Ren, Kai Wang, Peng Liao, Guan Yang, Yong Zhao, and Yang Zhou



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# Contents

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*Editorial*

# The Regulation of Innate Immunity by Nutritional Factors

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Recent years have witnessed growing interest in the biochemistry and physiology of nutrients for mammals, such as amino acids, fatty acids, polyphenols, and oligosaccharide. Notably, dietary nutrients have critical importance on immune function, especially in the pathogenesis of many immune related diseases including autoimmune diseases, inflammatory bowel disease (IBD), and cancer. These studies propose the way to manipulate immune associated diseases with a nutritional aspect.

This special issue provides us with a better understanding of the role of nutrition on immunity at the molecular, cellular, and organ level, which suggests possible implications in nutritional manipulations.

Mingxin Li et al. explored the effect of dietary methionine restriction on bone density and function of natural killer cells in mice. The results revealed that methionine-restricted diet decreases the bone mass and reduces the cytotoxicity of NK cells. Vitamin D has profound implications for animal and human health. However, the influence of the vitamin D signaling pathway on immunity and how it is regulated is only partially known which limits efforts to support immunity through the vitamin D pathway. R. Lin reviewed the recent knowledge on how immune signals regulate vitamin D metabolism and how innate immune responses are modulated by ligand-bound vitamin D receptor. Although osteopontin (OPN) is associated with the pathogenesis of osteoarthritis (OA), the underlying mechanism of OPN in the biology of OA remains to be known. Y. Li et al. demonstrated that OPN enhances the production of matrix metalloproteinase 13 (MMP13) and activates the NF-$\kappa$B pathway, while inactivation of NF-$\kappa$B pathway reduces the production of MMP13. Y. Zhou et al. found that Fetuin-A may improve the excessive activation of hepatic stellate cells by inhibiting the expression of Smad2 and Smad3 genes but upregulating the Smad7 gene expression.

The gastrointestinal tract is particularly responsive to stressors and inflammatory mediators. Oregano essential oil (OEO) has long been used to improve the health of animals and is widely known for its antimicrobial and anti-inflammatory effects. Y. Zou et al. investigated the effects of OEO in the intestine of pigs and they found that OEO promotes intestinal barrier integrity. Mechanically, this modulation is probably through regulating intestinal bacteria and immune status in pigs. Weaning is known to compromise the digestive, absorptive, and secretory capacity of the small intestine, which can cause morphological and histological changes of the small intestine. M. C. B. Tossou et al. showed that tryptophan (Trp) affects the tight junction barrier and intestinal health in weaned pigs. They found that 0.15% Trp supplementation did not affect pig performance, while 0.75% Trp supplementation negatively affects intestinal morphology and tight junction proteins in weaned pigs.

Chitosan is an attractive additive for animal feed because of its inherent antimicrobial and anti-inflammatory properties. G. Guan et al. explored relationships between low dose dietary supplementation of chitosan and body weight, feed intake, intestinal barrier function, and permeability in mice. They used the mouse model and demonstrated that 30 mg/kg dose of chitosan supplementation did not influence growth performance but compromised intestinal barrier integrity. M. Iser et al. also found that *Agave fourcroydes* powder can be used as a dietary supplement which had beneficial effects on increasing the growth performance and serum concentration of IgG, as well as improving the gut morphology without affecting the hematology parameters in broiler rabbits. L. Cheng et al. purified and characterized thermostable $\beta$-Mannanase from *Bacillus subtilis* BE-91 which will have potential applications as a dietary supplement in treatment of inflammatory diseases.

The research article by M. Brianza-Padilla et al. showed that chronic ingestion of sucrose in rats induces the upregulation of inflammation related microRNAs (miR-21 and miR-223) in plasma and extracellular vesicles. H. Ni et al. reported that isoquinoline alkaloids, derived from *Macleaya cordata* extract, are beneficial to swine and poultry growth by increasing feed consumption, body mass, and weight, as well as the concentration of serum amino acids. Isoquinoline alkaloid also boosts the innate immune system by regulating the concentration levels of haptoglobin and serum amyloid A. X. Chen et al. found that miR-166a is the most highly enriched exogenous plant miRNAs in the blood of mice fed with rapeseed bee pollen. The study also suggested that food-derived exogenous miRNAs from rapeseed bee pollen could be absorbed in mice and the abundance of exogenous miRNAs in mouse blood is dependent on their original levels in the rapeseed bee pollen.

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*Review Article*

# Analysis of the Impact of Isoquinoline Alkaloids, Derived from *Macleaya cordata* Extract, on the Development and Innate Immune Response in Swine and Poultry

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Medicinal extract has been chronicled extensively in traditional Chinese medicine. Isoquinoline alkaloids, extract of *Macleaya cordata* (Willd.) R. Br., have been used as feed additive in both swine and poultry. Dietary supplementation with isoquinoline alkaloids increases feed intake and weight gain. In addition, recent researches have demonstrated that isoquinoline alkaloids can regulate metabolic processes, innate immune system, and digestive functioning in animals. This review summarizes the latest scientific researches on isoquinoline alkaloids which are extracted from *Macleaya cordata* (Willd.) R. Br. This review specifically focuses on its role as a feed supplement and its associated impact on growth performance and innate immune system, as well as its capacity to act as a substitute for oral antibiotics.

## 1. Introduction

*Macleaya cordata* (Willd.) R. Br., also known as *Bocconia cordata* or plume poppy, belongs to the Papaveraceae family. It is an herbaceous perennial plant, ubiquitously dispersed in central and southeastern China. It is also found in the regions where the parasitic disease (schistosomiasis) is prevalent [1, 2].

*Macleaya cordata* (Willd.) R. Br. contains a number of important alkaloids, which include sanguinarine (SG), dihydroderivative (DHSG), chelerythrine (CH), protopine (PR), allocryptopine (AL), and phenolic acids [3, 4]. A small amount of other isoquinoline alkaloids have also been traced in this plant, such as chelirubine, macarpine, sanguidimerine, chelidimerine, homochelidonine, cryptopine, berberine, coptisine, chelilutine, bocconarborine A, bocconarborine B, oxysanguinarine, norsanguinarine, angoline, bocconoline, 6-ethoxychelerythrine, 6-ethoxysanguinarine, protopine-N-oxide, 6-methoxydihydrosanguinarine, 6-acetonyl-dihyrochelerythrine, and 6-acetonyl-dihydrosanguinarine [3].

*Macleaya cordata* (Willd.) R. Br. grow above the ground and have been used as traditional Chinese medicine for a long time. They are utilized for specific purposes, such as pain relief, modification of the immune system, and reduction of inflammation. The capacity to suppress the proliferation of bacteria, fungi, and viruses [5] has been ascribed to the quaternary benzo[c]phenanthridine alkaloids (QBA), SG and CH [2, 6, 7]. Furthermore, its positive effects on health are evidenced by its ability to inhibit the growth of microorganisms, to block the release or action of adrenaline at nerve endings, to decrease the excitation of sympathetic nervous system, to prevent from fungal infections, and to

be used in the treatment of cancer. It also can act as an antiseptic compound, a pesticide against molluscs, and an agent to destroy plant-parasitic nematode worms [2, 8–11].

More recently, food supplements derived from plants have been fed to farm animals. Gradually, they have evoked attention as a substitute to antibiotic growth promoters [12]. This is attributed to the fact that these plants and their extracts are natural substances. They are found to be beneficial in improving growth performance, digestive function, and the absorption of nutrients. They are also helpful in improving the ability of anti-infection and reducing the incidence of diarrhea [12–18].

Based on these properties, *Macleaya cordata* (Willd.) R. Br. showed up in the European Food Safety Authority (EFSA) database. It is employed as a feed additive in intensive livestock farming in an effort to elevate daily food consumption and growth performance [19–24]. According to Mellor [25] and Le Floc'h and Seve [26], sanguinarine can regulate the serotonin synthesis by employing tryptophan and finally lead to improvement in feed intake [20]. However, more studies are required to investigate the effects of extract of *Macleaya cordata* (Willd.) R. Br. on pigs fed with tryptophan-deficient diet [27, 28].

Some investigations have revealed that dietary supplementation with isoquinoline alkaloids reduced the diarrhea and improved gut health, immune system, and digestive function in nonruminant mammals [17, 18, 22, 29]. Therefore, the primary goal of this review was to discuss the impact of isoquinoline alkaloids, derived from extract of *Macleaya cordata* (Willd.) R. Br., on the growth and immune system in swine and poultry.

## 2. The Impact of Isoquinoline Alkaloids, Derived from Extract of *Macleaya cordata* (Willd.) R. Br., on the Growth of Animals

*2.1. Swine.* Phytobiotics can be defined as plant derived products added to feed in order to improve performance. It can be obtained through combining a large array of herbal-based products [30]. A number of researchers have claimed that some plants, as well as their extracts, are able to increase appetite and activate endogenous secretions of enzymes and hormones [13, 17, 31]. In the case of treatment of diseases, they have also been found to have the capacity to destroy microorganisms and parasitic worms in nonruminant animals. Moreover, they are able to retard the growth and reproduction of coccidian parasites [30].

Evidence is available from numerous studies to substantiate that adding phytochemical ingredients to the diet of pigs had beneficial outcomes, particularly in the treatment against growth retardation and disease. As antimicrobial agents, their efficacy is influenced by the concentration of additives and the pH in the animal's intestine [32]. Numerous researches revealed that phytochemical ingredients can reduce coliform bacteria in gastrointestinal tract (GIT) and decrease the diarrheal frequency or mortality rates among young pigs. Phytochemical additives also play an important role in deterring diarrhea and oedema in piglets during the weaning process [12].

Growth performance, as Kong et al. [13] and Jobgen et al. [33] stated, is a complicated progress involving the delicate interaction between metabolism and catabolism. But we may infer the potential physiological or biochemical effect of food additives on the animals through the investigation on the metabolites. For example, the metabolic properties of intracellular protein and the rate of fat deposits are valuable references for the determination of appropriate glucose and amino acid usage. It is no doubt that the metabolic processes are also modulated by hormones and other elements. Both antibiotics and extract of *Macleaya cordata* (Willd.) R. Br. can be used as growth promoters. When a comparative analysis was undertaken between them, the extract demonstrated similar effect as antibiotics on the intestinal health and growth performance [17, 29].

Using the extracts of *Macleaya cordata* (Willd.) R. Br. as feed additives at the concentration ranging from 15 to 50 mg/kg, increased weight gain was found [17, 29]. This outcome has been attributed to the positive influence of internal and external factors on animal production, particularly due to their antimicrobial properties and their capacity to modify immune system and the reduction of inflammation [34]. A number of bacteria located in the mouth cavity of humans were identified to have antimicrobial qualities. Some of these bacteria were classified among the species frequently situated in the GIT of swine [35, 36]. Feeding sanguinarine at minimal inhibitory concentration showed similar effect on bacteria. This may indicate that dietary supplements militate against the rapid multiplication of pathogen bacteria located in the GIT, which in turn impacts upon developmental progress.

From a scientific perspective, the primary contentious issue is about the effect of isoquinoline alkaloids from *Macleaya cordata* (Willd.) R. Br. on feed intake in farming animals. Some studies claimed that sanguinarine additives had no impact on feed consumption [28, 37]. Conversely, other researchers [25, 26] subscribed to the belief that sanguinarine could influence feed intake by regulating the pathway for the synthesis of serotonin by using tryptophan. One study showed that sanguinarine led to greater feed intake (increased by 7%) and acquisition of nourishment, compared to those fed with antibiotics [17]. Beneficial effect on nitrogen balance and growth performance was also found when sanguinarine was added to the diet of swine [20].

No toxicity was found when swine and mice ingested the plant *Macleaya cordata* (Willd.) R. Br., let alone its alkaloid extract, because most of the possible contaminants had been removed [35, 38, 39]. Thus, adding the herb or/and its extract into animal feed would not expose the consumer to dangers. Furthermore, no negative impact on health was detected [35].

In addition, the introduction of isoquinoline alkaloids has decreased the prevalence of diarrhea [18]. Typically, diarrhea is associated with rapid multiplication of *Escherichia coli* and other pathogens in the intestine. The abnormal proliferation of bacteria results in the excretion of water and electrolytes through the semifluid feces and urine [13]. Isoquinoline alkaloids in the extract were found to suppress or destroy

these microorganisms, as well as modulating vital functions, such as peristalsis and the pH of intestines [12].

Research conducted by Walker [8] and Newton et al. [9] confirmed that sanguinarine acts as an antimicrobial agent. They found that diet supplemented with sanguinarine had the potential to facilitate the establishment of beneficial bacteria in the GIT of swine, as well as the reinforcement of competitive exclusion principle by inhibiting the colonization of pathogenic bacteria. In addition, sanguinarine reduced the water loss in the epithelial cells of the intestines and/or enhanced the intestinal function in the absorption of water and nutrients [13]. The escalation of metabolic rates of biomolecules and the antioxidant capabilities in the small intestinal mucosa appeared to generate these effects [40].

One study demonstrated that the introduction of feed additives in the form of *Macleaya cordata* extract, containing isoquinoline alkaloids, increased the serum amino acids in swine [41]. And isoquinoline alkaloids can strengthen the capacity to assimilate and absorb ingested protein and AA. In addition, it is likely that this compound modulates the metabolism process in relation to the absorption of nutrients through signal transduction pathways. Nutrients augmentation in portal vein (and specifically AA) which derives from the small intestine may be adequate to stimulate tissue protein synthesis in animals, which has benefit impacts on the growth development [40, 42].

A correlation was found between feed additives and the enhanced movement of amino acids, leading to growth improvement. Greater volumes of essential amino acids, such as lysine, shield the intestine from pathogens and perform a crucial function in calcium absorption. They are also helpful in the preparation of muscle protein, hormones, enzymes, and antibodies [43, 44]. For example, arginine participates in various pathways, including the production of proteins, nitric oxide, polyamines, and creatine [45]. Methionine is another key intermediate in the biosynthesis of proteins and phospholipids. In addition, this amino acid, along with choline, contributes to transfer fat, thus decreasing the fat levels in liver. Methionine also has antioxidant property, and it comprises the element sulfur, which assists in neutralizing free radicals which emerge as a consequence of the diverse components of metabolism [40].

*2.2. Poultry.* Antibiotics as growth promoters have been withdrawn from the feedstuffs of poultry in most regions of the world. Therefore, an increasing demand for the exploration of other possible options is arising to sustain growth development. It is also important to ensure that beneficial microorganisms are predominant in the intestine to specifically prevent the proliferation of pathogenic bacteria. A number of plant additives have been extensively utilized to sustain or enhance the growth performance in poultry [46]. In addition, herb extracts may boost their immune system and decrease blood cholesterol levels [47].

Research has demonstrated that isoquinoline alkaloids prevent the spread of specific bacteria that generate gastrointestinal distress [48]. They also improve appetite and the growth performance [20]. In the case of broiler chickens and maturing turkeys, the recommended dose of *Macleaya cordata* in diet is 20 to 50 ppm [49].

Variations have emerged in the studies conducted to measure the impact of isoquinoline alkaloids on broiler chickens. One study found that when chickens (Cobb × Cobb, male) ingested isoquinoline alkaloids at the dose of 25 and 50 ppm, the body mass and feed conversion rate increased [36]. Notwithstanding this, another research focusing on maturing Ross 308 chickens did not reach a similar conclusion. It found that isoquinoline alkaloids administration at 20 mg/kg failed to influence the growth development and the protein utilization in the poultry [50].

Nevertheless, the introduction of isoquinoline alkaloids into the diet has been claimed to have impact on gastrointestinal performance and the fermentation metabolic process in terminal GIT. It has also been confirmed that isoquinoline alkaloids influence the gastrointestinal movements [51]. Jankowski et al. [52] reported that adding *Macleaya cordata* compounds to the diet of broiler chickens could decrease inordinate fermentation in the caecum without disturbing the pH levels in this area, leading to the enhancement of growth performance.

## 3. The Effects of Isoquinoline Alkaloids, Derived from *Macleaya cordata* Extract, on the Innate Immune Response

*3.1. Swine.* Young pigs, weaned prior to the usual period (ranging from 15 to 28 days old), were subjected to situational tension and nutritional deficits, resulting in the rapid multiplication of intestinal disease-inducing bacteria (e.g., *Escherichia coli*). In addition to growth retardation, it led to higher morbidity and mortality rates [13, 53]. This demonstrates that the innate immune system operating in young animals influences their performance levels, as well as their response to stimuli.

The innate immune system is an important subsystem of the overall immune system that comprises the cells and mechanisms that defend the host from infection by another organism. This implies that, within this immune system, cells identify and react to pathogen in a nonspecific manner. In contrast to the acquired immune system, it cannot endow immunity over a prolonged time period or defend its host. This innate immune system offers instantaneous protection from disease [54].

Throughout this phase, instantaneous defense is ensured by stimulating the inherent immune cells macrophages, as well as other cells such as the dendritic, polymorphonuclear, and epithelial. This occurs as a result of various toll-like receptors which identify crucial molecules on the outer layer of the bacteria [55]. Neutrophilic granulocytes consist of lysozyme, in primary as well as secondary granules. Its key role is to defend against pathogens and various foreign bodies surrounding the host [55]. This process is fully accomplished through phagocytosis and digesta. Therefore, the ingestion of isoquinoline alkaloids contained in extract of *Macleaya cordata* (Willd.) R. Br. was considered to be essential throughout crucial developmental phases, especially while the species is

primarily dependent on intrinsic immunity [35]. The compound activates phagocytes, hence stimulating the organism's defense mechanisms [29].

Intestinal barrier systems are rigorously managed by a meticulously construed epithelial junctional complex, commonly known as the "the tight junction" [56]. It comprises a number of different proteins, which include a transmembrane protein called occludin [57], various derivatives of the claudin group, a junctional adhesion molecule [58], and several linker proteins, for example, ZO-1. Three of the most crucial and beneficial proteins are occludin, ZO-1, and claudin-1, as they play an important role in the control of the tight junctions [59]. In connecting the C-terminal selections of $\beta$-actin and occludin [18], ZO-1 is a helpful linker protein in the tight junction.

Research has found that the ingestion of extract of *Macleaya cordata* (Willd.) R. Br. can increase the expression of ZO-1 and claudin-1. Thus, it is helpful in preventing allergenic and toxic matter entering the intestines and reducing risks [60]. This is indicates that the use of extract of *Macleaya cordata* (Willd.) R. Br. as a feed additive can promote intestinal mucosal growth and improve defense systems [18].

Recent research conducted by Kantas et al. [29] revealed that the introduction of alkaloids into the feedstuffs reduced the haptoglobin level in swine. This protein is found in blood plasma. It usually binds free hemoglobin and forms the hemoglobin-haptoglobin complex. Then the complex is withdrawn from circulation by the liver, whereupon it participated in a catabolic process in hepatic parenchymal cells. This study also demonstrated that dietary supplementation with alkaloids reduced the level of serum amyloid A (SAA). These proteins are a group of apolipoproteins produced in reaction to cytokines, which are stimulated by monocytes or macrophages. They are closely associated with inherent immunity. The long-established belief was that SAA performed a crucial function in relation to the biological mechanisms that lead to disease in amyloid A-type amyloidosis [61]. Hence, dietary supplementation with isoquinoline alkaloids extracted from *Macleaya cordata* (Willd.) R. Br. boosts the immune system and regulates metabolic process and finally promotes growth and development in swine.

*3.2. Poultry.* Antibiotics are usually replaced with probiotic, organic acids, and herbal extracts in poultry diet. Thus, it was necessary to clearly define the function of these compounds. Given these concerns, Yakhkeshi et al. [62] conducted a comparative analysis to determine the impact of herbal extracts, probiotics, organic acid, and antibiotics on the serum lipids, immune response, intestinal structures, and microbial population in broilers. No substantial variations were found in weight gain and feed conversion ratio when broilers were aged 1–14 and 14–28 days.

Furthermore, this study found that the alkaloids contained in these compounds substantially enhanced intestinal health and the absorption of nutrients. Unlike other interventions, the addition of sanguinarine to the diet resulted in a substantial rise in the heterophils to lymphocyte ratio (H/L). Evidence has shown that herbal extracts improve antibody titration against sheep red blood cells (SRBC). Studies have also demonstrated that herbal extracts trigger the immune system by boosting vitamin C levels. It has been recognized that isoquinoline alkaloids have the capacity to adjust or regulate immune functions [14]. In addition, this medicinal compound can activate phagocytosis, hence prompting defensive reactions by the host [63].

The introduction of isoquinoline alkaloids to the diet of broilers has been shown to considerably reduce the villus height of intestine and the depth of glandular layer [52]. But Vieira et al.[36] found no significant differences in villus height and crypt depth in broilers fed with and without sanguinarine. As far as we know, villus height and intestinal surface area are positively correlative to nutrients absorption and health in animals [64]. It was noted that cells situated in the villi (such as inflammatory cells or enterocytes) are also important when health problems exist. Typically, a greater volume of goblet and immunocyte are not directly correlated with nutrient absorption. But they were found to decrease absorption levels due to enhanced intestinal viscosity and the rate of passage of feeds.

Research undertaken by Pickler et al. [64] showed that the decrease of CD3 cells (this indicator relates to T lymphocytes cells) was detected in the duodenum, jejunum, and ileum of broilers fed with sanguinarine. More goblet cells were noted in the duodenum and ileum in control group compared with the group fed with sanguinarine. Sanguinarine was also found to alleviate the injury of mucosa, suggesting that it would be helpful to prevent enterobacterial infection.

This review has highlighted the idea that dietary supplementation with isoquinoline alkaloids, the extract of *Macleaya cordata* (Willd.) R. Br., is beneficial to swine and poultry. This compound increases feed consumption, body mass, and weight gain, as well as the concentration of serum amino acids. It boosts the innate immune system by regulating phagocytes, haptoglobin, and amyloid A. In addition, it promotes effective gastrointestinal movements, as well as carrying out an important intestinal barrier function by action of ZO-1 protein and claudin-1.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Hengjia Ni and Yordan Martínez contributed equally to this manuscript.

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*Research Article*

# Inflammation Related MicroRNAs Are Modulated in Total Plasma and in Extracellular Vesicles from Rats with Chronic Ingestion of Sucrose

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Circulating microRNAs (miRNAs) and the functional implications of miRNAs contained in extracellular vesicles (EVs) have gained attention in the last decade. Little is known about the regulation of the abundance of plasma miRNAs in response to chronic ingestion of carbohydrates. Therefore, we explored the circulating levels of miR-21, miR-146a, miR-155, and miR-223 in rats consuming sucrose in drinking water. Weanling Wistar rats were 25 weeks with 30% sucrose in drinking water, and miRNAs expression was determined in total plasma and in microvesicles, by RT-qPCR with TaqMan probe based assays for miR-21, miR-146a, miR-155, and miR-223, using cel-miR-39 (as spike in control and reference). Endotoxemia was also measured. Sucrose-fed animals showed higher body weight and retroperitoneal adipose tissue as well as higher glucose and triglyceride plasma levels than controls. Plasma endotoxin levels were low and not different among groups. Plasma miR-21 and miR-223 were higher in the sucrose group ($p < 0.05$), whereas miR-155 tended to be lower ($p = 0.0661$), and miR-146a did not show significant differences. In the plasma EVs the same trend was found except for miR-146a that showed significantly higher levels ($p < 0.05$). Overall, our results show that high carbohydrate ingestion modulates circulating miRNAs levels related to an inflammatory response.

## 1. Introduction

Chronic ingestion of high amounts of carbohydrates contributes to the obesity epidemic worldwide [1]. Experimental models have been widely used to explore this phenomenon. It is known that high fructose and sucrose-fed animals reproduce the features of metabolic syndrome (MS) [2, 3]. Although many of the signals participating in the response of an organism to the continued exposure to high carbohydrate ingestion are reported in the literature, molecular signals through circulating noncoding RNA, such as microRNAs (miRNAs), are giving new insights.

miRNAs are small, noncoding RNA molecules of approximately 22 nucleotides in length that act as posttranscriptional regulators of gene expression [4]. In 2008, miRNAs were found in human serum and plasma [3, 5, 6] and are now useful biomarkers in many inflammatory conditions including obesity [7]. Some circulating miRNAs are considered molecular players of the innate immune response, especially if they are contained in extracellular vesicles (EVs) [8]. In this context, some circulating miRNAs can participate in inflammatory pathways. Such is the case of miR-21, miR-146a, miR-155, and miR-223 [9–11]. As has been demonstrated for inflammatory and immunity molecules, miRNAs expression can also be regulated by nutrients [12]. Many dietary compounds may modify miRNAs in cells and tissues; thus, circulating miRNAs levels can also be biomarkers of exposure to particular nutrients [13].

Increased consumption of simple carbohydrates such as fructose and sucrose has been linked to many pathophysiological processes and their effect on health is still controversial [14]. In particular, rats with chronic ingestion of sucrose after weaning in the drinking water as an unlimited beverage may display many signs of metabolic abnormalities such as moderate elevation of blood pressure, hypertriglyceridemia, hyperinsulinemia, excessive retroperitoneal fat and whole body fat [6], renal damage [15], and high vascular reactivity and disruption of innate inflammatory mediators [16]. Because all these metabolic disorders have been associated with inflammation we hypothesized that miRNAs involved in innate immunity (known as inflamma-miRs) may be altered in parallel to the metabolic disturbances induced by chronic ingestion of sucrose; thus, we aimed to evaluate the effect of chronic ingestion of sucrose on the levels of miR-21, miR-146a, miR-155, and miR-223, in total plasma and plasma extracellular vesicles of rats.

## 2. Materials and Methods

*2.1. Animals.* Fourteen weanling male Wistar rats weighing 70–95 g were randomly allocated into two groups. Control group was supplied with tap water ad libitum, whereas high sucrose drink group received a 30% sucrose solution in water, as their only liquid source. Animal feeding during 25 weeks consisted of a standard rodent diet (Laboratory Rodent Diet 5001: protein 28.507%, fat 13.496%, HCO 57.996%, from which sucrose 3.7%, fructose 0.3%, glucose 0.22%, PMI Nutrition International, Brentwood, MO). All animals were housed under artificial 12-hour light/dark cycles and a mean temperature of 22°C. The experiments in animals were approved by the Laboratory Animal Care Committee of our Institution and were in compliance with international ethical guidelines for animal research.

*2.2. Serum Measurements.* After 25 weeks, rats from both groups were weighed, fasted 12 h, and sacrificed. Blood samples were collected using K+EDTA as anticoagulant. Retroperitoneal adipose tissue was collected and weighed. Plasma was obtained by blood centrifugation (3000 rpm during 15 minutes at 4°C) and stored at −70°C until needed. Glucose was measured with a commercial enzymatic kit (DCL-glucose oxidase Diagnostic Chemical Limited de Mexico, Mexico). Insulin was determined with a commercial rat specific radioimmunoassay kit (Linco Research, Inc., Missouri, USA) with 0.1 ng/mL sensitivity and intra- and inter-assay coefficients of variation of 5 and 10%, respectively. Triglycerides and cholesterol were determined with commercially available procedures (Spinreact cholesterol-LQ and triglycerides-LQ; Spinreact S.A. Girona, Spain). HDL-cholesterol was measured by enzymatic procedures (Hitachi 902 analyzer; Hitachi LTD, Tokyo, Japan). Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance as recommended by the Centers for Disease Control and Prevention (Atlanta, GA, USA). Plasma endotoxin levels were determined with GenScript Toxin Chromogenic LAL Endotoxin according to the manufacturer's instructions.

*2.3. Plasma RNA Isolation.* From the collected blood sample in K+EDTA, 100 $\mu$L of plasma was processed for the isolation of RNA using the miRNeasy serum/plasma kit adding cel-miR-39 ($1.6\times10^8$ copies) spike in control (Qiagen) and 1 $\mu$L of bacterial ribosomal RNA (Roche) according to the provider recommendation. Extracted RNA isolated from samples was stored at −70°C until processing.

*2.4. Extracellular Vesicles RNA Isolation.* 500 $\mu$L of plasma was processed for the isolation of RNA using the exoRNeasy serum/plasma midi kit. During the RNA purification step the same amount mentioned above of cel-miR-39 spike in control was added (QIagen) according to the provider recommendations and previous publication [17]. Extracted RNA isolated from EVs was immediately converted to cDNA as described below.

*2.5. Determination of miRNAs by RT-qPCR.* The miRNAs were detected and quantified using two-step RT-qPCR with RT-primer specific assay in combination with TaqMan probes (Applied Biosystems). Each RT-reaction used 1.5 $\mu$L from the 14 $\mu$L eluted RNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The RT-reaction program consisted of 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C. The miRNAs were detected and quantified using miRNAs Assays hsa/mus/rno-miR-21, miR-146a, miR-155, and miR-223 primers and probes (Applied Biosystems). The 2 $\mu$L of RT-reaction was amplified in 15 $\mu$L

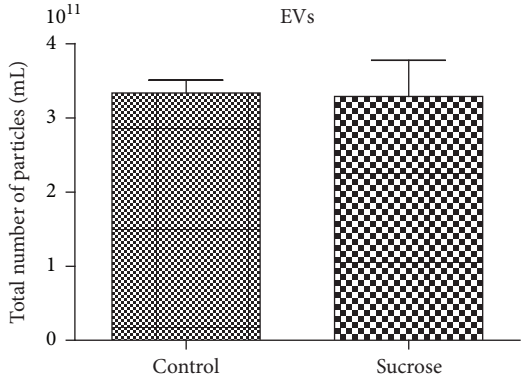


Figure 1: EVs assessed in plasma by particle number estimation in 3 control and 3 sucrose-fed rats. Means ± SE are shown and no differences were observed by Mann-Whitney $U$ test ($p > 0.05$).

reactions. PCR cycling conditions were initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, at 60°C for 60 s, and at 72°C for 1 s. PCR was performed using a LightCycler TM 480 II System (Roche Applied Science, Basel, Switzerland) with the LightCycler 480 Probes Master kit (Roche Applied Science). miRNAs relative concentrations were normalized with Ct values of cel-miR-39 and values were calculated using $2^{-\Delta\Delta Ct}$ and $2^{-\Delta Ct}$ formulas. All Ct values for cel-miR-39 ranged from 20 to 22 cycles both for total plasma and for EVs RNA isolations.

*2.6. Particle Number Estimation.* One mL of plasma was pipetted into a 1.5 mL tube and 400 $\mu$L of PBS was added to each sample. The tubes were loaded into a fixed angle rotor (TLA 100.3; Beckman Coulter) for ultracentrifugation (Optima MAX Ultracentrifuge; Beckman Coulter) at 120,000 ×g at 4°C for 90 min. The pellets were resuspended in PBS and centrifuged again at 120,000 ×g for 90 min. The final pellet was resuspended in 50 $\mu$L of PBS for nanoparticle tracking analysis (NTA). NanoSight NS300 was used to determine vesicle size and concentration (Malvern Instruments Ltd). Dilutions of 1 : 200 in PBS of each sample were injected into the NanoSight chamber. The camera gain was set at a constant value of 10 and the threshold value for vesicle detection was set at 5.

*2.7. Statistical Analysis.* Data are presented as means and standard errors. Data were tested for normality and equal variances. Accordingly, differences between groups were assessed by unpaired $t$-test or Mann-Whitney $U$ test ($p < 0.05$) using the Graph Pad Prism software version 5.

## 3. Results

Rats in the high sucrose drink group had higher body weight and had almost three times more retroperitoneal fat than controls ($p = 0.05$ for both variables). Also, the sucrose-fed rats showed higher glucose levels and triglycerides than controls ($p = 0.001$). No differences between groups were observed for plasma insulin, endotoxins, and total HDL and LDL cholesterol ($p > 0.05$) (Table 1).

Previously in 3 controls and 3 sucrose-fed rats, we determined the amount of total extracellular vesicles and no differences between groups were observed. Since quantification of EVs is complex we supposed that the amount of EVs does not change with chronic sucrose ingestion as seen by total particle assessment (Figure 1).

The relative levels for the miR-21 and miR-223 were 2.7- and 3-fold higher, respectively, more abundant in the sucrose-fed animal groups when compared to the control group ($p < 0.01$). The plasma levels of miR-155 from the animals fed with sucrose had a nonsignificant tendency to be 40% downregulated when compared to the control group ($p = 0.066$). The levels of miR-146a were not different when compared to the control group ($p > 0.05$) (Figure 2).

In plasma EVs the miRNA levels of miR-146a and miR-223 were found higher in the sucrose drink group as compared to the control group ($p < 0.05$ and $p < 0.01$, resp.). The miR-155 levels in the EVs had lower levels in the sucrose drink animals than in the control group ($p < 0.05$). For the miR-21 levels, only a trend for higher abundance was found in the sucrose group ($p = 0.057$) (Figure 3).

The relative abundance in total plasma as compared to the same amount of cel-miR-39 spike in control was miR-223 > miR21 > miR146a > miR-155. The relative abundance of miRNAs present in plasma EVs was miR-223 > miR-21 > miR-155 > miR-146a (Figure 3).

## 4. Discussion

In our study, chronic ingestion of sucrose induced changes in the concentrations of inflammation related miRNAs both in plasma and in plasma EVs. In agreement with previous findings in sucrose-fed rats by other groups [2] and by us [6], these rats had also higher body weight and visceral fat, as well as glucose and triglycerides levels. Insulin levels, total cholesterol, HDL, and LDL cholesterols were not found to be modified by sucrose. Because an endotoxemia secondary to changes in microbiota has been described in rats following a high fat diet [18], we measured plasmatic levels of endotoxin to assess if any changes of on miRNAs levels could be explained by this fact. No differences in endotoxemia were observed between groups, indicating that our findings may not be attributed to a similar phenomenon. Also, in a preliminary experiment we determined vesicle size and concentration in both rat groups, and the results were not different ($p > 0.05$). Thus, we assumed that EVs were not affected by chronic sucrose.

The changes observed in miR-21 total plasma and EVs, upon sucrose chronic exposure, are likely associated with the increased adipose tissue mass. Previous reports show that miR-21 levels increase in the white adipose tissue of mice with high fat diet-induced obesity and during human adipocyte stem cells proliferation [19]. Also, upregulated miR-21 levels in serum are associated with nonalcoholic fatty liver disease, especially in men [20]. Accordingly, 20% consumption of sucrose has been reported associated with mild liver steatosis in rats [21]. This miRNA may have a role in sustaining adipose tissue expansion as reported in a study using miR-21 antagomiRs in the db/db mice [22].

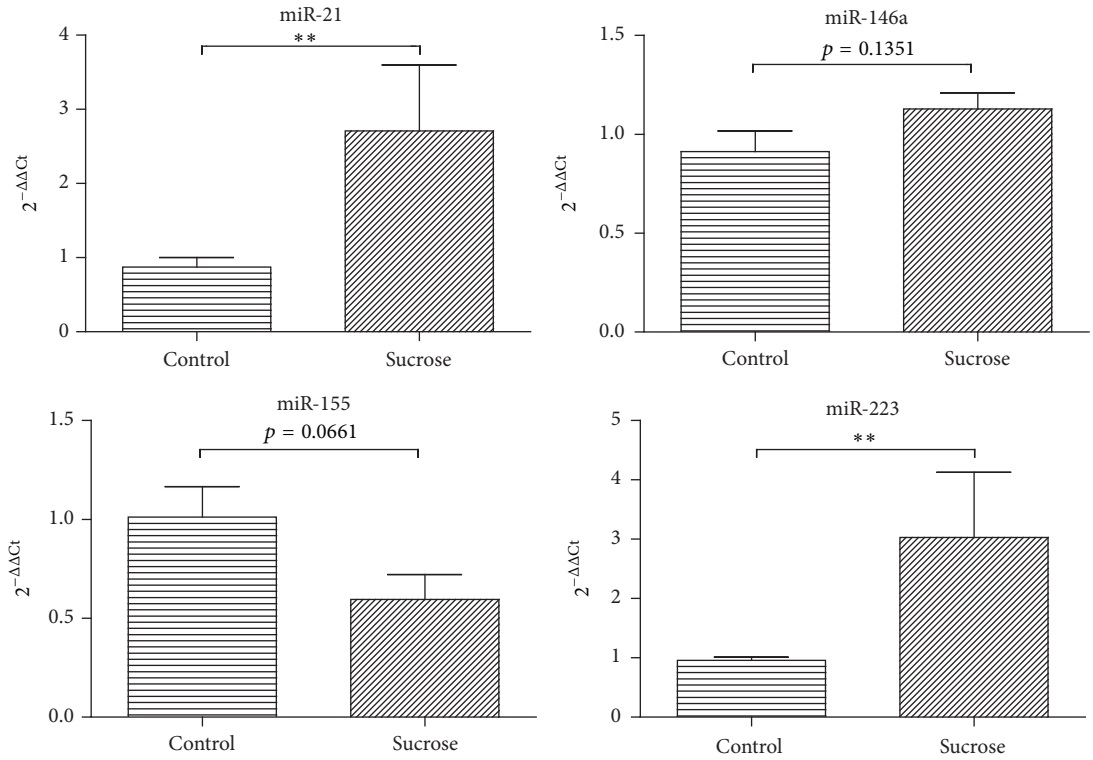


Figure 2: Plasma miRNAs levels in sucrose-fed rats (means ± SE). miR-21, miR-146a, miR-155, and miR-223 were measured in 7 animals per group by RT-qPCR using cel-miR-39 as a reference for the $2^{-\Delta\Delta Ct}$ method. Differences were tested by unpaired $t$-test or Mann-Whitney $U$ test. $^{**}p < 0.01$.

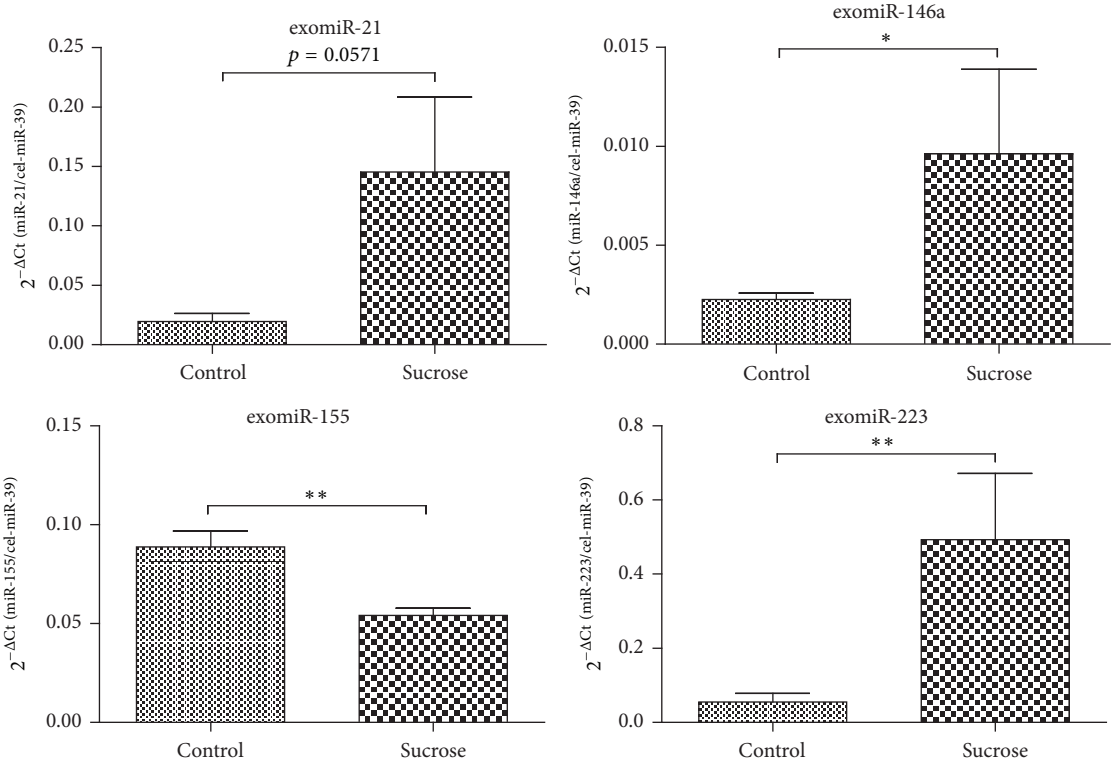


Figure 3: miRNAs levels in plasma extracellular vesicles of chronic sucrose-fed rats (means ± SE). RNA was isolated from plasma EVs, and the miR-21, miR-146a, miR-155, and miR-223 levels were measured in 4 animals per group by RT-qPCR using cel-miR-39 spike as a reference for the $2^{-\Delta Ct}$ method. Differences were tested by unpaired $t$-test or Mann-Whitney $U$ test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 1: Body weight central adiposity and biochemical means (±SE) related to metabolic syndrome.

| | Control | Sucrose drink | $p$ value* |
|---|---|---|---|
| Weight (g) | 460 ± 18.4 | 565 ± 27.4 | 0.05 |
| Blood pressure (mmHg) | 124 ± 5.6 | 132.3 ± 10.5 | n.s. |
| Retroperitoneal fat deposits (g) | 5.25 ± 0.8 | 14.02 ± 2.4 | 0.05 |
| Glucose (mg/dL) | 87.7 ± 8.6 | 105 ± 6.2 | 0.05 |
| Triglycerides (mg/dL) | 58.5 ± 12.7 | 117.8 ± 17.3 | 0.001 |
| Cholesterol (mg/dL) | 51.2 ± 4.9 | 52.9 ± 4.1 | n.s. |
| HDL-cholesterol (mg/dL) | 39.4 ± 3.7 | 36.0 ± 1.8 | n.s. |
| LDL-cholesterol (mg/dL) | 6.2 ± 0.9 | 7 ± 1.3 | n.s. |
| Insulin ($\mu$UI/mL) | 11.5 ± 2.3 | 12.0 ± 2.3 | n.s. |
| Endotoxin (EU/mL) | 0.0276 ± 0.0048 | 0.0332 ± 0.0088 | n.s. |

*Means were separated by unpaired $t$-test or Mann-Whitney $U$ test.

The higher levels of miR-146a observed only in the RNA from the plasma EVs in the sucrose group may consider that miR-146a levels are associated with several diseases, including diabetes [23, 24]. Since in our experiment the sucrose group rats had a mild hyperglycemia, we think that, as others have suggested, miR-146a upregulation through EVs may be an anti-inflammatory mechanism important in the controls of insulin sensitivity induced by inflammatory mediators [25]. Thus, it is possible that upregulation of circulating miR-146a on hyperglycemia may start in EVs, as seen in our chronically exposed rats. In patients with newly diagnosed type 2 diabetes miR-146a is elevated [24] and may diminish as disease progresses [23]. We also found lower levels of miR-155 in plasma EVs, correlated with total plasma levels. This reduction may be explained by the expansion of the adipose tissue found in our sucrose group of rats. Accordingly Chen and collaborators showed that miR-155 and C/EBP$\beta$ constitute a bistable system for the regulation of adipogenesis [26]. In inflammation, evidence so far presented on miR-155 function indicates that it is likely to be pro- rather than anti-inflammatory [27]. Although, it has been recently reported by Li and collaborators that miR-155 is overexpressed in the plasma from patients with atherosclerosis and may have a key role in the anti-inflammation activity of macrophages, attenuating foam cell formation [28].

The changes seen in the expression of miR-146a and miR-155 may reflect part of the functional adaptations after a chronic exposure to high sucrose, in this case probably related to the innate immune response. In a model of endotoxemia in mice, it has been reported that exosomal miR-146a inhibits while miR-155 promotes the inflammatory response in some contexts [29]. Thus, the alternated increase of miR-146a and reduction miR-155 in plasma EVs could be part of the miRNA-mediated modulation of the inflammatory response.

We found miR-223 upregulated in both plasma and plasma EVs from the sucrose group of rats. These results are opposed to others previously reported in obese [30, 31] and type 2 diabetic individuals [32], in whom downregulation of miR-223 was found. Another study, however, found that levels were unchanged in diabetic subjects [33]. Previous studies using also chronic ingestion of sucrose found high levels of adiponectin [6, 16]. In the adipose tissue miR-223 suppresses proinflammatory activation of macrophages [34] and probably contributes to the results showing high levels of adiponectin in sucrose ingestion [6]. Also, this upregulation of miR-223 may in part account for the unchanged levels of circulating IL-1$\beta$ in six months and its downregulation after 12 months [16]. It has been recognized that miR-223 negatively regulates NLRP3 and therefore IL-1$\beta$ production [35].

Our results suggest that high sucrose consumption may induce a low grade inflammatory state characterized by a decrease in miR-155 with the increase of miR-21, miR-146a, and miR-223 in EVs. The results presented herein gain relevance in light of recent evidence showing that a horizontal vesicle-mediated transfer of miRNAs allows the intercellular dissemination of gene expression regulatory messages, which may modify the function of target cells. Interestingly, exosome produced by macrophages upon administration to mice migrate into the adipose tissue [36]. Further studies are needed to clarify the cells originating the changes in EVs miRNA composition upon chronic consumption of sucrose.

## 5. Conclusions

Chronic ingestion of sucrose induced the upregulation of miR-21 and miR-223 in plasma and EVs. Interestingly, the combined upregulation of miR-21 and downregulation of mR-155 may possibly be responsible of high carb diets (in this case sucrose) mediating the adipose tissue expansion. Thus, we hypothesize that inflammatory modulation triggered by the high availability of simple carbohydrates from early life may force the organism to seek homoeostatic mechanisms including regulation by inflamma-miRs.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Malinalli Brianza-Padilla and Roxana Carbó participated equally in this work.

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*Research Article*

# Effect of Exogenous Fetuin-A on TGF-$\beta$/Smad Signaling in Hepatic Stellate Cells

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*Objective.* To explore the effects of low concentration of exogenous fetuin-A intervention on TGF-$\beta$1 induced LX2 cells through detection of the expression of mRNA and protein of Smad2, Smad3, and Smad7. *Methods.* MTT assay was used to detect the LX2 cells proliferation and the regression equation calculating software was applied to determine $IC_{50}$ of fetuin-A. RT-PCR was used to determine the relative content of Smad2, Smad3, and Smad7 mRNA in LX2 cells. Western blot was used to detect the LX2 cells relative content of Smad2, Smad3, Smad7 protein expression, respectively. *Results.* The analysis from RT-PCR and western blot showed that when compared with the other groups TGF-$\beta$1 + fetuin-A group increased the expression of Smad2 and Smad3 while decreased the expression of Smad7 ($P < 0.05$). *Conclusion.* Fetuin-A may improve the excessive activation of hepatic stellate cells which is caused by an enhanced positive regulation of Smad2 and Smad3 protein and the deficiency in negative regulation of Smad7 protein. This is through inhibiting the expression of Smad2 and Smad3 gene and promoting the expression of Smad7 gene. As a result, the development of liver fibrosis will be reduced.

## 1. Introduction

Fetuin-A, discovered in 1944, is a 59 kDa glycoprotein [1]. It is mainly synthesized by hepatic stellate cells (HSC) and thus closely related to liver. It works as a rare negative acute phase protein, downregulating the activity of macrophages, and has a strong anti-inflammatory effect [2]. Inflammation is one of the major factors that leads to liver fibrosis; thus anti-inflammatory effect of fetuin-A may influence the progress of hepatic fibrosis. At the same time, fetuin-A is also known as a natural TGF-$\beta$ antagonist [3] and is closely associated with TGF-$\beta$/Smad signaling pathway, which plays a key role in the process of liver fibrosis. Therefore, we inferred that fetuin-A may inhibit the process of liver fibrosis through TGF-$\beta$/Smad signaling pathway.

## 2. Materials and Methods

### 2.1. Materials

*2.1.1. Cultivation of Human Hepatic Stellate (LX2) Cell Line.* Human hepatic stellate (LX2) cell lines were obtained from Xiangya central laboratory of Central South University. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which is a modification of Basal Medium Eagle (BME), with a higher concentration of amino acids and vitamins than BME and additional supplemental components. It is also supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 $\mu$g/mL of streptomycin.

*Conditions.* The plates were cultured in a 5% $CO_2$ and 100% humidity cell culture box.

*Reagents.* Trizol, Invitrogen, #15596-026; RevertAid™ H Minus First Strand cDNA Synthesis Kit, Fermentas #K1631; Deoxyribonuclease I (DNase I), Fermentas #EN0521 were used.

RiboLock™ Ribonuclease Inhibitor, Fermentas #EO0381; SYBR Green PCR Master Mix, ABI 4309155, were used.

*First Antigen.* Mouse Smad7 antibody (1 : 800), SANTA, SC-365846; rabbit TGF$\beta$1 antibody (1 : 400), SANTA, SC-146; rabbit Smad2/3 antibody (1 : 400), CST, #3102; mouse fetuin-A

antibody (1 : 800), SANTA, SC-133146; mouse GAPDH antibody (1 : 800), SANTA, SC-365062, were used.

*Second Antigen.* Goat anti-mouse IgG/HRP (1 : 80000); goat anti-rabbit IgG/HRP (1 : 40000); goat anti-rabbit IgG/HRP (1 : 40000); goat anti-mouse IgG/HRP (1 : 80000); goat anti-mouse IgG/HRP (1 : 80000) were used.

*2.1.2. Establishment of Four Experimental Groups*

10% FCS + DMEM culture liquid

TGF-$\beta$1 experimental group: 10% FCS + DMEM culture liquid + final concentration of 5 ng/mL TGF-$\beta$1

TGF-$\beta$1 + fetuin-A experimental group: 10% FCS + DMEM culture liquid + final concentration of 5 ng/mL TGF-$\beta$1 + 10 ng/mL fetuin-A

TGF-$\beta$1 + asialoglycoprotein + fetuin-A experimental group: 10% FCS + DMEM culture liquid + final concentration of 5 ng/mL TGF-$\beta$1 + 10 ng/mL fetuin-A treated with asialoglycoprotein

*2.2. Methods*

*2.2.1. Determination of Fetuin-A Concentrations Intervention.* Cells were added in 96-well microtiter plates (100 $\mu$L/hole, approximately $1 \times 10^4$) and were cultured at 37°C in a 5% $CO_2$ humidified incubator for 24 hours which were then mixed with the appropriate concentration of tested compounds. The plates were cultured in a 5% $CO_2$ and 100% humidity cell culture box. Each hole was added with 50 $\mu$L 1x MTT and incubated for 4 hours. Discard supernatant, and 150 $\mu$L DMSO was added to each hole to dissolve the armour and was shaken. The optical density of each hole at was detected at 570 nm. The temperature will remain the same during the whole process.

*2.2.2. RNA Isolation and Purification and Real-Time PCR.* Total RNA was extracted from cells using Trizol reagent with the instructions of Invitrogen. Reverse transcription was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. Real-time PCR samples were prepared with SYBR Green PCR Master Mix (ABI 4309155) and real-time PCR was performed with an ABI Prism 7500 Detector System. The Housekeeping Gene GADPH was used as an internal control. The real-time PCR primers were from Gene Bank (BCOl2678, mouse fetuin-A cDNA). Primer sequences are as follows: Smad2 gene (180 bp) upstream primer: 5-cggtagaaatgacaagaagg-3, downstream primer: 5-tcttcagattacagcctggt-3; Smad3 gene (155 bp) upstream primer: 5-gtccagtctcccaactgtaa-3, downstream primer: 5-aactggtagacagcctcaaa-3; Smad7 gene (169 bp) upstream primer: 5-atgatctacctcaggggaat-3, downstream primer: 5-gacttgatgaagatggggta-3; $\beta$-actin gene (208 bp) upstream primer: 5-cattaaggagaagctgtgct-3, downstream primer: 5-gttgaaggtagtttcgtgga-3.

The real-time PCR system contains a template of 1 $\mu$L; Primer A 100 nm; Primer B 100 nm; 2x SYBR Green PCR Master mix 12.5 $\mu$L; DDW 25 $\mu$L. Parameters are as follows: 94°C 5 min; 94°C 20 s, 61°C 20 s, 72°C 20 s, 40 cycles; 72°C 5 min; 55°C 10 s; +0.5°C/cycle 10 s (80 cycles). The melting curve was analyzed after the amplification (detection between 60–95°C) to determine DEGC. The conditions were set at incremental increase of 0.5°C and 5 s each cycles. After agarose gel electrophoresis and ethidium bromide coloration, the data obtained from the assays were analyzed with eagle eye II gel imaging and analysis system for digital conversion. To show the relative expression of Smad2, Smad3, and Smad7, fold change of expression was then calculated using the $2^{-\Delta\Delta Ct}$ method [4].

*2.2.3. Western Blot Analysis.* Western blot analysis was conducted according to previous studies [5, 6]. The cellular lysates extracted from the cells were used for protein assays. Protein concentration was detected by a spectrophotometer using a BCA protein assay kit. Equal amounts of protein were subjected to SDS-PAGE on a 10% poly-acrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane with blotted protein was blocked for 1 hour at room temperature with blocking buffer containing 5% BSA, followed by incubation with antibodies overnight at 4°C. GAPDH was used as an internal control. After SDS-PAGE, the proteins were transferred onto the PVDF membrane and hybridized with specific primary antibodies and were incubated with HRP-conjugated sheep anti-mouse IgG. Bands were viewed using the ECL kit (Amersham, Piscataway, NJ) according to the manufacturer's instruction.

*2.2.4. Statistical Analysis.* The data was presented as a mean ± SD. Using SPSS 15.0 software (SPSS, Chicago, IL), one-way analysis of variance (ANOVA) test was employed for the comparison among all groups. Levene's test was then applied to compare the statistical difference between the two groups. Tamhane's T2 test was used when the variance was unequal. All tests were two-sided and a $P$ value $< 0.05$ was considered to be statistically significant. The comparison of means between groups was made up by ANOVA.

## 3. Results

*3.1. The Determination of the Interfering Concentration of Fetuin-A.* According to the results of MTT Assays (Figure 1), the concentration was set at fetuin-A 10 ng/mL.

*3.2. Real-Time PCR.* To monitor for endogenous fetuin-A retention and exogenous fetuin-A supplement, the RQ value of each group were measured. Statistical analysis (Figure 2) suggests that the gene transcription of Smad2 in samples in group C was lower than that of the samples in groups A, B, and D. Both groups A and D were negative control groups ($P < 0.0001$). The values of group C were significantly lower than group B sample (0.123 ± 2.02% versus 1.441 ± 2.70%). The analysis of Smad3 shows similar results, whereas the results for Smad7 were conversely represented. In summary, mRNA expressions of Smad2 and Smad3 in group C were decreased, while Smad7 was increased. The differences were all statistically significant.

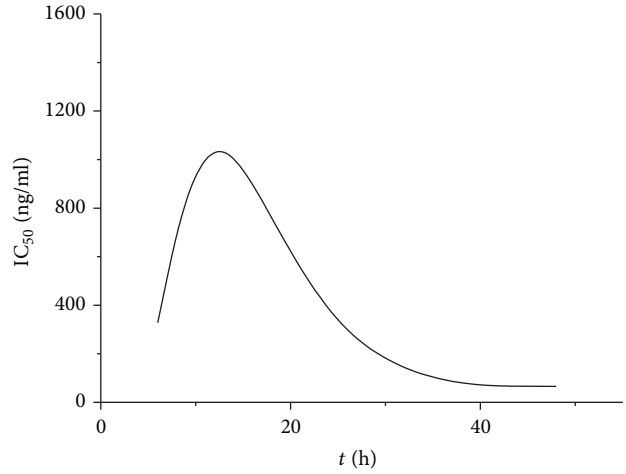


Figure 1: Time-$IC_{50}$ of fetuin-A.

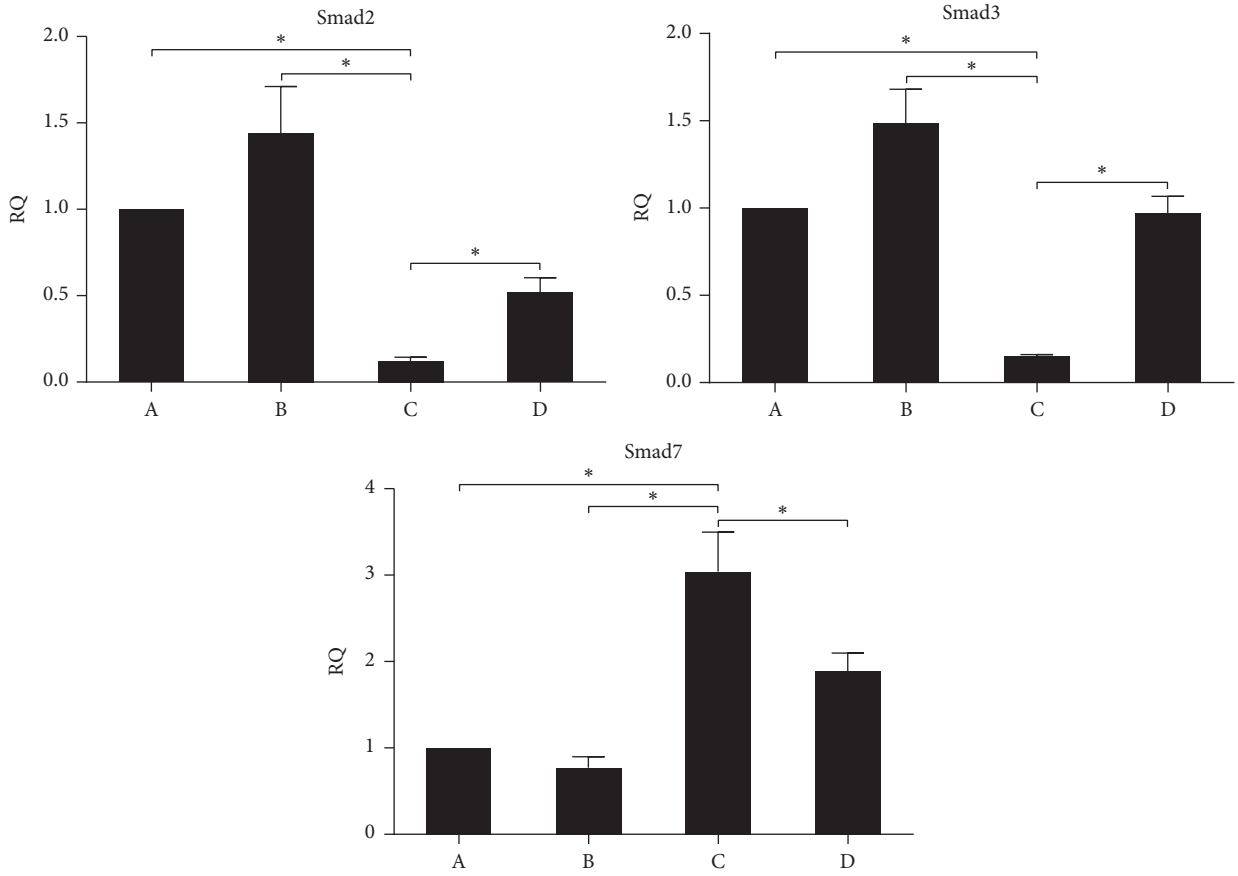


Figure 2: Relative expression of Smad2, Smad3, and Smad7. A: blank control group; B: TGF-$\beta$1 experimental group; C: TGF-$\beta$1 + fetuin-A experimental group; D: TGF-$\beta$1 + asialofetuin-A experimental group. The relative expression of Smad2, Smad3, and Smad7 was analyzed by RT-PCR. Experimental treatments were analyzed in triplicate. Data were represented as mean ± SD. Statistics were analyzed with a one-way analysis of variance (ANOVA) test. Asterisk represents $P < 0.05$.

*3.3. Western Blot.* To confirm well established functional consequence of exogenous fetuin-A, we analyzed differences in the translation of Smad2, Smad3, and Smad7 genes involved in TGF-$\beta$/Smad signaling pathway. The results of western blot (Figure 3) determine that mRNA translation of Smad2 and Smad3 in group C was significantly downregulated (0.662±2.03% versus 1.404±3.36%, 0.481±1.19% versus 1.421 ± 2.65%), while Smad7 was significantly increased (1.522 ± 4.53% versus 0.677 ± 1.23%). The differences showed statistical significance. The housekeeping gene GADPH was

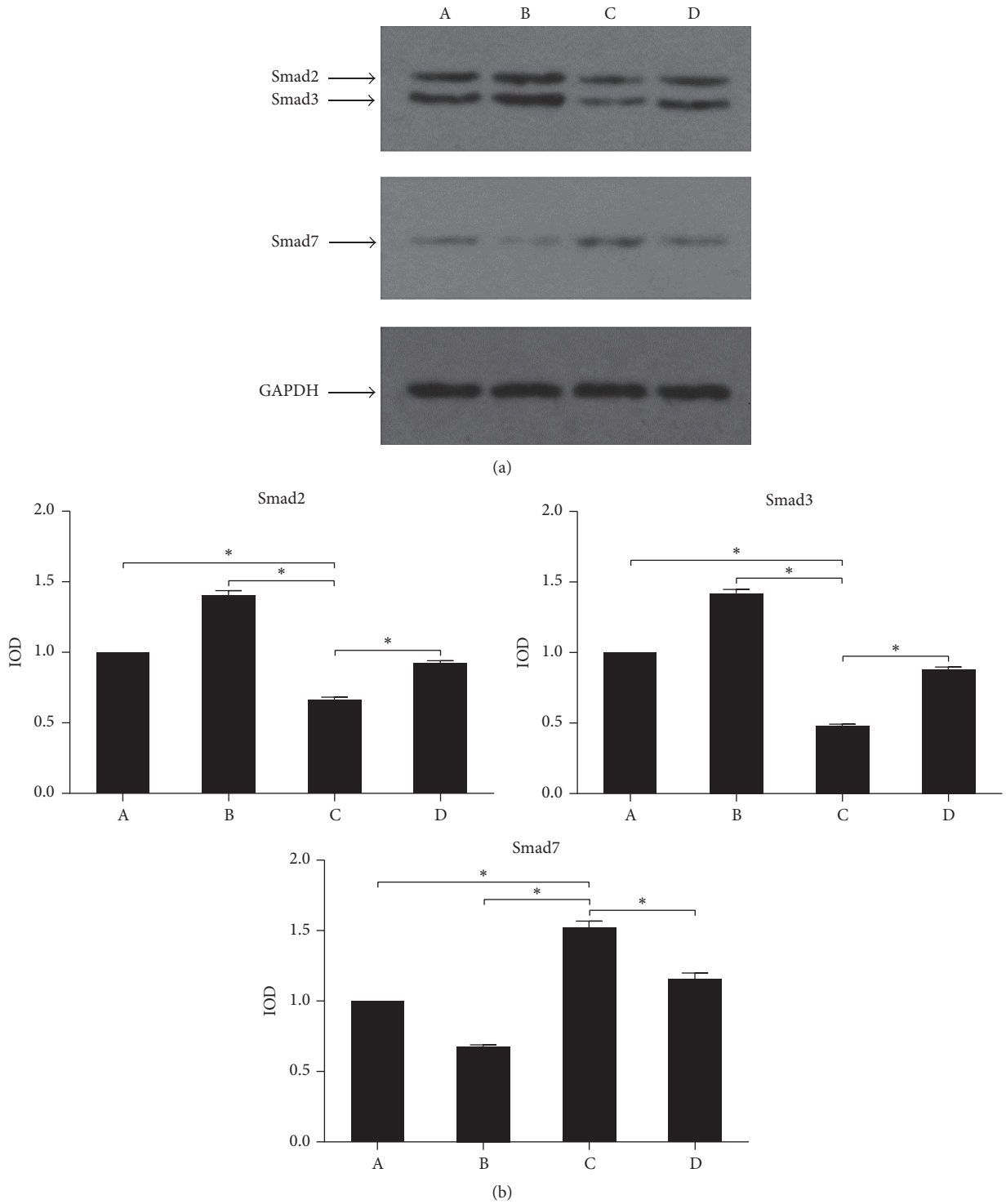


Figure 3: The protein abundance of Smad2, Smad3, and Smad7. (a) Representative bands showing the changes of Smad2, Smad3, Smad7, and GAPDH. Lane A: blank control group; Lane B: TGF-$\beta$1 experimental group; Lane C: TGF-$\beta$1 + fetuin-A experimental group; Lane D: TGF-$\beta$1+ asialofetuin-A experimental group. (b) Densitometric analysis of Smad2, Smad3, Smad7, and GAPDH. Relative protein expression was analyzed by western blot. Experimental treatments were analyzed in quadruplicate. The positive bands were quantitatively analyzed by Gel Pro 4.0 Analysis software after their integrated optical density (IOD) was measured. The housekeeping gene (GADPH) was used as an internal control. Data were represented as mean ± SD. Statistics were obtained with a one-way analysis of variance (ANOVA) test. Asterisk represents $P < 0.05$.

used as an internal control and the differences between each experimental group were not statistically significant.

## 4. Discussion

Liver fibrosis is the excessive accumulation of extracellular matrix proteins, such as collagen. It is considered as a wound healing response to chronic liver injury. It always indicates the onset of progressive disease, which may eventually lead to cirrhosis and end-stage liver disease [7, 8]. HSC has a major role in the accumulation of extracellular matrix (ECM) which contributes to fibrogenesis. This is activated mainly through TGF-$\beta$/Smad pathway. Therefore, this experiment has attempted to explore how fetuin-A affects the gene expression of Smad2, Smad3, and Smad7, researching for how fetuin-A affects the pathway as well as its potential influence on HSC activation.

TGF-$\beta$ works through multiple signaling pathways, affecting cell proliferation, apoptosis, recession, differentiation, and migration [9]. It is known as a major cytokine, with a complex function in HSC activation accelerating liver fibrosis [10, 11]. Its signaling pathways include Smad dependent and Smad independent pathways. Recent studies show that Smad proteins are important substrate for TGF-$\beta$ receptor intracellular kinase. Furthermore, they effectively mediated in the TGF-$\beta$ in the intracellular signal transduction [12, 13].

The classical TGF-$\beta$/Smad pathway is a highly conserved linear cascade process. Firstly, TGF-$\beta$ was combined with TGF$\beta$ type II receptor (TGF$\beta$RII) on cell membrane, providing it with kinase activity, and then combined with TGF$\beta$I receptor (TGF$\beta$RI). Activated TGF$\beta$RI was then combined with restriction Smad (R-Smad), phosphorylating the COOH end of Smad2 or Smad3. This formed R-S heteromeric transcription complexes with Smad4 which shifted towards the nucleus and bonded with sequence specific DNA. This complex plays a role in transcriptional regulation [14], mediating HSC transforming to fibroblast (myofibroblaster, MFB) and leading to the development of fibrosis [15–18]. Smad7 gene is an inhibitor of TGF-$\beta$ signaling pathway (inhibition Smad, I-Smad). Smad7 inhibits the phosphorylation of Smad2 and Smad3 through the activation of TGF$\beta$RI, while inhibiting Smad2 and Smad3 combination with receptors. This formed a negative feedback loop in TGF-$\beta$ signaling and effectively antagonizied fibrogenesis [19, 20]. On the other hand, I-Smad can enhance the interaction of E3 ubiquitin protein ligase, Smad ubiquitin regulatory factor 1/2 (Smurf 1/2) and receptor complex, to regulate receptor update [21, 22].

When liver injury occurs, TGF-$\beta$ enables HSC activation, proliferation, and differentiation into MFB, promoting collagen production. TGF-$\beta$ mediates wound healing response through ALK/Smad2 and Smad3 signaling pathway [23]. The ongoing presence of the damaging factors alters the phenotypic conversion of HSC. As a result, the inhibition effect of Smad7 on the TGF-$\beta$ negative feedback will be reduced. Consequently, HSC is excessively activated, causing the ECM metabolic imbalance. Eventually, the progression of liver fibrosis is deteriorated [24, 25]. Compared with the blank control group, the gene expression of Smad2 and Smad3 in the TGF-$\beta$1 experimental group was increased while Smad7 was decreased, indicating that TGF-$\beta$/Smad pathway may be involved in the process of excessive activation of HSC and hepatic fibrosis.

Fetuin-A is mainly synthesized and secreted by the liver. During the fetal period, the concentration of fetuin-A in serum is high and drops significantly after an inflammatory injury [26–28]. Fetuin-A is also a natural TGF-$\beta$ antagonist [29], which can be applied in the TGF-$\beta$/Smad signaling pathway. Fetuin-A and TGF$\beta$RII have 18~19 amino acid sequence in common. Fetuin-A can be combined with TGF-$\beta$ related cytokines such as bone morphogenetic proteins 2, 4, and 6 and TGF-$\beta$1 and TGF-$\beta$2, thus competitively blocking the combination of TGF-$\beta$ and its TGF$\beta$RII on the cell surface. A study conducted by Carol confirmed that fetuin-A can inhibit the combination of TGF-$\beta$ and its receptor and thus blocking the phosphorylation and nuclear translocation of Smad2 and Smad3 [30]. Judging from the close relationship between TGF-$\beta$/Smad signaling pathway and liver fibrosis, we can speculate that fetuin-A has the potential to affect the progression of liver fibrosis via the TGF-$\beta$/Smad signaling pathway.

MTT assay was used to detect the proliferation of LX2 cells. The half inhibitory concentration ($IC_{50}$) of fetuin-A was then calculated by the regression equation software. The optimal concentration of fetuin-A was 10 ng/mL (Figure 1). At this concentration, the results of RT-PCR (Figure 2) and western blot (Figure 3) showed that, in comparison to the other groups, the gene expression of Smad2 and Smad3 in TGF-$\beta$1 + fetuin-A experimental group was enhanced while expression of Smad7 was suppressed. These differences were all statistically significant. Fetuin-A may reduce HSC hyperactivity and the severity of hepatic fibrosis. This is achieved by the suppression of Smad2 and Smad3 gene expression whereas the gene expression of Smad7 was promoted.

In conclusion, fetuin-A could be a protective agent for hepatic fibrogenesis through the TGF-$\beta$/Smad signaling pathway. Supplementation with exogenous fetuin-A could alter the balance between inflammation and liver fibrosis by reducing the inflammatory effect. Given that fetuin-A represents biological homology, a high affinity, and no significant side effects, the administration of fetuin-A confers protection against hepatic fibrosis. Therefore, further studies are required to explore the therapeutic potential of fetuin-A in the clinical management of acute liver failure.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contributions

Yulai Zhou and Shuang Yang contributed equally to this work and share the first authorship.

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*Research Article*

# Effect of Methionine Restriction on Bone Density and NK Cell Activity

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Methionine restriction (MR) is proven to increase the lifespan; and it also affects the bone density and the innate immune system. The aim of this study is to explore the effect of methionine restriction on bone density and natural killer (NK) cells. C57BL/6J mice were subjected to either basal diet (BD, containing 0.80% methionine) or methionine-restricted diet (containing 0.14% methionine). Mice with MR diet displayed reduced bone mass and decrease in the cytotoxicity of NK from the spleen, compared to BD animals. Also, mice with MR diet had an inferior body weight ($P < 0.05$) and higher plasma levels of adiponectin and FGF21 ($P < 0.05$) but lower concentrations of leptin and IGF-1 ($P < 0.05$). Overall, the investigation shows that methionine affects bone density and NK cell cytotoxicity.

## 1. Introduction

In modern times, a person's health and fitness have become an important factor in everyday life, and researchers are pursuing ideas that enable people to improve their vitality and prolong their life. Experiments occurring in the last thirty years have used the restriction of certain macronutrients to promote a healthier lifestyle. While methionine restriction (MR) is a new invention to improve lifespan and vitality, protein restriction (PR) has been used for a lengthier period [1]. Also, caloric restriction (CR) has been a positive source to extend the average lifespan, plus increasing the maximal age [2]. This important and significant increase in lifespan can be principally accounted for due to age-related illnesses appearing later than in previous generations, plus the downregulation of oxidative stress [3]. Also, when dietary methionine content is restricted, energy metabolism is altered [4], and it can increase a rodent's lifetime by thirty percent [5]. This alone is interesting, but MR can also intensify concentrations of glutathione, which is an antioxidant, in blood. Also MR can lessen visceral adiposity and induce insulin receptiveness [3, 5].

There is a worry with MR that it causes reduced bone strength. This side-effect was verified in rats given MR resulting in stunted growth, making them light and diminutive [6]. MR could have an impact on bone growth/development, tissue material properties, and bone metabolism when related to alternative diets which use dietary restrictions. This idea comes from the fact that the consumption of MR, by rats, for a lengthened period of time results in the intake of more sustenance per unit of body weight (BW), when related to rats in control groups.

Gene expression can go through alterations if DNA-methylation patterns, facilitated by *S*-adenosylmethionine, when subjected to methionine-deficient diets [7]. Androgen-independent prostate cancer cells, when exposed to methionine created apoptosis, signify affected motility [8]; plus methionine reduces mitochondrial oxidative stress [9]. As we become older, methylation deteriorates, which can result in the reduction of T cells and NK cells. Unfortunately, this can alter the actions of the immune system, but methylation can reverse this and prevent this by assisting in the construction of natural killer (NK) cells. These cells signify a distinct lymphocyte subset, playing an important part in innate

immunity. Findings in humans and mice propose that NK cells are a significant tool for influencing the functions of the adaptive immune response [10] and, due to their cytotoxic role, NK cells can fight against pathogens and tumors, in addition to other vital inception [11]. Thus, in this study, we examined correlation between MR and bone density and the cytotoxic activity of NK cells in C57BL/6J mice.

## 2. Material and Methods

*2.1. Animal Care.* During this project, procedures set out by the Laboratory Animal Ethical Commission of Tianjin Hospital were followed. In the beginning, we obtained, from the Tianjin Laboratory Animal Center (Tianjin, China), forty-seven-week-old C57BL/6J mice and accommodated them in typical housing, which was regulated at 22 ± 3°C and 53 ± 10% comparative humidity. They were given nourishment and water (pH 2.8) as needed and kept in a light cycle of twelve hours of light, followed by twelve hours of a dark photoperiod. When the fourteen-week experiment began, the mice were randomly divided into two groups, with one set given the basal diet (BD) consisting of 0.80% methionine and the other group receiving the methionine-restricted sustenance, consisting of 0.14% methionine. The researchers measured the body mass and the food intake of the mice, twice per week, with the average daily food eaten by every mouse being calculated to find the cumulative food amount. When the experiment drew to a climax, the mice were subjected to a four-hour fasting period, starting at the introduction of the light cycle. This was to assign a physiological baseline, before being sacrificed. After that, blood was obtained via the retroorbital plexus, a sample of plasma was flash-frozen and kept at −80°C, and femur and spleen bones were attained, flash-frozen, again at −80°C, and saved for examination.

During the study, enzyme-linked immunosorbent assay (ELISA) kits were utilized when recording the amount of apo B, leptin, adiponectin, insulin, homocysteine, IGF-1, and FGF21. Also, an Abbott® Freestyle glucometer and glucose strips were the choice to detect the amount of blood glucose. Colorimetric assays were needed to detect plasma triglycerides (TG), total cholesterol (TC), LDL, and HDL. Finally, a Beckman Synchron CX5 system distinguished the quantity of plasma alanine aminotransferase.

*2.2. Preparation of Target Cells and NK Cell Degranulation and Cytotoxicity.* Total RPMI 1640 medium, primed as stated above, was used to cultivate YAC-1 cells, which are murine T-lymphoma cell lines, receptive to NK cell killing. The habitat was kept at 37°C in 5% $CO_2$, and cells were subcultured once a day to guarantee that the log-phase was displayed. During the essay, the cells were coloured with 10 nM of carboxyfluorescein succinimidyl ester in darkness for ten minutes. Then, they were cleaned thoroughly twice in PBS with 2% heat-inactivated fetal bovine serum, before being subjected to another solution of $10^6$ cells/mL in medium. The methods of preparation of NK cells and the cytotoxicity of these cells were gained during the flow cytometric assay defined by Cao et al. [12].

*2.3. Statistical Analyses.* The information is displayed as the means ± the standard error of the mean (SEM), and the statistical research was collated and scrutinised, by use of a SPSS 25.0 (Chicago, IL, USA). The significant differences between the sets, recorded by a Student's $t$-test, were defined at $P < 0.05$.

## 3. Results

To test whether MR diet can inhibit body weight gain, average daily food intake, body weight, and body weight gain were measured. As shown in Figure 1, average daily food intake was almost the same in both BD and MR mice during the whole period (Figure 1(a)). However, significantly lower body weight was found in MR mice compared to that in the BD group ($P < 0.05$) from three weeks after the start of the experiment (Figure 1(b)). Meanwhile, in the MR mice, a significantly lower weight gain ($P < 0.05$) was observed (Figure 1(c)). Data of plasma lipids indicated TG level was significantly lower in MR mice compared to that of BD mice ($P < 0.05$). Meanwhile, the levels of TC, HDL, LDL, and apo B were not altered in both groups (Table 1). As represented in Table 1, the study of plasma levels of hormones linked to the resistance of insulin was carried out. Mice with MR diet had higher ($P < 0.05$) level of adiponectin and FGF21 but lower concentration of leptin and IGF-1 ($P < 0.05$).

Table 2 confirmed the concern that an MR diet altered the density and structure of bones, as investigations into bone consistency revealed that MR mice were not as long as the BD mice ($P < 0.05$). Also, MR animals had left femurs which were diminished when compared to BD mice ($P < 0.05$). Supplementary evidence comes in the form of the MR group displaying diminished amounts of BMC and BMD ($P < 0.05$), in a bone mineral density examination, by means of DEXA. Also, in contrast to BD mice, MR animals had slighter diameters of mediolateral and anteroposterior shafts, and the third trochanter shafts were also prominently diminished ($P < 0.05$).

Another area which is found to be pointedly lessened in MR animals, in contrast to BD mice, is NK cell cytotoxicity, which has cell ratios of 20 : 1 for MR mice and 10 : 1 in BD animals (Figure 2). The NK cell cytotoxicity, consuming BD diet or MR diet, is so diverse; this is because the cytotoxicity assay records the action of both NK and NKT cells. As shown in Figure 3, the spleens of both diet sets recorded a similar percentage of NK and NKT cells ($P > 0.05$).

## 4. Discussion

In this experiment, methionine was restricted in, not eradicated from, the diet of the mice, similarly to other MR examinations, where the experiments were performed on rats, and proved to prolong their lifespan [6, 13]. Furthermore, the restrictive consumption of methionine decreased mitochondrial reactive oxygen species in rats [14] indicated that

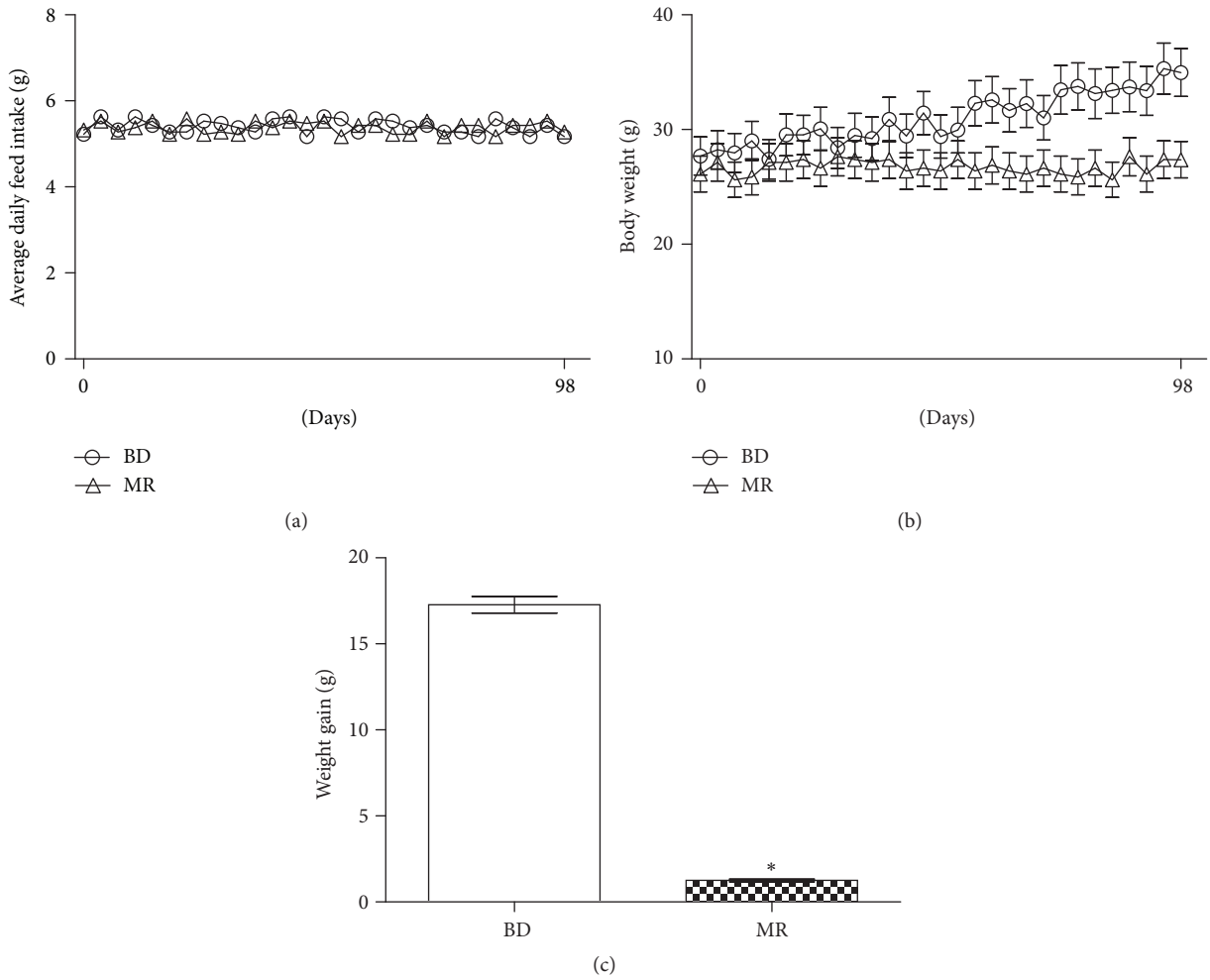


Figure 1: MR mice have lower body weight and lower body weight gain. (a) Average daily feed intake for 14 weeks. (b) Body weight was measured twice a week for 14 weeks. (c) Body weight gain. $^{*}P < 0.05$. $n = 10$.

their levels of lipogenic, lipolytic, and conceivably hypercholesterolemia progressed [15]. When witnessing the rats, we were conscious to note that the rodents did not suffer any ill effects while being subjected to the MR diet.

In this experiment, comparisons between the different sets of mice were varied. The typical daily food consumption was comparable in MR and BD groups, while the animals in the MR set displayed a pointedly diminished body weight when contrasted, three weeks into the study and thereafter, with the BD group. Moreover, there was a connection found with the reduction of bone density. In contrast, MR mice, which were given high fat diet, signified a reduction in body weight gain, albeit developing hyperphagia, which is known to occur in rats and mice, consuming low-fat foods [13]. The hyperphagia could be seen in the animals because of their bodies producing elevated levels of glutamic acid, in a need to compensate for the decline in methionine consistencies [16]. However, ultimately, the movement and physical planes of the rats consuming a reduced methionine diet and the mice who were given the total required amount of amino acids were comparable.

The result of the MR diet found in the configuration of the C57BL/6J mice was comparable with plasma adiponectin, which had elevated levels, and also with the diminished quantities of IGF-1, leptin, and insulin [4]. Heightened levels of adiponectin were recorded in the mice in the MR group, and these levels have been known to potentiate insulin receptive properties, via the initiation of PPAR$\gamma$ signaling [17]. FGF21 is a hormone, newly identified and capable of immense interaction with glucose homeostasis [18]. The MR mice, in this study, indicated heightened planes of FGF21, within their plasma, when compared to BD grouped animals, and when analysed further, MR mice show a similar hormone make-up to those of insulin sensitive animals. FGF21, when subjected to MR, can clarify, to some extent, the reductions in growth witnessed in the MR animals [19]. There are surprising correlations between the research carried out in this study and alternative explorations, such as the interpretations of Inagaki and company, where FGF21 transgenic animals displayed not only a smaller mass, but also declined amounts of plasma IGF-1 in comparison to the wild-type versions [20]. Furthermore, these researchers found that the influence

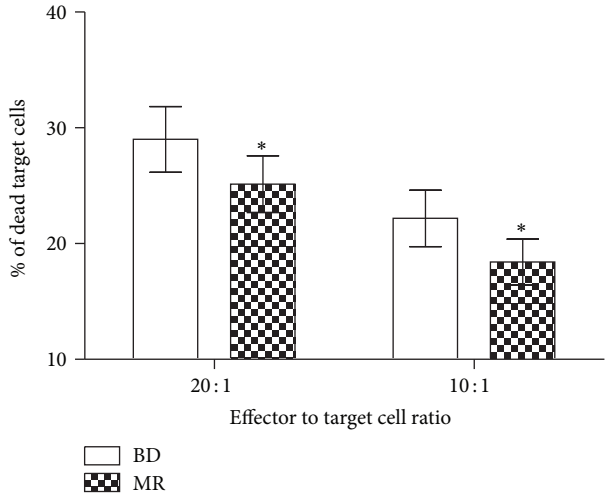


Figure 2: The consequence of the MR diet on NK cell cytotoxicity. Splenocytes were secluded before being gestated with YAC-1 target cells at 20 : 1 and 10 : 1 ratio of effector to target cells. Dead target cells were enumerated by flow cytometry, and the quantities are presented as means ± SEM, $n = 8$. $^*P < 0.05$.

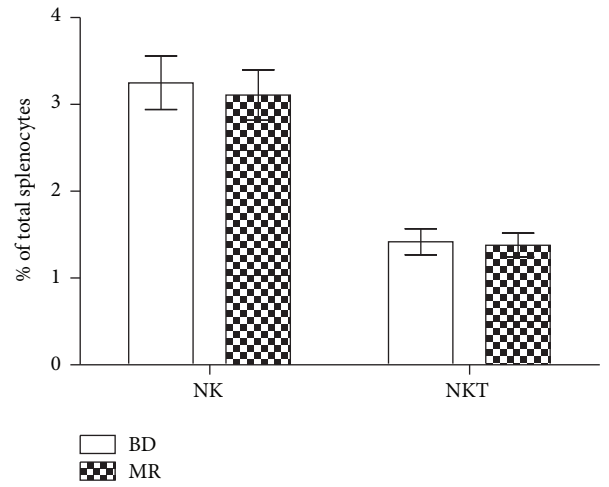


Figure 3: The influence the MR diet has on aged mice within NK cells. Splenocytes are recorded as percent of NK or NKT cells in entire splenocytes enumerated. Data are presented as means ± SEM, $n = 8$.

of FGF21 was moderated by phosphorylated Stat5 being heightened and Jak2 phosphorylation being diminished [20]. This brings about an interesting point concerning our data, in the context of FGF21 being the agent which disturbs the growth hormone signaling downstream of Jak2. In this alternative study, wild-type mice suffered augmented bone deterioration, by means of the PPAR$\gamma$ agonist, rosiglitazone, whereas no change was recorded in the FGF21 knockout mice, possibly concluding that FGF21 could be the attribute responsible for diminished bone density in PPAR$\gamma$ signaling [21]. Therefore, it is plausible that the lessening of bone mass in MR mice could be happening due to heightened FGF21 involvement.

Table 1: Plasma biochemistry of BD mice and MR mice ($n = 8$, $^*P < 0.05$).

| | BD group | MR group |
|---|---|---|
| TG (mg/dL) | 67.25 ± 8.12 | 55.78 ± 4.86* |
| TC (mg/dL) | 145.83 ± 12.47 | 149.32 ± 13.27 |
| LDL (mg/dL) | 32.76 ± 2.89 | 33.14 ± 2.43 |
| HDL (mg/dL) | 106.37 ± 9.54 | 105.38 ± 8.89 |
| Apo B ($\mu$g/dL) | 30.1 ± 3.5 | 30.9 ± 3.3 |
| Adiponectin (ng/dL) | 3.67 ± 0.42 | 5.78 ± 0.76* |
| FGF21 (pg/mL) | 78.76 ± 7.12 | 97.48 ± 10.54* |
| IGF-1 (pg/mL) | 424.85 ± 54.18 | 281.24 ± 31.19* |
| Leptin (pg/mL) | 210.11 ± 23.42 | 120.56 ± 14.55* |

Table 2: Bone parameters of femurs from BD mice and MR mice ($n = 8$, $^*P < 0.05$).

| | BD group | MR group |
|---|---|---|
| Femur length (mm) | 16.23 ± 0.54 | 15.43 ± 0.48* |
| Mediolateral shaft diameter (mm) | 2.12 ± 0.13 | 1.91 ± 0.13* |
| Anteroposterior shaft diameter (mm) | 1.36 ± 0.11 | 1.21 ± 0.08* |
| Third trochanter diameter (mm) | 2.85 ± 0.17 | 2.53 ± 0.28* |
| BMD (g/cm$^2$) | 0.061 ± 0.008 | 0.049 ± 0.006* |
| BMC (g) | 0.031 ± 0.006 | 0.024 ± 0.005* |

The MR diet's influence on NK cytotoxicity was then further investigated, with no major variances in NK cells percentage amongst dietary sets. NK cell cytotoxicity and immune activity benefit from receiving satisfactory methionine, and this project has proved that methionine restriction is related to reduced NK cell function. For these years, there have been ongoing studies into the correlations between an intensified risk of cancer and reduced NK cytotoxicity [22]. The results indicated a reduced amount of NK cell cytotoxicity, because of restricted methionine, increased the risk of cancer and viral infections, particularly in older generations.

In conclusion, the animals subjected to the MR diet did not display any unhealthy side-effects except the inferior body weight and lower bone density. The accumulation of information concerning the study exhibited reduced NK cell cytotoxicity, which could be due to weakening or damage to NK cells while maturing. Additional studies are needed regarding the impact made on NK cell cytotoxicity by restricted methionine and the effects for improving health in humans.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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*Research Article*

# Purification and Characterization of a Thermostable $\beta$-Mannanase from *Bacillus subtilis* BE-91: Potential Application in Inflammatory Diseases

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$\beta$-mannanase has shown compelling biological functions because of its regulatory roles in metabolism, inflammation, and oxidation. This study separated and purified the $\beta$-mannanase from *Bacillus subtilis* BE-91, which is a powerful hemicellulose-degrading bacterium using a "two-step" method comprising ultrafiltration and gel chromatography. The purified $\beta$-mannanase (about 28.2 kDa) showed high specific activity (79, 859.2 IU/mg). The optimum temperature and pH were 65°C and 6.0, respectively. Moreover, the enzyme was highly stable at temperatures up to 70°C and pH 4.5–7.0. The $\beta$-mannanase activity was significantly enhanced in the presence of $Mn^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Ca^{2+}$, $Mg^{2+}$, and $Al^{3+}$ and strongly inhibited by $Ba^{2+}$ and $Pb^{2+}$. $K_m$ and $V_{max}$ values for locust bean gum were 7.14 mg/mL and 107.5 $\mu$mol/min/mL versus 1.749 mg/mL and 33.45 $\mu$mol/min/mL for Konjac glucomannan, respectively. Therefore, $\beta$-mannanase purified by this work shows stability at high temperatures and in weakly acidic or neutral environments. Based on such data, the $\beta$-mannanase will have potential applications as a dietary supplement in treatment of inflammatory processes.

## 1. Introduction

Mannan consists of a series of complex polysaccharides, which are found in the cell wall of marine algae [1]. The backbone is comprised of $\beta$-1,4-linked mannose residues. Konjac glucomannan is a randomly arranged polymer of $\beta$-1,4-linked glucose and mannose residues at ratio of 1.0 : 1.6. Both the backbones of mannan and Konjac are modified by $\alpha$-1,6-linked galactosyl residues to form galactomannan and galactoglucomannan, respectively [2].

$\beta$-mannanase (EC 3.2.1.78) is a hemicellulase that attacks the internal glycosidic bonds of mannan backbone to release the condensed $\beta$-1,4-manno-oligosaccharides [3]. $\beta$-mannanases are widely applied in pulp and paper processing [4], feed [5], food [6], pharmaceutical [7], oil, and textile industries [8] to randomly hydrolyze the $\beta$-1,4 mannopyranoside linkage in mannan, galactomannan, glucomannan, and galactoglucomannan.

$\beta$-mannanase is widely produced by bacteria [9, 10], actinomycetes [11], fungi [12], plants, and animals [13]. Among them, $\beta$-mannanase from bacteria is wildely used because of numerous advantages, including extracellular secretion, economic production and purification, and novel characteristics, such as tolerance to heat and alkaline conditions [14].

Although multiple $\beta$-mannanase-producing bacteria have been reported [15, 16], they are far from the diverse industry needs. Currently, acidic and alkaline $\beta$-mannanase has been proposed to meet the industrial demands [17]. However, the requirements of high energy in production and the environmental impact limit their development. Neutral and weakly acidic $\beta$-mannanase with lower energy for production has attracted considerable interest over the past few years; however, it has rarely been characterized. It is clarified that $\beta$-mannanase with high activity in short fermentation time confers lower costs during the production procedures. Therefore, the exploitation of strains producing high $\beta$-mannanases activity is valuable and profitable. In current study, we isolated and preserved a powerful hemicellulose-degrading bacterium (BE-91). Then we explored

the efficient purification process and characterized the enzymatic properties of its $\beta$-mannanase.

## 2. Materials and Methods

*2.1. Microorganism, Media, and Fermentation Conditions.* *B. subtilis* BE-91, a strain used for herbaceous fiber extraction, was identified and preserved by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science (Changsha, Hunan, China). *B. subtilis* BE-91 was cultured in Petri dish containing 0.5% yeast extract, 1% NaCl, 0.5% Konjac glucomannan, 1% bacto tryptone, 0.05% trypan blue, and 1.5% agar. The seed medium was mainly composed of 0.1% glucose, 0.4% Konjac glucomannan, 0.3% beef extract, 0.2% yeast extract, 0.5% peptone, and 0.5% NaCl. The fermentation medium primarily consisted of 0.2% yeast extract, 0.7% Konjac glucomannan, 0.5% peptone, 0.3% beef extract, and 0.5% NaCl. *B. subtilis* BE-91 was first activated in the seed medium at 35 ± 1°C for 5.5 h. Subsequently, the suspension was serially diluted, spread onto Petri dishes, and incubated at 35 ± 1°C for 18 h. The single colony exhibiting the largest hydrolytic halo was transferred into an Erlenmeyer flask with the seed medium and cultured at 35 ± 1°C for 6 h at 180 rpm. Consequently, 2% culture was inoculated in the fermentation medium and cultured for 6 h at 35 ± 1°C at 180 rpm [18].

*2.2. Classification of Strain BE-91.* The 16S rDNA of strain BE-91 was PCR amplified from genomic DNA using the Bacterial Identification PCR Kit (TaKaRa, Japan). The obtained 16S rDNA was sequenced by the ABI 3730XL 96-capillary DNA analyzer. The primers were as follows: P1 5′-AGAGTTTGATCMTGGCTCAG-3′ and P2 5′-TACGGYTACCTTGTTACGACTT-3′. The resulting sequence aligned closely with the related standard sequences of other bacteria retrieved from GenBank. Neighbor-joining clusters were constructed by Mega 6.0 [19].

*2.3. Enzymatic Assays.* $\beta$-mannanase activity was estimated by initiating the reaction at 65°C for 10 min in 0.05 mol/L citric acid/0.1 mol/L $Na_2HPO_4$ buffer (pH 6.0) with 0.2% (w/v) Konjac glucomannan as substrate. The amounts of reducing sugar in the reaction were quantified based on a standard curve generated with mannose using the 3,5-dinitrosalicylic acid (DNS) method. One unit (IU) of $\beta$-mannanase activity was defined as the amount of protein producing 1 $\mu$mol/L of reducing sugar per minute (e.g., mannose) under standard conditions [20].

*2.4. Purification of β-Mannanase.* The bacterial $\beta$-mannanase was purified using a two-step process involving ultrafiltration (Sartorius, Germany) and gel filtration. The fermentation liquid was fractionated orderly by 100 kDa, 50 kDa, and 5 kDa membrane thresholds. The solution filtered with 5 kDa < MW < 50 kDa was further purified on a Sephadex G-100 gel column (Φ1.6 cm × 100 cm, Pharmacia). The eluate was obtained at a rate of 0.5 mL/min and collected in 5 mL fractions. $\beta$-mannanase activity was determined by the DNS method, whereas the protein was quantified by the Coomassie brilliant blue staining against bovine serum albumin (BSA) standard [21].

*2.5. The Determination of Apparent Molecular Weight.* The molecular mass of the $\beta$-mannanase was determined by SDS-PAGE (Bio-Rad, USA), with 3% stacking gel and 12% separating gel [22]. The protein bands were stained with 0.01% Coomassie brilliant blue R-250 and destained with a water-methanol-acetic acid (9 : 9 : 2) solvent. Zymogram analysis was performed by the method of Chanhan [17]. The molecular weight of $\beta$-mannanase was derived from the relative mobility of molecular weight markers resolved simultaneously.

*2.6. The Effect of Temperature on the Activity and Stability of β-Mannanase.* The activity of $\beta$-mannanase was assayed at a range of temperatures between 50 and 70°C in 0.05 mol/L citric acid-0.1 mol/L $Na_2HPO_4$ buffer at pH 6.0. The thermostability was assessed by preincubating the enzyme, without a substrate, at different temperatures varying over 20–80°C for 30 min. The residual activity was promptly measured by the DNS method. The $\beta$-mannanase activity was considered to be 100% when preincubated at 4°C.

*2.7. The Effect of pH on the Activity and Stability of β-Mannanase.* $\beta$-mannanase activity was evaluated by incubating the purified enzyme at different pH conditions ranging from 4.0 to 8.0 in 0.05 mol/L citric acid-0.1 mol/L $Na_2HPO_4$ buffer at 4°C. The stability at a particular pH was tested by preincubating the purified enzyme, without a substrate, for 30 min in various 0.05 mol/L citric acid-0.1 mol/L $Na_2HPO_4$ buffers at pH 3.0–8.5 at 4°C. The residual $\beta$-mannanase activity was immediately measured after treatment by the DNS procedure.

*2.8. The Effect of Metal Ions on the Activity of β-Mannanase.* In order to examine the effects of metal ions on the activity of $\beta$-mannanase, the enzyme was incubated for 30 min at 4°C in the presence of various 1.0 mmol/L metal ions, $CaCl_2{\cdot}2H_2O$, $ZnCl_2$, $FeCl_3$, $PbCl_2{\cdot}2H_2O$, $MnCl_2{\cdot}4H_2O$, $MgCl_2{\cdot}6H_2O$, KCl, $CuCl_2{\cdot}2H_2O$, $AlCl_3$, $BaCl_2$, and $NH_4Cl$. The residual $\beta$-mannanase activity was measured at a specific condition and that of the treatment in the absence of additives as a control.

*2.9. Substrate Specificity and Kinetic Parameters.* Various glycans, such as Konjac glucomannan [23], locust bean gum from *Ceatonia siliqua* seeds (Sigma, G0753), carob galactomannan (Megazyme, P-GALML), guar galactomannan (Megazyme, P-GGMMV), ivory nut mannan (Megazyme, P-MANIV), 1,4-beta-D-mannan (Megazyme, P-MANCB), wheat arabinoxylan (Megazyme, P-120601a), beechwood xylan (Megazyme, P-141101a), and carboxymethyl cellulose (Megazyme, P-CMC4M) were examined. In brief, 0.2% (w/v) glycans were incubated with $\beta$-mannanase at 65°C for 10 min in 0.05 mol/L citric acid-0.1 mol/L $Na_2HPO_4$ buffer at pH 6.0, and the reducing sugars were measured by DNS. The Michaelis-Menten kinetic parameters, $V_{max}$ and $K_m$, were calculated for $\beta$-mannanase. The assays of the purified enzyme were carried out by the standard DNS procedure, as described

Table 1: $\beta$-mannanase activities of five bacteria.

| Bacterium number | Activity (IU/mL)[a] | Specific activity (IU/mg)[a] |
|---|---|---|
| BE-23 | 0 | 0 |
| BE-78 | 191.5 ± 4.5 | 879.8 ± 13.2 |
| BE-46 | 83.2 ± 2.1 | 311.6 ± 9.4 |
| BE-83 | 70.7 ± 1.6 | 119.7 ± 25.5 |
| BE-91 | 273.7 ± 6.5 | 2,319.2 ± 26.3 |

[a]Data are mean ± SD, $n = 3$.

above, using 1–5 mg/mL locust bean gum and 0.5–2.5 mg/mL Konjac glucomannan as substrates. The kinetic constants were determined from the Michaelis-Menten equation by directly inputting the initial rates from Lineweaver-Burk plots or the nonlinear regression [24].

*2.10. Statistical Analysis.* Each $\beta$-mannanase activity experiment was performed in triplicate and expressed as mean ± SD (standard deviation). The statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago IL, USA). One-way or two-way analysis of variance (ANOVA) was used to compare various treatment groups.

## 3. Results and Discussion

*3.1. Screening of the High $\beta$-Mannanase Activity Producing Strain.* Four bacteria were stochastically selected for the $\beta$-mannanase activity assay. Figure 1 exhibited the halos produced on the screening plate. Table 1 summarized the $\beta$-mannanase activity of the four bacteria (strain BE-23 without $\beta$-mannanase activity was used as a negative control). Strain BE-91 fermented for 9 h exhibited the highest activity, up to 273.7 IU/mL. Wild-type *B. subtilis* MA139 yielded a maximum $\beta$-mannanase activity of 170 IU/mL after 3 days of fermentation, and the maximum enzyme activity of *B. subtilis* TJ-102 was 205.3 IU/mL at 38 h [25, 26]. Notably, BE-91 secreted $\beta$-mannanase with higher activity in shorter time.

*3.2. Classification of B. subtilis BE-91.* The 1,508 bp sequence of 16S rDNA of strain BE-91 was analyzed by a phylogenic tree (Figure 2). The homology between BE-91 16S rDNA (gi 260159552) and *B. subtilis* 16S rDNA (gi 530330588 and gi 341831474) was 99%. It was confirmed that the similarity of *B. subtilis* type strains about 16S rRNA gene sequence is higher than 98% [27, 28]. We also obtained ≥98% similarity to 16S rRNA gene sequences of *B. subtilis* isolates.

*3.3. Isolation and Purification of $\beta$-Mannanase.* 2,000 mL of fermentation liquor was purified by ultrafiltration and chromatography. Specific activity, recovery, and multiple purifications at each step were summarized in Table 2. The recovery of $\beta$-mannanase in *B. subtilis* BE-91 exceeded 66.0%; multiple purifications achieved 32.9-fold pure $\beta$-mannanase activity, and the specific activity of the purified enzyme reached 79,859.2 IU/mg. The purified $\beta$-mannanase was shown to be homogeneous judged by SDS-PAGE analysis (Figure 3). Compared with the previous separation and purification methods [29, 30], the two-step method has the advantages of high efficiency, high yield, and easy operation.

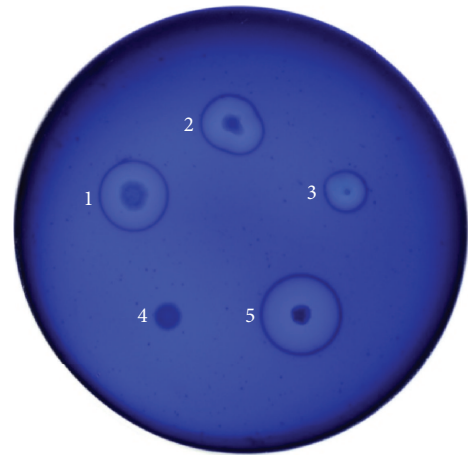


Figure 1: Clear halos produced by control and active colonies with $\beta$-mannanase activity 1, BE-78; 2, BE-46; 3, BE-83; 4, BE-23 (negative control); 5, BE-91.

*3.4. Apparent Molecular Weight of $\beta$-Mannanase.* The apparent molecular weight of $\beta$-mannanase was 28.2 kDa (Figure 3), lower than those of the most known $\beta$-mannanases from *Bacillus* spp. (*Bacillus licheniformis* THCM 3.1, 40 kDa; *B. subtilis* WY34, 39.6 kDa; *B. subtilis* Z-2, 38 kDa; *Bacillus circulans* CGMCC1554, 32 kDa) [28, 31–34]. Similarly, the molecular weights of $\beta$-mannanases from *Penicillium occitanis* Po16 and *Bacillus halodurans* PPKS-2 were 22 and 18 kDa, respectively [30, 31]. Due to low molecular weights, these enzymes may rapidly penetrate the lignocellulose systems and depolymerize the mannans more efficiently [35].

*3.5. Optimal Temperature and Thermostability of $\beta$-Mannanase.* The purified $\beta$-mannanase was maximally active at 65°C (Figure 4) and remained more than 80% active at 70°C (Figure 5). Compared with the optimal temperatures obtained for other $\beta$-mannanases (40°C for *Penicillium occitanis* Pol6; 50°C for both *Bacillus circulans* TN-31 and *B. subtilis* B36; 60°C for *Paenibacillus* sp. DZ3) [29, 31, 36], $\beta$-mannanase of BE-91 showed a pronounced activity at higher temperatures. As compared to the thermostability of the $\beta$-mannanase from wild-type *B. subtilis* BCC41051 (60°C for 30 min) [37], this $\beta$-mannanase retains 80% residual activity after incubation at 20–70°C for 30 min, indicating enhanced thermostability.

*3.6. Optimal pH and Stability of $\beta$-Mannanase.* The optimal pH and the stability of BE-91 $\beta$-mannanase were measured at various pHs. The optimum enzyme activity was obtained at pH 6.0 (Figure 6), and more than 80% maximal activity was retained at pH 4.5–7.0 (Figure 7). Interestingly, the optimal pH of BE-91 $\beta$-mannanase was the same as that of *B. subtilis* MA139 (pH 6.0), an enzyme that can potentially be used

Table 2: Purification of $\beta$-mannanase by ultrafiltration and gel chromatography.

| Purification step | Total activity (IU) | Total protein (mg) | Specific activity (IU/mg) | Recovery (%) | Purification multiple (fold) |
|---|---|---|---|---|---|
| Fermentation liquor | 429,650.8 | 176.7 | 2,431.4 | 100 | 1 |
| Ultrafiltration | 328,317.4 | 8.6 | 38,070.2 | 76.4 | 15.6 |
| Gel chromatography | 283,500.2 | 3.6 | 79,859.2 | 66.0 | 32.9 |

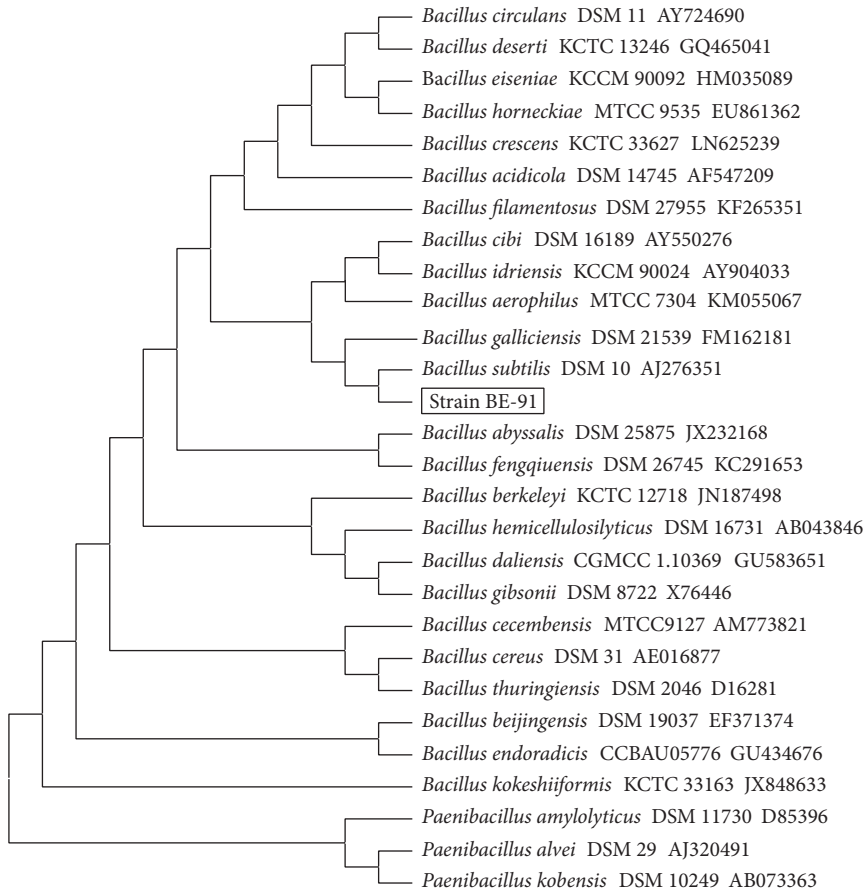


Figure 2: Phylogenetic tree based on 16S rDNA sequences of strain BE-91 and other bacteria by Mega 6.0 using neighbor-joining analysis with 1000 bootstrap replicates.

as a feed additive for monogastric animals [25]. At pH < 4.0, the $\beta$-mannanase activity was negligible, retaining <80% of its maximal value obtained after incubation at pH > 7.5, 4°C for 30 min. A relatively broad zone of optimum activity was observed. Therefore, BE-91 $\beta$-mannanase can be considered a weakly acidic and neutral enzyme, thereby rendering suitability for animal feed industry [38].

*3.7. The Effect of Metal Ions on β-Mannanase Stability.* The effect of a variety of metal ions on $\beta$-mannanase activity was measured (Table 3). The highest induction was achieved with $Mn^{2+}$, which showed 168% baseline activity, followed by $Al^{3+}$, $Ca^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Mg^{2+}$, and $NH_4^+$, respectively. $K^+$ and $Fe^{3+}$ had no obvious effects on $\beta$-mannanase activity in these conditions. $Ba^{2+}$ and $Pb^{2+}$ greatly inhibited the enzyme activity to a final rate of 83% and 74%, respectively. This suggests that BE-91 $\beta$-mannanase should not be contaminated by $Ba^{2+}$ and $Pb^{2+}$.

Table 3: Effects of different metal ions (1 mmol/L) on $\beta$-mannanase activity.

| Metal ions | Relative activity (%)[a] |
|---|---|
| Blank | 100 |
| $K^+$ | 99 ± 3.2 |
| $NH_4^+$ | 103 ± 2.7 |
| $Ca^{2+}$ | 117 ± 3.6 |
| $Zn^{2+}$ | 115 ± 2.9 |
| $Mn^{2+}$ | 168 ± 4.5 |
| $Cu^{2+}$ | 116 ± 2.1 |
| $Mg^{2+}$ | 107 ± 2.8 |
| $Ba^{2+}$ | 83 ± 3.1 |
| $Pb^{2+}$ | 74 ± 2.9 |
| $Fe^{3+}$ | 99 ± 3.6 |
| $Al^{3+}$ | 121 ± 4.3 |

[a]Data are mean ± SD, $n = 3$.

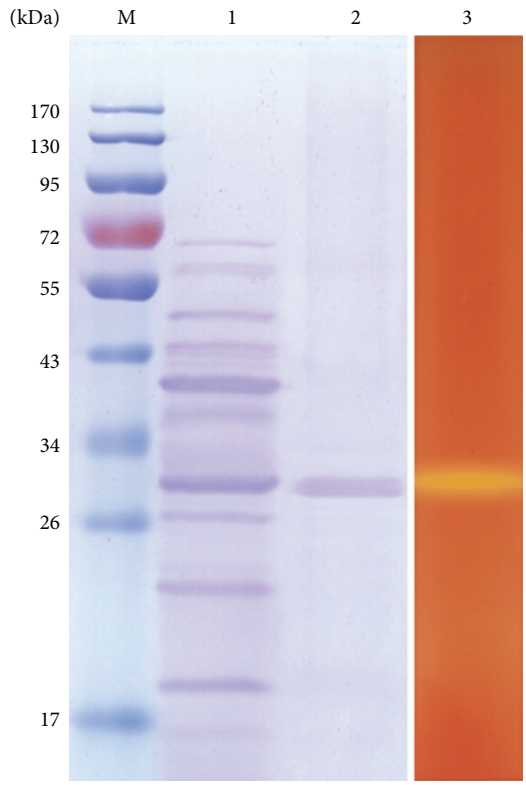


Figure 3: SDS-PAGE analysis of $\beta$-mannanase. Lane M: protein molecular weight standard; Lane 1: culture broth; Lane 2: purified $\beta$-mannanase; Lane 3: zymogram of purified $\beta$-mannanase.

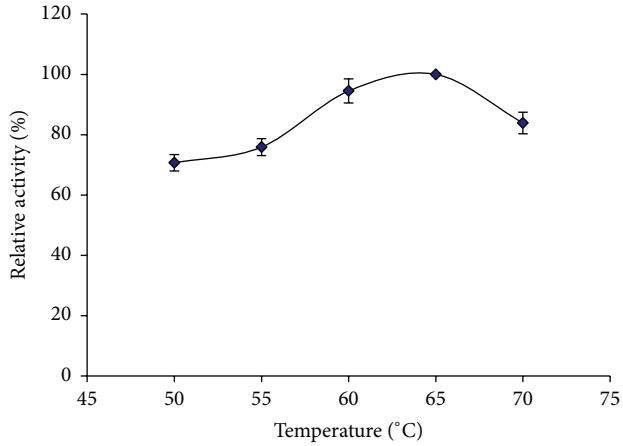


Figure 4: Optimum temperature curve of $\beta$-mannanase.

*3.8. Kinetic Parameters.* The purified enzyme hydrolyzed Konjac glucomannan but only slightly hydrolyzed ivory nut mannan, guar galactomannan, and 1,4-beta-D-mannan. Wheat arabinoxylan, beechwood xylan, and CMC were barely hydrolyzed, as shown in Table 4. This $\beta$-mannanase exhibited the highest activity with Konjac glucomannan, enriched in glucose units. This finding suggests that $\beta$-mannanase of BE-91 preferentially hydrolyzes the $\beta$-1,4-linkage of the glucosylated mannan backbone.

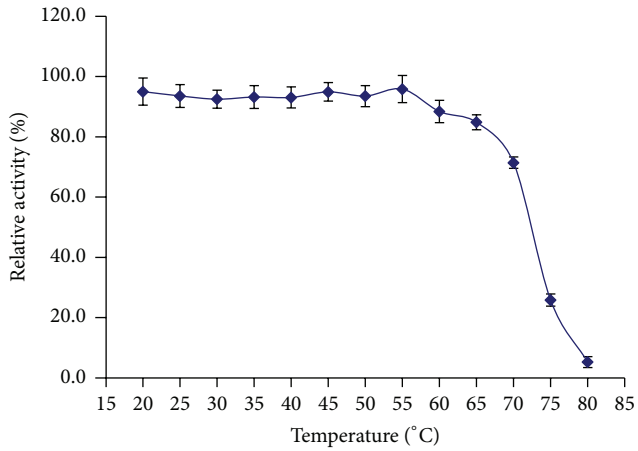


Figure 5: Thermal stability curve of $\beta$-mannanase.

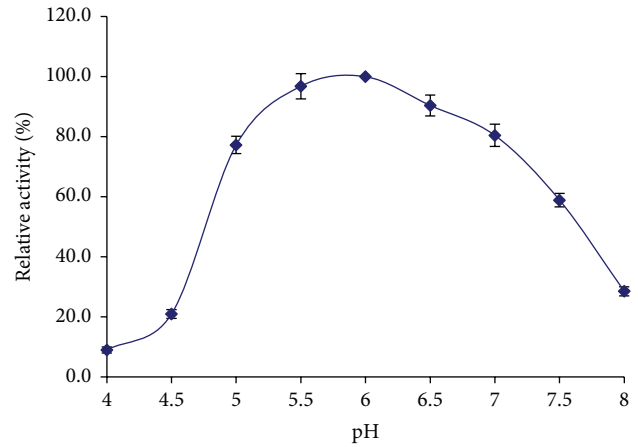


Figure 6: Optimum pH curve of $\beta$-mannanase.

Table 4: Hydrolytic activity of the purified enzyme on different polysaccharides.

| Substrate (0.5%, w/v) | Relative activity (%)[a] |
|---|---|
| Konjac glucomannan | 100 |
| Locust bean gum | 88.15 ± 1.8 |
| Carob galactomannan | 91.85 ± 1.7 |
| Guar galactomannan | 35.70 ± 0.6 |
| Ivory nut mannan | 32.74 ± 0.3 |
| 1,4-Beta-D-mannan | 46.22 ± 0.4 |
| Wheat arabinoxylan | 0 |
| Beechwood xylan | 0 |
| Carboxymethyl cellulose | 0 |

Assays were carried out at 65°C at pH 6.0 for 10 min in 0.05 mol/L citric acid-0.1 mol/L $Na_2HPO_4$ buffer.
[a]Data are mean ± SD, $n = 3$.

$K_m$ and $V_{max}$ values of this $\beta$-mannanase estimated by the Lineweaver-Burk plot were 7.14 mg/mL and 107.5 $\mu$mol/min/mL, respectively, for locust bean gum, versus 1.749 mg/mL and 33.45 $\mu$mol/min/mL for Konjac glucomannan,

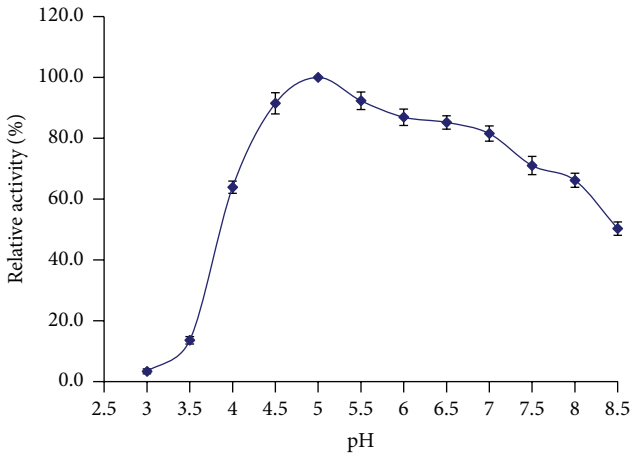


Figure 7: pH stability curve of $\beta$-mannanase.

respectively. These results displayed higher affinity of $\beta$-mannanase towards natural Konjac glucomannan ($V_{max}/K_m$, 19.1 $\mu$mol/min/mg) than the locust bean gum ($V_{max}/K_m$, 15.0 $\mu$mol/min/mg), similar to the values obtained for *Penicillium pinophilum* C1 and *Penicillium freii* F63, hence constituting it as an adequate candidate in food industry for the production of oligosaccharides [17, 18, 39].

## 4. Conclusion

*B. subtilis* bacteria are abundant, moderately stable, and mostly nonpathogenic microorganisms. Our results indicated that *B. subtilis* BE-91 could be considered a prominent candidate for the production of extracellular $\beta$-mannanase. In addition, this study developed an advanced purification approach, "two-step method," with high efficiency, high yield, and easy operation. Furthermore, the $\beta$-mannanase purified from BE-91 was extremely stable at relatively high temperatures and various weak acidic or neutral pHs. Finally, the enzyme showed a higher affinity towards natural Konjac glucomannan, a major functional food material. Therefore, this $\beta$-mannanase, purified and characterized from *B. subtilis* BE-91 for the first time, is suitable for inflammatory diseases.

## Competing Interests

The authors declare that they have no competing interests.

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*Research Article*

# The Effects of *Agave fourcroydes* Powder as a Dietary Supplement on Growth Performance, Gut Morphology, Concentration of IgG, and Hematology Parameters in Broiler Rabbits

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This study was conducted to determine the effects of *Agave fourcroydes* powder as a dietary supplement on the growth performance, gut morphology, serum concentration of IgG, and the hematology parameters of broiler rabbits. A total of 32 rabbits [New Zealand × Californian] were weaned at 35 days. They were randomly selected for two dietary treatments (eight repetitions per treatment), which consisted of a basal diet and a basal diet supplemented with 1.5% dried-stem powder of *A. fourcroydes*. On day 60 from the initiation of treatment, gut histomorphology (duodenum and cecum), serum concentration of IgG, and hematology parameters were all measured. The results showed that *A. fourcroydes* powder supplementation improved ($P < 0.05$) the ADFI, ADG, and final BW. Correspondingly, this treatment increased ($P < 0.05$) the muscle and mucosa thickness and height and width of villi. However, duodenum crypts depth was lower ($P < 0.05$) when rabbits were fed with this natural product, compared with the basal diet treatment. Results also indicated that the *A. fourcroydes* powder increased ($P < 0.05$) the serum concentration of IgG but did not change the hematology parameters. This data indicates that *A. fourcroydes* powder, as a supplement, had beneficial effects on increasing the growth performance and serum concentration of IgG, as well as improving the gut morphology without affecting the hematology parameters in broiler rabbits.

## 1. Introduction

The constant antibiotic applications, as additives for animal feed, could provoke an increase of the number of resistant strains, as well as an immense risk of crossed resistance spreading to other microorganisms [1]. Although antibiotics have been used to a lesser extent in rabbits, compared with poultry and pigs, the use of antibiotics as zinc-bacitracin has been applied to reduce the proliferation of *Clostridium perfringens* and other pathogens, mainly after weaning [2].

In the interests of public health, especially the prevention of the indiscriminate use of dietetic antibiotics, which have been the subject of worldwide concerns, some antibiotic-alternatives that have had positive effects on growth performance and product quality should be used in the diets of nonruminant animals [3]. Natural products are considered better alternatives to Antibiotic Growth Promoters (AGP), from the point of view of biosafety and low residue [4].

The *Agave* genus, a part of the Agavaceae family, is native to Mexico. They have been cultivated since the pre-Columbian era for the production of textile fibers, alcohol, molasses, pulp, and fodder, as well as for erosion control and soil conservation [5]. Specifically, the stem of the *Agave fourcroydes* has a high oligosaccharide content, which forms a polydisperse mixture [6]. Also, our previous studies have demonstrated the presence of flavonoids, anthocyanins, saponins, coumarins, reducing sugars, and tannins in this plant material [7].

On the other hand, the dried-stem powder of *Agave tequilana*, which are rich in fructans type inulin, used as a supplement in the diets of swine, enhances immunity, microbiology, and intestinal morphology, as well as promoting the growth performance and meat quality [8]. Other studies have also shown that using extracts of *A. tequilana* and *A. fourcroydes* with the diets of mice decreased the cholesterol and glucose serum levels, as well as increasing the production of volatile fatty acids and the growth of lactic acid bacteria [6, 9].

There are many chemical benefits of *Agave* spp. for animal feed. However, there have been few studies done on the dietary use of dried-stem powder of *A. fourcroydes* in animal nutrition, especially on the diet of rabbits. It can be hypothesised from previous studies that dietary supplementation with this natural product may have the potential capability of stimulating the immune system and modulating the intestinal integrity, thereby improving the growth performance in rabbits. Thus, the objective of the current study was to evaluate the effects of dried-stem powder of *Agave fourcroydes* on growth performance, gut morphology, serum concentration of IgG, and hematology parameters of broiler rabbits.

## 2. Materials and Methods

*2.1. Animal, Housing, and Treatment.* This study was carried out in accordance with the Mexican guidelines for animal welfare and experimental protocol, which is approved by the Animal Care Committee, University of Guadalajara, Mexico. A total of 32 rabbits [New Zealand × Californian] that were weaned at 35 days with an initial BW of 768 to 769 g were randomly selected to two dietary treatments.

The dietary treatments consisted of a basal diet (BD) and a basal diet supplemented with 1.5% dried-stem powder of *A. fourcroydes*. There were 16 rabbits in each treatment, with two rabbits per pen. The pen is 76-cm long, 76-cm wide, and 45-cm high.

Feed and water were freely available during the entire experimental period, which lasted for 60 days. The temperature was kept at 22 (±2)°C, and relative humidity was maintained between 60 and 65%. BD was prepared according to the nutritional requirements of broiler rabbits [11]. Ingredients of the diets are summarised in Table 1. The dried-stem powder of *Agave fourcroydes* was kindly provided for the study by the Study Center of Animal Production, Faculty of Veterinary Medicine, University of Granma, Cuba.

Table 1: Compositions and nutrient levels in basal diets (as-fed basis).

| Ingredients | Content (%) |
|---|---|
| Alfalfa hay | 12.00 |
| Wheat straw | 17.5 |
| Barley grain | 19.00 |
| Wheat bran | 24.00 |
| Sunflower meal, 30% CP | 12.00 |
| Soybean meal, 44% CP | 11.0 |
| Soybean oil | 2.88 |
| Sodium chloride | 0.50 |
| Monocalcium phosphate | 0.50 |
| HCL-lysine | 0.09 |
| L-Threonine | 0.08 |
| DL-Methionine | 0.05 |
| Premix[1] | 0.50 |
| *Calculated composition*, % *as fed* | |
| Crude protein | 16.70 |
| Digestible energy (MJ/kg) | 9.92 |
| Neutral detergent fibre | 29.10 |
| Lysine | 0.77 |
| Methionine + cystine | 0.59 |
| Threonine | 0.65 |
| Ashes | 5.37 |

[1]Each kg contains vitamin A 12,000 IU, vitamin D3 2,000 IU, vitamin B2 4160 IU, niacin 16 700 IU, pantothenic acid 8200 IU, vitamin B6 3,420 IU, folic acid 0.980 g, vitamin B12 16 mg, vitamin K 1560 IU, 16 g vitamin E, 8.5 g BHT, 0.750 g cobalt, copper 3.5 g, 9.86 g iron, manganese 6.52 g, 0.870 g sodium, 4.24 g zinc, and selenium 6.67 g.

*2.2. Growth Performance.* Rabbits were weighed (BW) on days 35 and 95. Feed intake (g/rabbit/day) was measured daily. Average daily gain (ADG), average daily feed intake (ADFI), feed/gain ratio (F/G), and viability were calculated for the period of 1 day to 60 days during the trial.

*2.3. The Analysis of Gut Morphology.* At the end of the experiment, rabbits (one rabbit/pen) were killed to sample gut tissues. The gastrointestinal tract (GIT) was divided into two segments: duodenum and cecum. Approximately 5 cm of intestinal tissue was cleaved, removed, and fixed at 10% formalin in PBS, at 4°C for histomorphological analysis.

The formalin-fixed samples, duodenum and cecum tissues were initially dehydrated in a graded series of ethanol. These light microscopic observations were followed by the samples being embedded in paraffin wax. Then, the tissues were sectioned at 5 $\mu$m thickness and mounted in slides.

Table 2: Effects of dietary supplementation of *Agave fourcroydes* powder on growth performance of broiler rabbits (95 days old).

| Items | Treatments | | SEM ± | *P* value |
|---|---|---|---|---|
| | No additive | 1.5% *A. fourcroydes* powder | | |
| Finish BW, g | 2398.38 | 2462.31 | 12.507 | 0.001 |
| ADFI, g/d | 119.91 | 123.12 | 0.625 | 0.001 |
| ADG, g/d | 27.16 | 28.22 | 0.285 | 0.013 |
| F/G | 4.42 | 4.37 | 0.042 | 0.461 |

The experiment lasted for 60 days; $n = 16$.
(i) BW: body weight.
(ii) ADFI: average daily feed intake.
(iii) ADG: average daily gain.
(iv) F/G: feed/gain ratio.
SEM: standard error of the mean.

After dewaxing, hydrating, and staining the tissues with Hematoxylin-Eosin, the thickness of muscle and mucous membrane, the width and depth of the crypts, and the height and width of villi of the duodenum and cecum were determined by using an Axiostar microscope (Carl Zeiss, Oberkochen, Germany) connected to a computer with Analysis-Opti Basic and soft imaging system software. Images to 500x and 100x were obtained [12]. Then, the villus height/crypt depth ratio was calculated.

*2.4. The Determination of Hematology Parameters and Serum Concentration of IgG.* Blood samples were collected from the jugular vein of eight rabbits, one rabbit per pen per treatment, on the day of euthanasia at 95 days old; one part of the samples was collected in 20 mL tubes and stood for one hour. Then, the serum was separated by centrifugation at 10,000 rpm for 25 minutes, at 20°C, using Eppendorf centrifuge 5804, USA. The other blood sample (whole blood) was placed in 2 mL tubes with sodium heparin, which was added at a ratio of 2 : 1. Both samples were stored at −20°C until analyses in the laboratory.

Leukocytes were analysed by blood smear and Giemsa dye. The hemoglobin was analysed by the HemoTest. Hematocrit was determined according to Wintrobe. Total proteins were measured by Biuret using spectrophotometer Shimadzu UV-Visible 160 A (Japan). Erythrocytes and platelets were analysed by cell counting using a Neubauer chamber and the automatic platelets counter, respectively.

The Concentration of the Mean Corpuscular Hemoglobin (CMCH), the Mean Corpuscular Hemoglobin (MCH), and the Mean Corpuscular Volume (MCV) were determined by the following formulas:

(i) CMCH = Hb (g/100 mL) × 100/Ht (%).

(ii) MCH: (Hgb ∗ 10)/leukocytes.

(iii) MCV: Ht (%) ∗ 10/RBCs (millions/$mm^3$).

The serum concentration of IgG was determined using a commercially available 125I Radio Immunoassay analyser kit with $\gamma$-calculating instrument GC-300 (Beijing, China), according to the manufacturer's instructions. The kits are commercially available from Kemeidongya Biotechnology Company (Beijing, China).

*2.5. Statistical Analysis.* Data was subjected to analysis of variance (ANOVA) for simple classification of completely randomised design. Prior to the analysis of variance, the normality of the data and the uniformity of variance were verified by Kolmogorov-Smirnov and the Bartlett test, respectively, using the statistical software SPSS version 17.1.

## 3. Results

*3.1. Growth Performance.* All rabbits were healthy and grew well throughout the entire experimental period of 60 days. *A. fourcroydes* powder supplementation improved ($P < 0.05$) the final BW, ADG, and ADFI compared with BD (Table 2). However, F/G did not show significant differences ($P > 0.05$) among treatments.

*3.2. Gut Morphology.* Table 3 illustrates the data from the analysis of the gut morphology of broiler rabbits at 95 days old. In the duodenum and cecum, the *Agave fourcroydes* powder increased ($P < 0.05$) the muscle and mucosa thickness compared with BD, as well as improving the ($P < 0.05$) height and width of villi. However, the duodenum crypts depth of *A. fourcroydes*-treated group was lower ($P < 0.05$) than that of BD group (Table 3). Meanwhile, the width and depth of cecum did not show significant differences ($P > 0.05$) amongst the treatments.

*3.3. Hematology Parameters and Serum Concentration of IgG.* Dietary supplementation with *A. fourcroydes* powder did not influence ($P > 0.05$) the hematology parameters of broiler rabbits according to Table 4. It can also be seen that these parameters of *A. fourcroydes*-treated rabbits were in the normal range according to previous reports [10]. In addition, the serum concentration of IgG was higher ($P < 0.05$) when rabbits were fed with the *A. fourcroydes* powder as feed additives (Figure 1) compared with the BD group.

## 4. Discussion

The viability (100%; data not shown) was excellent for both treatments, which indicated the innocuousness of the product for a 60-day course. The present results showed that *A. fourcroydes* powder supplementation at 1.5% led to a higher

Table 3: Effects of dietary supplementation of *Agave fourcroydes* powder on gut morphology of broiler rabbits (95 days old).

| Items, $\mu$m | Treatments | | SEM ± | $P$ value |
|---|---|---|---|---|
| | No additive | 1.5% *A. fourcroydes* powder | | |
| *Duodenum* | | | | |
| Muscle thickness | 117.12 | 154.80 | 5.757 | <0.001 |
| Mucosal thickness | 1070.40 | 1351.52 | 32.83 | <0.001 |
| Height of villi | 892.72 | 1027.96 | 26.07 | <0.001 |
| Width of villi | 108.04 | 142.48 | 7.310 | <0.001 |
| Crypts depth | 98.56 | 71.68 | 7.417 | <0.001 |
| Width depth | 66.92 | 63.93 | 4.082 | 0.424 |
| Villus height/crypt depth | 9.06 | 14.35 | 1.102 | <0.001 |
| *Cecum* | | | | |
| Muscle thickness | 284.00 | 335.60 | 30.06 | <0.001 |
| Mucosal thickness | 426.56 | 438.20 | 3.370 | <0.001 |
| Crypts depth | 243.36 | 204.20 | 11.40 | <0.001 |
| Width depth | 121.48 | 86.52 | 6.890 | <0.001 |

The experiment lasted 60 days; $n = 8$. SEM: standard error of the mean.

Table 4: Effects of dietary supplementation of *Agave fourcroydes* powder on hematology parameters of broiler rabbits (95 days old).

| Items | Treatments | | SEM ± | $P$ value | Reference values* |
|---|---|---|---|---|---|
| | No additive | 1.5% *A. fourcroydes powder* | | | |
| RBCs, millions/mm$^3$ | 6.53 | 5.92 | 0.492 | 0.178 | 4.5–7.0 |
| Leucocytes, thousands/mm$^3$ | 6.69 | 6.00 | 0.476 | 0.150 | 6.00–9.30 |
| Hemoglobin, g/dL | 13.44 | 12.70 | 0.578 | 0.392 | 8–15 |
| Hematocrit, % | 40.50 | 37.58 | 1.814 | 0.288 | 30–50 |
| MCH, pg | 20.80 | 19.94 | 1.321 | 0.658 | 19–30 |
| MCV, fL | 62.14 | 58.80 | 3.613 | 0.532 | 40–80 |
| MCHC, g/dL | 33.16 | 33.20 | 0.076 | 0.720 | 32–38 |
| Platelets, thousands/mm$^3$ | 449.21 | 410.02 | 15.84 | 0.118 | 400–700 |
| Total protein, g/dL | 7.28 | 7.30 | 0.082 | 0.867 | 5.2–7.8 |

The experiment lasted for 60 days; $n = 8$. *Giusti et al. [10].
(i) MCH: Mean Corpuscular Hemoglobin.
(ii) MCV: Mean Corpuscular Volume.
(iii) MCHC: Mean Corpuscular Hemoglobin Concentration.
SEM: standard error of the mean.

BW compared with the BD group, suggesting that this material may contain some compounds that could enhance the growth performance of the rabbits.

Fructans, which are recognized as one kind of oligosaccharides presented in the *A. fourcroydes,* are not digestible by the digestive enzymes of the host but can be metabolised by microorganisms in the large intestine, which is beneficial for the synthesis of short-chain fatty acids. Thus, *A. fourcroydes* is capable of positively affecting the growth performance by increasing the production of short-chain fatty acids [13, 14].

In rabbits, fructans were reported to increase the population of lactic acid bacteria, particularly *Lactobacillus* spp. and *Bifidobacterium* spp., which provokes a competitive exclusion against pathogenic bacteria in the GIT, as well as a beneficial influence on body weight [15]. This study coincides with the results of Abdel-Aziz et al. [16], which reaffirms the importance of *Lactobacillus* spp. in the efficiency of the digestive process.

Also, this natural product has a high presence of flavonoids, a polyphenolic compound with the ability to inhibit the production of nitric oxide (NO), interleukin-6 (IL-6), and prostaglandin E2 (PGE2) in LPS-induced macrophage cells [17], which ensures improvement in the absorption of nutrients, subsequently increasing the body weight [18, 19].

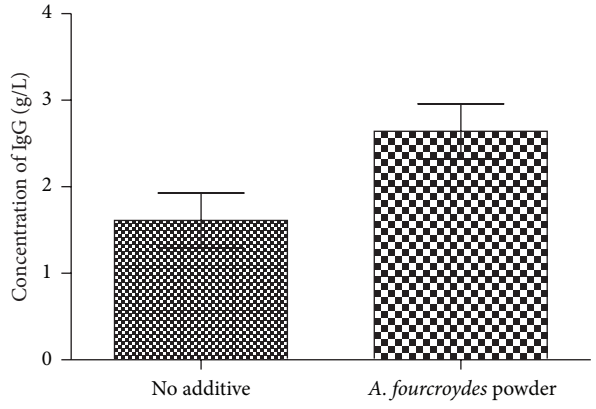


Figure 1: Effect of dietary supplementation of 1.5% *Agave fourcroydes* powder on concentration of IgG of broiler rabbits (95 days old) (SEM ± 0.319; $P$ value = 0.049). The experiment lasted for 60 days; $n = 8$.

In addition, other secondary metabolites detected in *A. fourcroydes* powder, such as tannins, reducing sugars, anthocyanins, and saponins [7], could have beneficial effects on growth performance, especially for the control of pathogenic microflora and the modulation of innate and humoral immune response [3, 20]. Some studies have found a positive relationship between the incorporation of small concentrations of beneficial secondary metabolites in diets and productive behavior [21, 22].

However, other studies have found that the extracts of *Agaves* (*tequilana* and *fourcroydes*) were unable to increase the growth performance of laboratory mice [6, 9]. It is possible that the effects of products rich in fructans on body weight depend on the concentration of fructans in the diet, as well as the animal species under study.

An important discovery in this study is that the supplementation of *A. fourcroydes* increases the thickness of muscle and mucosa in broiler rabbits and subsequently improves intestinal health [12]. The competitive exclusion in the GIT reduces the adhesion of pathogenic bacteria, which consequently results in a decrease in intestinal lesions and has a positive effect on the thickness of these layers [23]. In our previous studies, using the *A. fourcroydes* powder as a supplement increased the proliferation of lactic acid bacteria, as well as decreasing cecal pH [7].

de Blas et al. [23], studying the relationship between microorganisms and intestinal health, found a marked reduction of mucosa thickness induced by the proliferation of *C. perfringens*, *Campylobacter* spp., and *Helicobacter* spp., which in turn has a negative impact on the viability and biological responses of rabbits. It is well known that the intestinal layer plays an important role in the defense against infectious diseases of the host. The improvement of an intestinal barrier function, though modulating the intestinal microbial community diversity, is mostly beneficial for the animal's health [24]. Furthermore, Raj et al. [25] found that intestinal permeability is directly related to the thickness of the intestinal mucosa. They found that a reduction of the intestinal mucosa would lead to the uncontrolled access of toxins, chemicals, microorganisms, and macromolecules, which could cause enteric problems and reduction of growth performance [23].

In addition, the mucosal thickness of duodenum is greater than that of cecum, due to its great absorptive capacity. However, the muscle thickness of cecum is more beneficial in transporting the large and heavy feces in rabbits due to caecotrophy [26].

Similarly, Jiang et al. [12] reported that the morphology of the villi and crypts has been associated with bowel function and growth performance of animals. Our results are consistent with these findings, as it was observed that *A. fourcroydes* powder supplementation (1.5%) increased ($P < 0.05$) the height and width of the villi, due to suitable intestinal conditions. These conditions include higher proliferation of lactic acid bacteria, decrease of cecum pH [7], and thickening of the intestinal mucosa, which denoted a more mature tissue.

Other studies with diets rich in fructans found similar results to the structure of the villi [27, 28], suggesting that there may indeed be an association between the intestinal health status and the absorptive capacity. However, Mourão et al. [29] found no significant effects of fructooligosaccharide on the intestinal structure in rabbits ($P > 0.05$).

Understanding the relationship between villi and crypt is helpful, in order to estimate the nutrient digestion and absorption capacity of the small intestine [30]. The higher the ratio of villi/crypt, the better efficiency there is in the digestive process [31]. In addition, a reduction of villi and crypt results in less absorption cells and more secretory cells [32]. From this point of view, dietary supplementation of *A. fourcroydes* powder can be beneficial in promoting a better productive response (Table 2) through upregulating ($P < 0.05$) the ratio of villi/crypt (5.29) related to the control treatment.

Furthermore, rabbits from the BD group had a higher width and depth of crypts than those from the *A. fourcroydes* group. It is reported that the migration of specialised cells to the villi, especially with the decrease of villus height, would elevate the depth of crypts [33]. However, other studies that use mannan-oligosaccharides as growth promoters did not find any significant changes in the intestinal morphology [26]. However, there is no doubt that the gut can undergo rapid epithelial renewal by shortening the crypt depth [34].

Hematological parameters are currently used as indicators of health in humans and animals. Variations of these indicators may reflect bacterial, viral, parasitic, or fungal infections, as well as intoxication, dehydration, or blood clotting problems [10].

Usually, it is necessary to determine whether the newly introduced feed additive would induce the comprehensive immune response in a body. Some compounds, such as nonenzymatic feed additives, may induce changes in polymorphonuclear leukocytes (neutrophils and eosinophils), mainly by activating the immune system to eliminate the exogenous material and/or the possible toxic and allergenic compounds [35, 36]. Thus, the body's immune response to *A. fourcroydes* powder was studied. After the 60-day treatment, it was found that *A. fourcroydes* powder, with high fructan concentrations and secondary metabolites, did not cause adverse symptoms or diminish the defenses (white blood cells) in rabbits.

The supplementation of low concentrations of *A. fourcroydes* powder did not influence the absorption of iron, as illustrated by the value of hemoglobin in Table 4. This is possibly because this additive did not give rise to any excess of tannins, which could inhibit the absorption of this mineral and induce iron deficiency, causing anemia [36]. On the other hand, the hematocrit value suggests that the animals were under suitable conditions of hydration. Once the hemoconcentration occurred due to water deficit, the hematocrit value would increase [37].

The amount of serum antibody is an indicator of humoral immunity in all mammals [38]. The rabbits can generate abundant antibodies or proteins and promote the proliferation of B lymphocytes to defend against any parasitic or pathogenic infections [39]. Curiously, IgG represents about 80% of the immunoglobulins in serum, which participated in humoral immunity against bacteria and pathogens [20, 40]. Thus, *A. fourcroydes* powder supplementation may improve

antitoxic and antibacterial immune responses through the elevation of serum concentration ($P < 0.05$) of IgG in rabbits.

Several studies have shown that certain functional foods can improve the phagocytic activity of the intestinal leukocytes, as well as promoting the proliferation of leukocytes B and secretion of immunoglobulins A and G [41]. Other studies on feed additives in nonruminants have also found similar results when animals were fed with foods rich in secondary metabolites [20, 40, 42].

In summary, feeding with 1.5% of *A. fourcroydes* powder improved the growth performance, as well as the serum concentration of IgG and gut morphology, in broiler rabbits. Supplementation of this product did not affect the hematology parameters, suggesting that it can be used safely as a food additive at a dose of 1.5% for broiler rabbits.

## Competing Interests

The authors declare that there were no conflicts of interests regarding the publication of this article.

## Authors' Contributions

Maidelys Iser, Yordan Martínez, and Hengjia Ni contributed equally to this manuscript.

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*Research Article*

# Osteopontin Promotes Expression of Matrix Metalloproteinase 13 through NF-$\kappa$B Signaling in Osteoarthritis

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Osteopontin (OPN) is associated with the severity and progression of osteoarthritis (OA); however, the mechanism of OPN in the pathogenesis of OA is unknown. In this study, we found that OA patients had higher abundance of OPN and matrix metalloproteinase 13 (MMP13). In chondrocytes, we showed that OPN promoted the production of MMP13 and activation of NF-$\kappa$B pathway by increasing the abundance of p65 and phosphorylated p65 and translocation of p65 protein from cytoplasm to nucleus. Notably, inhibition of NF-$\kappa$B pathway by inhibitor suppressed the production of MMP13 induced by OPN treatment. In conclusion, OPN induces production of MMP13 through activation of NF-$\kappa$B pathway.

## 1. Introduction

Osteoarthritis (OA) is regarded as the most prevalent chronic joint disease. OA is characterized by a group of mechanical abnormalities including degradation of articular cartilage, thickening of subchondral bone, synovial inflammation, and formation of osteophytes, leading to chronic pain and functional restrictions in affected joints, substantial morbidity, physical disability, and reduced quality of life [1–3]. Data from the World Health Organization (WHO) estimated that about 10% of men and 18% of women, as well as 65% of all those with age more than 60 years, have symptomatic OA [4]. Although the etiology of OA is complex, including reasons from the genetic, constitutional, and biomechanical aspects, we have growing knowledge and understanding on the pathogenesis of OA [3].

OPN (osteopontin) is a 44~75 KD multifunctional phosphoprotein and is known as early T cell activation gene-1 (Eta-1) [5, 6]. OPN is secreted by many types of cells, including macrophages, lymphocytes, epithelial cells, vascular smooth muscle cells, and even chondrocytes as well as synoviocytes [7–10]. OPN is highly abundant in the extracellular fluids at sites of inflammation, extracellular matrix of mineralised tissues, and even in the bone [7, 9, 11]. In the bone, OPN regulates the cell-matrix and cell-cell interaction, the cartilage-to-bone transition in fracture repair, and the attachment of osteoclasts to the bone matrix [6, 12, 13]. Interestingly, mRNA expression and protein abundance of OPN are associated with the pathogenesis of OA. At the beginning, a study found that mRNA expression of OPN isolated from human OA cartilage is higher compared to the normal cartilage [14]. Subsequently, increased abundance of OPN in the plasma, synovial fluid, and articular cartilage in OA patients were found [3, 15, 16], indicating that expression of OPN is associated with progressive joint damage and the severity and progression of OA.

The most remarkable biochemical change in OA is the progressive loss of articular cartilage, which contains two

main extracellular matrix macromolecules: type II collagen and aggrecan, a major component of the cartilage-specific proteoglycans [17–19]. The matrix metalloproteinases (MMPs), including collagenases (MMP1 and MMP13), gelatinases (MMP2 and MMP9), and stromelysin (MMP3), mediate cartilage collagen breakdown, whereas aggrecanases, which are members of the A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family including ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, and ADAMTS15, mediate loss of cartilage aggrecan. Thus, the importance of MMPs and ADAMTS in the pathogenesis and development of OA is widely demonstrated [20–23]. Intriguingly, recent investigations have shown that OPN affects the expression of MMP13 [17, 24]. However, the mechanism by which OPN regulates expression of MMP13 still remains to be known. NF-$\kappa$B pathway has critical roles in the expression of inflammatory factors, including MMP13 [25–28]. Thus, the hypothesis of this study is that OPN regulates expression of MMP13 through NF-$\kappa$B pathway.

## 2. Materials and Methods

*2.1. Cartilage Acquisition and Assessment.* The study was approved by the institutional review board and ethics committee of Xiangya Hospital affiliated to Central South University, which conformed with the regulations of medical ethics. An informed consent about this experiment was obtained from all subjects. A written informed consent to participate in this study was provided by participants. The normal cartilage tissues from non-OA patients and degenerated cartilage tissues from OA patients were obtained in previous studies [15, 17, 29, 30]. The cartilage tissues were assessed with hematoxylin-eosin (HE) and safranin-O staining and a modified Mankin grading system in previous studies [15, 17, 29, 30].

*2.2. Cell Isolation and Culture Conditions.* The chondrocytes were isolated and cultured according to previous studies [17, 29, 30]. Briefly, samples were minced into pieces of less than 1 $mm^3$, followed by sequential digestion at 37°C with 0.15% collagenase II (Invitrogen, Carlsbad, CA, USA) for 5-6 h with stirring every 20 min after 2 h. Chondrocytes were isolated after centrifugation and cultured in DMEM-F12 containing 10% fetal bovine serum (FBS) and antibiotics for 5–7 days before use. For OPN treatment, OPN (recombinant human osteopontin, R&D Systems, Minneapolis, MN, USA) was added to the medium with the dosage of 0, 0.5, 1, 2, and 4 $\mu$g/mL for 24, 48, or 72 hours. For OPN inhibition, siRNA targeting OPN was transiently transfected into cells using Lipofectamine™ 2000 reagent (Invitrogen Life Technologies, San Diego, CA, USA) according to previous studies [29, 31]. siRNA sequences were OPN-siRNA, with sequence as 5′-CCU GUG CCA UAC CAG UUA ATT-3′ and antisense 5′-UUA ACU GGU AUG GCA CAG GTT-3′ [29]. For p65 protein translocation inhibition, pyrrolidinedithiocarbamic (PDTC) acid was used with 100 $\mu$M.

*2.3. RT-PCR.* RT-PCR analysis was performed according to previous reports [25]. Briefly, total RNA was isolated from liquid nitrogen frozen samples using TRIzol regent (Invitrogen, USA) and then treated with DNase I (Invitrogen, USA) according to the manufacturer's instructions. Synthesis of the first strand (cDNA) was performed using oligo (dT) 20 and SuperScript II Reverse Transcriptase (Invitrogen, USA). Primers used in this study were designed with Primer 5.0. Sequences of all primers used were as follows: MMP13-F: 5′-CTTAGAGGTGACTGGCAA AC-3′; MMP13-R: 5′-GCCCATCAAATGGGTA GAA G-3′; OPN-F: 5′-GTGGGA AGG ACA GTT ATG AA-3′; OPN-R: 5′-CTG ACT TTG GAA AGT TCC TG-3′; GAPDH-F: 5′-TGA CTT CAA CAG CGA CAC CCA-3′; and GAPDH-R: 5′-CAC CCT GTT GCT GTA GCC AAA-3′. GAPDH was used as an internal control to normalize target gene transcript levels.

*2.4. Immunoblotting.* Western blot analysis was conducted according to previous study [25]. Briefly, SDS-PAGE is used to separate protein obtained from samples. Then, the separated protein is transferred to PVDF membranes (Millipore, MA, USA) and incubated with primary antibodies overnight at 4°C after the blockage with 5% nonfat milk in Tris-Tween buffered saline buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 3 h. Following is the information for antibodies: anti-OPN (AP11567a, Abgent, CA, USA), MMP13 (ALS10395, Abgent, CA, USA), anti-p65 (BS4135, Bioworld, MN, USA), anti-p-p65 (#3031, CST, USA), and anti-COL2A1 (sc-28887, Santa Cruz Biotechnology, Texas, USA). Further, HRP-conjugated secondary antibodies were incubated for 1 h at room temperature before analysis the signal intensity using AlphaImager 2200 software (Alpha Innotech Corporation, CA, USA).

*2.5. Immunofluorescence.* Immunofluorescence staining was performed at room temperature according to previous studies [32, 33]. Firstly, the cells were fixed by 4% paraformaldehyde (PFA, Sigma-Aldrich) and then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) solution in phosphate buffered saline (PBS) and incubated with blocking solution (3% bovine serum albumin (BSA, Sigma-Aldrich) diluted in 0.1% Triton X-100). Then, before washing with PBS, cells were incubated firstly with polyclonal rabbit antibodies against anti-NF-$\kappa$B p65 (BS4135, Bioworld, MN, USA) for 24 h (1 : 250) and then with FITC-conjugated goat anti-rabbit IgG for 1 h. Further, coverslips were mounted with GEL/MOUNT (Biomeda, Foster City, CA, USA) and then visualized the fluorescence under a confocal fluorescence microscope (Carl Zeiss, Göttingen, Germany).

*2.6. Statistical Analyses.* Data shown are the means ± the standard error of the mean (SEM). All statistical analyses for data were performed using SPSS 16.0 software (Chicago, IL, USA). Data were analyzed between two groups using Student's $t$-test, while among more than two groups data were analyzed by the one-way ANOVA method. Differences of $P < 0.05$ were considered significant.

## 3. Results

*3.1. OPN Promotes Expression of MMP13.* Expressions of OPN and MMP13 were evaluated in normal cartilage tissues obtained from non-OA patients and degenerated cartilage tissues from OA patients. The cartilage tissues from OA patients have significantly ($P < 0.05$) higher expression of OPN compared to the normal cartilage tissues (Figure 1(a)). Also, expression of MMP13 significantly ($P < 0.05$) increased in the cartilage tissues from OA patients, compared to the normal cartilage tissues (Figure 1(b)). To characterize the cause and effect relationship between OPN and MMP13, we treated the chondrocytes with different dosage of OPN. As indicated in Figure 1(c), OPN treatment significantly ($P < 0.05$) induced mRNA expression of MMP13 in chondrocytes. Indeed, similar result was found in protein abundance of MMP13 in chondrocytes (Figures 1(d) and 1(e), $P < 0.05$). Also, we analyzed expression of MMP13 in chondrocytes, treated with OPN at the dosage of 1 $\mu$g/mL. OPN supplementation remarkably promoted mRNA expression of MMP13 at 24, 48, and 72 hours after supplementation (Figure 1(f), $P < 0.05$). Similarly, we also observed that OPN supplementation significantly increased protein abundance of MMP13 in chondrocytes at 24, 48, and 72 hours after supplementation (Figures 1(g) and 1(h), $P < 0.05$). Collectively, expression of OPN increases in cartilage tissues from OA patients, and OPN promotes expression of MMP13.

*3.2. OPN Promotes Expression of MMP13 through NF-Kappa B Signaling.* To characterize the mechanism by which OPN regulates expression of MMP13, we focused on the NF-kappa B pathway in chondrocytes because this signaling has a pivotal role in the expression of inflammatory factors after stimulation [25, 26, 34]. We transfected the chondrocytes with OPN targeting siRNA, which significantly ($P < 0.05$) lowered mRNA expression and protein abundance of OPN in chondrocytes (Figures 2(a) and 2(b)). As the control, control siRNA has little effect on mRNA expression and protein abundance of OPN in chondrocytes (Figures 2(a) and 2(b)). In the chondrocytes, OPN treatment significantly ($P < 0.05$) increased the abundance of p65 and phosphorylated p65, compared to the nontreatments (Figures 2(c) and 2(d)). However, the abundance of p65 and phosphorylated p65 in chondrocytes with OPN-siRNA treatment was significantly ($P < 0.05$) lower compared to the controls (Figures 2(c) and 2(d)). Also, we found that OPN treatment significantly promoted translocation of p65 protein from the cytoplasm to the nucleus, while OPN-siRNA treatment inhibited this translocation, compared to the controls (Figure 2(e)). As the control, PDTC treatment inhibited translocation of p65 protein from the cytoplasm to the nucleus (Figure 2(e)). In the chondrocytes, OPN treatment significantly ($P < 0.05$) increased protein abundance of MMP13, while OPN-siRNA treatment lowered ($P < 0.05$) protein abundance of MMP13 (Figure 2(f)). Although OPN treatment enhanced the protein abundance of MMP13, PDTC treatment reversed ($P < 0.05$) abundance of MMP13 caused by OPN treatment (Figure 2(f)). Indeed, PDTC along with treatment also decreased protein abundance of MMP13 in the chondrocytes (Figure 2(f)). OPN treatment significantly ($P < 0.05$) decreased protein abundance of COL2A1 in the chondrocytes, while OPN-siRNA treatment increased ($P < 0.05$) protein abundance of COL2A1 (Figure 2(e)). Although OPN treatment inhibited protein abundance of COL2A1 in the chondrocytes, PDTC treatment alleviated ($P < 0.05$) the lower abundance of COL2A1 induced by OPN treatment (Figure 2(e)). Summarily, OPN activates the NF-kappa B pathway, which in turn promotes expression of MMP13.

## 4. Discussion

Although most investigations have shown that increased OPN is associated with the progressive joint damage and the disease severity and progression of OA [15, 35], some reports have a contradictory conclusion. For example, one study has found that OA patients have lower concentration of OPN compared to the healthy controls [36]. Also, with OPN-deficient mice, OPN deficiency results in aging-associated and instability-induced OA [24], suggesting that it is required for cartilage homeostasis and preventing OA progression. These compelling findings are suggesting that OPN has complex roles in joint homeostasis and in the pathogenesis of OA. Indeed, this is supported by our discovery that OPN could regulate the expression of various factors associated with the pathogenesis of OA, including hypoxia-inducible factor-2$\alpha$ [29], ADAMTS4 [30], tissue inhibitors of metalloproteinases [37], interleukin-6 and interleukin-8 [38], and even caveolin-1 [39]. Similar to the previous study, we found that mRNA expression of OPN increases in the cartilage tissues from OA patients. Also, increased mRNA expression of MMP13 was found in the cartilage tissues from OA patients. This leads to the hypothesis that increased OPN promotes expression of MMP13. Indeed, in the chondrocytes, we found that OPN increases mRNA expression and protein abundance of MMP13. Similarly, a previous study has found that both OPN and phosphorylated OPN promote expression of MMP13 at both mRNA and protein levels [17].

NF-$\kappa$B pathway has critical roles in the expression of inflammatory factors [25, 26]. In unstimulated situation, the NF-$\kappa$B dimers are sequestered in the cytoplasm by the inhibitor of $\kappa$Bs (I$\kappa$B), which keeps NF-$\kappa$B proteins in the cytoplasm by masking the nuclear localization signals of NF-$\kappa$B proteins with the ankyrin repeat domains of I$\kappa$B [34]. Upon activation, I$\kappa$B was phosphorylated, leading to targeting for the proteasomal degradation and to release the NF-$\kappa$B proteins [34]. Subsequently, the NF-$\kappa$B proteins are moved into the nucleus and turn on the expression of specific genes that have NK-$\kappa$B binding elements in their promoter or other sites [34]. Interestingly, NF-$\kappa$B pathway has been reported to regulate the expression of MMP13. For example, high-mobility group box chromosomal protein 1 (HMGB1) or lipopolysaccharide (LPS) significantly promotes the expression of MMP13 and the activation of NF-$\kappa$B pathway by increasing the I$\kappa$B phosphorylation, while it inhibits the activation of NF-$\kappa$B pathway by NF-$\kappa$B inhibitor (Bay 11-7085) which remarkably inhibits expression of MMP13 induced by HMGB1 and LPS [27]. CpG oligodeoxynucleotides treatment

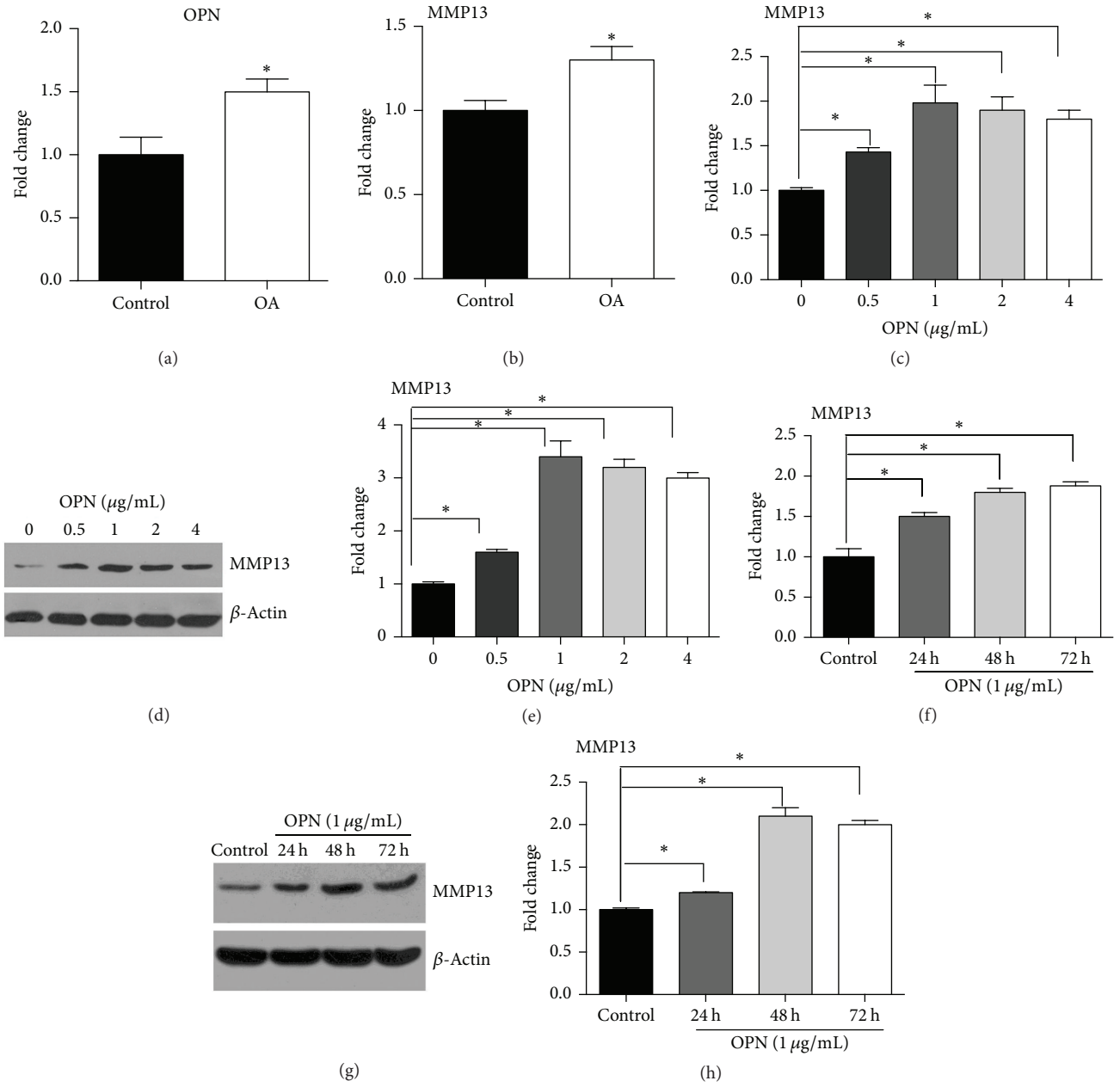


Figure 1: OPN promotes expression of MMP13. ((a)-(b)) The expression of OPN ((a), $n = 13$) and MMP13 ((b), $n = 13$) in normal cartilage tissues obtained from non-OA patients and degenerated cartilage tissues from OA patients. ((c)–(e)) OPN treatment at indicated concentrations promotes expression of MMP13 at mRNA (c) and protein levels ((d) and (e)) in the chondrocytes. (d) Immunoblotting of MMP13. (e) Quantification of relative MMP13 abundance from data shown in (d). ((f)–(h)) OPN treatment at indicated time promotes expression of MMP13 at mRNA (f) and protein levels ((g) and (h)) in the chondrocytes. (g) Immunoblotting of MMP13. (h) Quantification of relative MMP13 abundance from data shown in (g). Data are representative of two independent experiments with 4–6 repeats per group. $*$ indicates a statistically significant difference between two groups ($P < 0.05$). OA: osteoarthritis; OPN: osteopontin; and MMP13: matrix metalloproteinase 13.

promotes the expression of MMP13 and the activation of NF-$\kappa$B pathway in the murine odontoblast-lineage cell line, while the treatment with inhibitors of NF-$\kappa$B pathway, including NF-$\kappa$B inhibitors (PDTC), I$\kappa$B$\alpha$ phosphorylation inhibitors (Bay 11-7082), or I$\kappa$B protease inhibitor (L-1-tosylamido-2-phenylethyl chloromethyl ketone, TPCK), markedly suppresses MMP13 expression induced by CpG ODN [28]. Notably, OPN has been shown to induce the activation of NF-$\kappa$B pathway and the expression of NF-$\kappa$B pathway-dependent factors in breast cancer cells [40]. Similarly, OPN promotes expression of MMP2 and MMP9 through NF-$\kappa$B pathway [41]. In line with these investigations, this study shows that OPN treatment promotes activation of NF-$\kappa$B pathway by increasing the abundance of p65 and phosphorylated p65

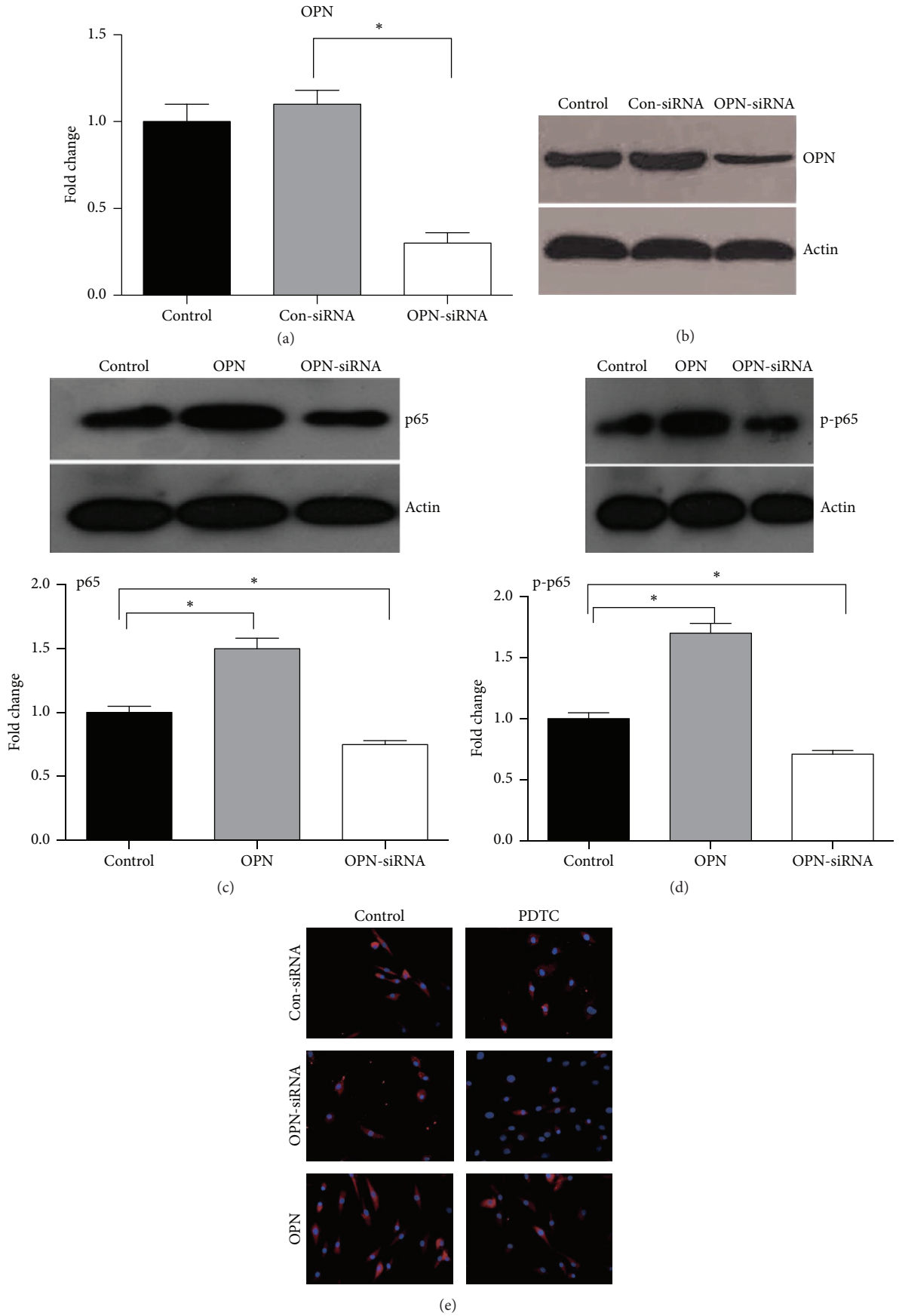


Figure 2: Continued.

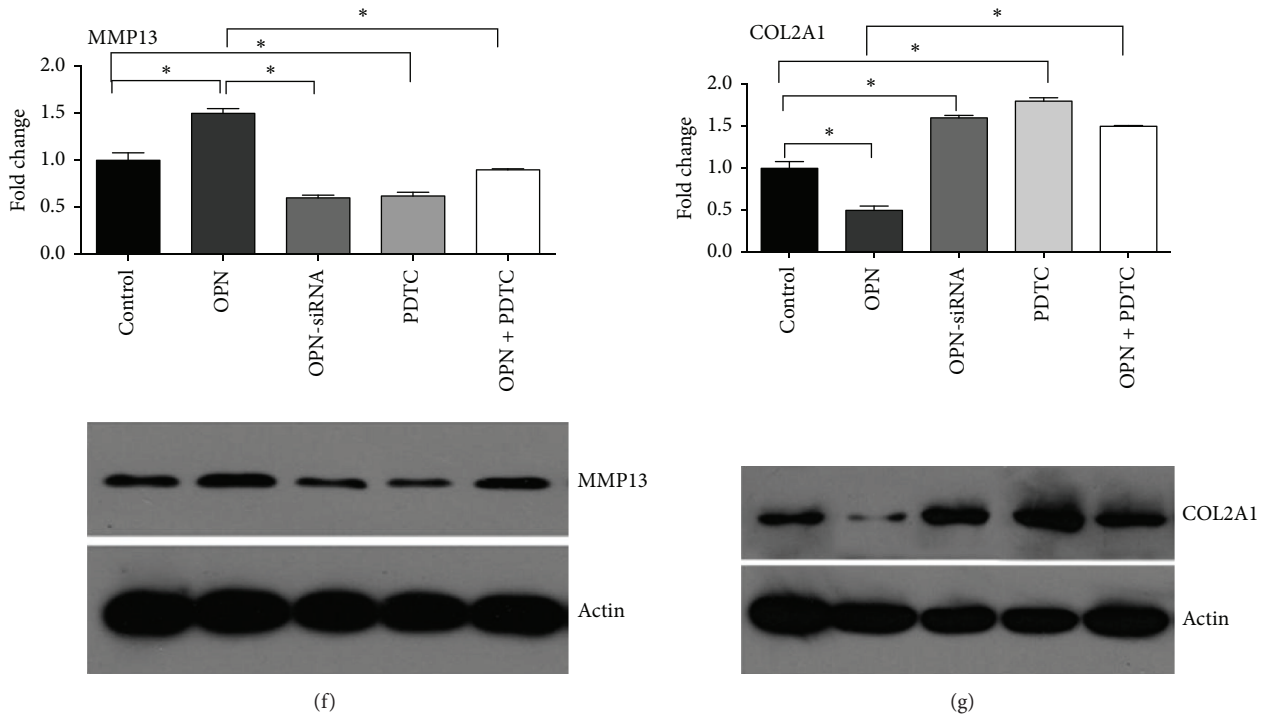


Figure 2: OPN promotes expression of MMP13 through NF-kappa B signaling. ((a)-(b)) The expression of OPN after OPN targeting siRNA in chondrocytes from mRNA expression (a) and protein levels (b). ((c)-(d)) The abundance of p65 (c) and phosphorylated p65 (d) after OPN treatment or OPN-siRNA treatment. (c) Immunoblotting of p65 (upper part) and quantification of relative p65 abundance (bottom) from data shown in upper part. (d) Immunoblotting of p-p65 (upper part) and quantification of relative p-p65 abundance (bottom) from data shown in upper part. (e) The translocation of p65 protein from the cytoplasm to the nucleus in indicated situations (×100). ((f)-(g)) The protein abundance of MMP13 (f) and COL2A1 (g) in indicated situations. (f) Immunoblotting of MMP13 (bottom) and quantification of relative p65 abundance (upper part) from data shown in bottom. (g) Immunoblotting of COL2A1 (bottom) and quantification of relative p65 abundance (upper part) from data shown in bottom. Data are representative of two independent experiments with 4–6 repeats per group. $*$ indicates a statistically significant difference between two groups ($P < 0.05$). OPN: osteopontin and MMP13: matrix metalloproteinase 13.

and translocation of p65 protein from the cytoplasm to the nucleus. Inhibition of expression of OPN inactivates the NF-$\kappa$B pathway. Importantly, the current study found that OPN increases expression of MMP13 through activation of NF-$\kappa$B pathway, which is similar to a previous conclusion that OPN activates NF-$\kappa$B pathway and promotes the expression of NF-$\kappa$B- dependent factors [40, 41]. However, besides the NF-$\kappa$B pathway, other signal pathways are also associated with expression of MMP13. For example, SB203580, the inhibitor of p38 signal pathway, significantly reduces expression of MMP13 induced by TNF-$\alpha$ treatment [42] or others [27]. Thus, it is interesting to know whether OPN also regulates MMP13 expression through other signaling pathways.

In conclusion, current study verified that expression of OPN and MMP13 is increased in OA, and OPN promotes expression of MMP13 through activation of NF-$\kappa$B pathway in OA. The discovery of this study has great potentials for understanding the pathogenesis of OA and treatment of OA through manipulation of NF-$\kappa$B pathway.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Yusheng Li and Wei Jiang contributed equally. Yusheng Li and Guanghua Lei designed the experiment. Yusheng Li, Wei Jiang, Chao Zeng, and Hua Wang performed the experiment. Yusheng Li, Zhenhan Deng, and Min Tu analyzed the data. Liangjun Li, Wenfeng Xiao, and Shuguang Gao helped in experiment. Yusheng Li, Wei Jiang, and Guanghua Lei wrote the manuscript.

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*Research Article*

# Low Dosage of Chitosan Supplementation Improves Intestinal Permeability and Impairs Barrier Function in Mice

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The purpose of this study was to explore relationships between low dose dietary supplementation with chitosan (COS) and body weight, feed intake, intestinal barrier function, and permeability in mice. Twenty mice were randomly assigned to receive an unadulterated control diet (control group) or a dietary supplementation with 30 mg/kg dose of chitosan (COS group) for two weeks. Whilst no significant differences were found between the conditions for body weight or food and water intake, mice in the COS group had an increased serum D-lactate content ($P < 0.05$) and a decreased jejunal diamine oxidase (DAO) activity ($P < 0.05$). Furthermore, mice in COS group displayed a reduced expression of occludin and ZO-1 ($P < 0.05$) and a reduced expression of occludin in the ileum ($P < 0.05$). The conclusion drawn from these findings showed that although 30 mg/kg COS-supplemented diet had no effect on body weight or feed intake in mice, this dosage may compromise intestinal barrier function and permeability. This research will contribute to the guidance on COS supplements.

## 1. Introduction

Chitosan (COS) is an alkaline glucosamine polymer derived from hydrolysed chitosan [1]. In common with other plant polysaccharides, chitosan molecules have a number of biological activities, such as antibacterial property, immune effect, and disease prevention [2].

Typically, peptides and proteins are not well absorbed through the intestinal tract; chitosan has been demonstrated to promote intestinal absorption of macromolecules significantly. The mechanisms behind the enhanced absorption are thought to relate to mucoadhesion and relaxing intercellular tight junctions [3]. Mucoadhesion describes the adhesion between two materials, where one is a mucosal surface. The mucus membrane bears negatively charged sialic acid groups with interacting with chitosan's positively charged amino groups [4].

Chitosan is an attractive additive for animal feed [5] because of its inherent antimicrobial properties [5], but it is restrictively used due to high viscosity and solubility [6]. Studies of the effect of chitosan on piglet growth attributed the influence on immune ability, morphology of the intestine, and microflora function [7, 8]. The results were variable and the dosages used in the studies were large (100 mg/kg–5 g/kg). Our earlier study used low dose chitosan (30 mg/kg) to supplement the diet piglets, which resulted in small intestine oxidative and immune stress responses and a reduction in intestinal barrier efficiency [9]. For this study, we predicted that a low dose chitosan supplement would disturb the intestinal permeability and function by the determination of genes expression of barrier function and proteins in mice.

## 2. Materials and Methods

*2.1. Experiment Design.* This study was conducted in accordance with the guidelines of the Laboratory Animal Ethical Commission of the Chinese Academy of Sciences. Six-week-old ICR mice were bought from SLAC Laboratory Animal Central (Changsha, Hunan, China). The mice were kept in clean animal colonies (temperature, 25°C, relative humidity, 53%; 12-h dark/12-h light); the mice had *ad lib* access to water and a typical rodent diet [10]. After three days, mice were randomly allocated to COS group ($n = 10$) or control group

($n = 10$). For two weeks, control group mice received basal diet [10], whilst the diet in the experimental group was supplemented with 30 mg/kg chitosan. Chitosan was obtained from the Dalian Chemical and Physical Institute (Chinese Academy of Sciences, Dalian, China); molecules were composed of 5 oligomers with an average molecular weight of 1,000 to 2,000 Da, a minimum sugar content of 85%, and 99% water soluble. After two weeks, the mice were sacrificed and the jejunum was recovered. Samples were frozen and kept at −80°C until required.

*2.2. Analysis of Intestinal Permeability.* The serum level of D-lactate was assessed using a commercial kit from Sino-German Beijing Leadman Biotech Ltd., Beijing, China, and a Beckman CX4 chemistry analyser (Beckman Coulter, CA, USA). Serum diamine oxidase activity experiment was established by assay kits, which were used as per the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

*2.3. RT-PCR of Tight Junction Genes.* Complete RNA was recovered from ground jejunum using TRIzol reagent (Invitrogen, USA) and treated with DNase I (Invitrogen, USA) as per the instructions provided by the manufacturer. cDNA synthesis was carried out using oligo (dT) 20 and Superscript II reverse transcriptase (Invitrogen, USA). Primers were selected based upon previous research [10]. $\beta$-actin was chosen as a reference gene.

*2.4. Immunoblotting of Tight Junction Proteins.* Following the instructions provided with the total protein extraction kit (KGP200, Keygen Biotech, Nanjing, Jiangsu, China), the total protein was isolated. Equal quantities of intestinal mucosa proteins were isolated using a polyacrylamide gel before being moved onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). These were then incubated with primary antibodies (goat polyclonal claudin-1 antibody, rabbit polyclonal ZO-1 antibody, and $\beta$-actin rabbit antibody) (Santa Cruz Biotechnology, CA, USA) for 12 h at 4°C. PVDF membranes were subsequently incubated with the secondary antibodies (goat anti-rabbit IgG-HRP and rabbit anti-goat IgG-HRP) (Santa Cruz Biotechnology, CA, USA) for 120 min at 25°C. Western blots were visualised using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA) and photographed with Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA). $\beta$-actin reference proteins were equally distributed between the groups. The value of protein expression was determined as the densitometry ratio of tight junction proteins and $\beta$-actin.

*2.5. Statistical Analyses.* The statistical data analyses were carried out using SPSS 22.0 (Chicago, IL. USA). Student's $t$-test was employed to determine differences between the groups and significance was determined at $P < 0.05$. Data was presented as the means ± the standard error of the mean (SEM).

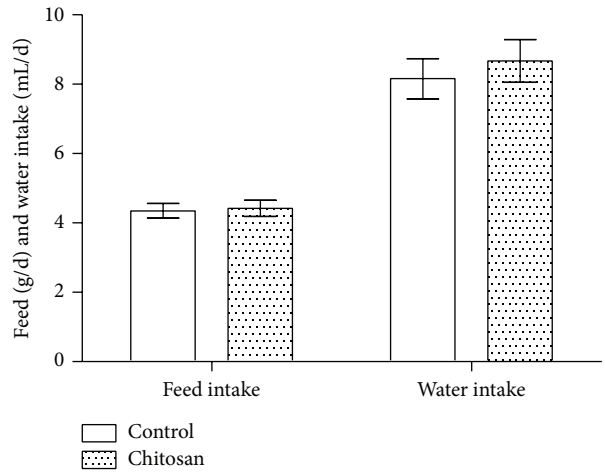


Figure 1: In the mouse model, 30 mg/kg chitosan supplement has no effect on food and water intake. Average intakes for each group are indicated. For two weeks, control group ($n = 10$) mice received a basal diet and the COS group of mice ($n = 10$) received 30 mg/kg chitosan supplemented diet.

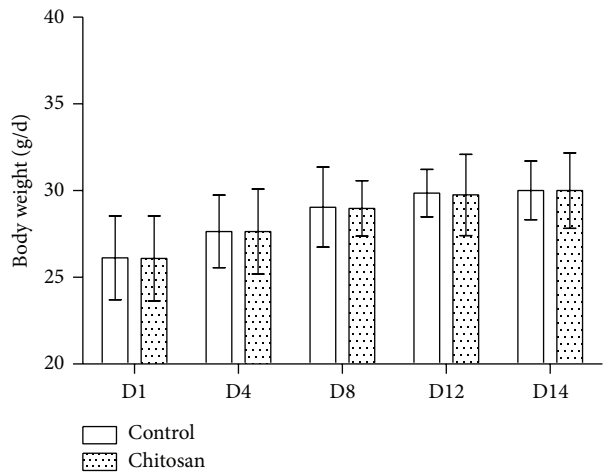


Figure 2: Body weight of mice for the control group and COS group averaged over the 14-day period. Control group ($n = 10$) mice received a basal diet and the COS group of mice ($n = 10$) received 30 mg/kg chitosan supplemented diet for two weeks.

## 3. Results

*3.1. Low Dosage Chitosan Has No Effect on Body Weight and Food Intake.* The effect of a COS-supplemented diet on mice body weight, food, and water intake was determined. After two weeks, no difference in food and water intake (Figure 1) and body weight (Figure 2) was detected between the groups.

*3.2. Low Dosage Chitosan Increases Intestinal Permeability.* To appraise the integrity of the intestine, the biomarkers of serum D-lactic acid level and jejunal diamine oxidase were measured. Figure 3 indicates that serum D-lactate in the COS group was greater than it was in the control group ($P < 0.05$)

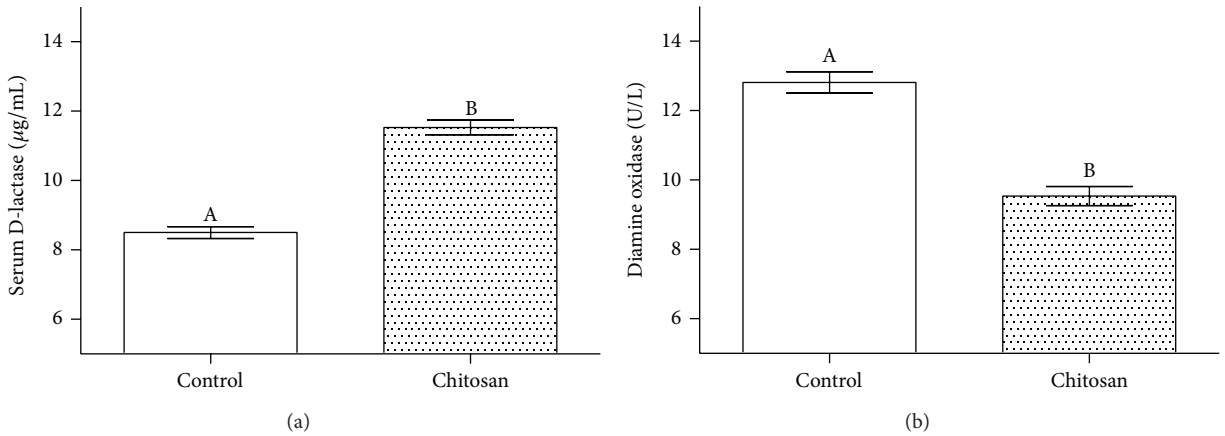


Figure 3: The effects of chitosan dietary supplement on (a) serum D-lactase and (b) jejunum mucosal diamine oxidase. A and B: different letters indicate a statistical difference between the control group and COS group.

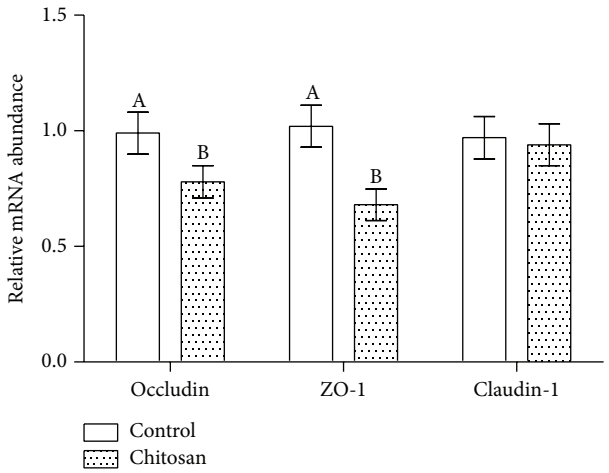


Figure 4: Effect of dietary chitosan on mRNA levels of occludin, ZO-1, and claudin-1 in the control ($n = 10$) and COS groups ($n = 10$). A and B: different letters indicate a statistical difference between the control and COS group.

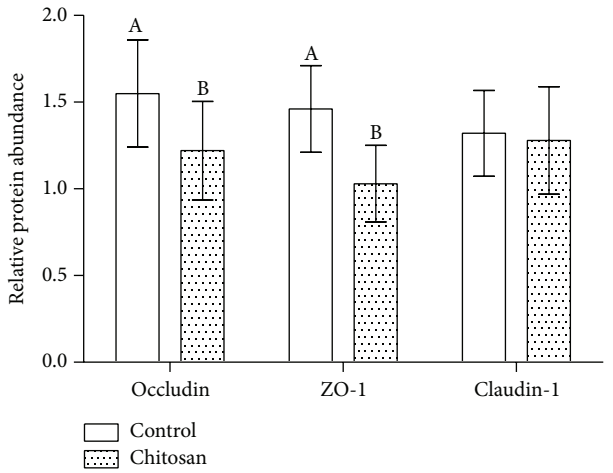


Figure 5: The relative abundance of tight junction proteins in the control and COS groups. A and B: different letters indicate a statistical difference between the control and COS groups.

and jejunal diamine oxidase activity in the COS group was lower than that in the control group ($P < 0.05$).

*3.3. Low Dosage Chitosan Decreases the Expression of Tight Junction Genes and Proteins.* ZO-1, claudin-1, and occludin are intestinal tight junction proteins, essential to maintain tight junction stability, and barrier function. In this study, the jejunal mucosa levels of ZO-1, claudin-1, and occludin mRNA were evaluated. The mRNA expression levels for all intestine segments are shown in Figure 4. Compared to the control group, jejunal expression of ZO-1 and occludin in COS group was significantly lower in the experimental group ($P < 0.05$). The same trend was observed in western blots via estimating the relevant protein levels (Figure 5). Compared to mice fed with control diet, the protein levels in the jejunum of ZO-1 and occludin were significantly reduced in the COS group ($P < 0.05$).

## 4. Discussion

Chitosan is the second most abundant polymer in nature [11] (cellulose being the first), which is also one of the few positively charged alkaline polysaccharides. Chitosan as a dietary supplement has been explored for its potential as an antimicrobial growth promoter and has been shown to promote growth in broiler chickens [12, 13] and pigs [14]. This could be ascribed to higher feed intake, serum growth hormone, and IGF-1 concentrations [15]. In this study, we found that a low dosage COS-supplement diet did not have any impact upon

food and water intake or body weight in mice. This echoes the finding of our earlier study that no effect on the average feed and body weight was found via using the same 30 mg/kg dose of chitosan to supplement piglet diets [9]. Huang et al. also reported that no difference in growth performance was found in a broiler chicken study that used a high chitosan dose (150 mg/kg) [12]. Differences between the dosage, molecular weight, purity, or solubility of the chitosan used in this experiment and others may account for the contrary results [16].

To determine the effect of chitosan on intestinal integrity, we evaluated the concentration of serum D-lactate and jejunal diamine oxidase in accordance with these biomarkers which are identified as useful to determine gut integrity [17]. Reduced intestinal diamine oxidase activity and elevated serum D-lactate levels corresponded with cell and tissue injury [18]. We used the same biomarkers to evaluate the permeability of the intestine. Our results indicated an increase in the serum level of D-lactate and activity of diamine oxidase in response to 30 mg/kg dose of chitosan; this suggests that intestinal integrity is compromised.

The epithelium of the intestine forms a selectively permeable barrier that is key in preventing pathogenic invasion. Tight junctions are central to the barrier's functionality and are responsible for its integrity [19]. Tight junction proteins encompass a number of integral membrane proteins that are bound to cytoplasmic plaque proteins.

Occludin, claudin-1, and ZO-1 are tight junction proteins that vary in their molecular structures and function; they are collectively important to maintain the tight junction's structure and function. Occludin and claudin-1 are transmembrane proteins that regulate the tight junction's function and integrity [20]. It has been demonstrated by Shen et al. that the cytoskeleton regulates permeability of the leak pathway via mechanism that includes occludin and ZO-1 [19]. On the other hand, Rosenthal et al. demonstrated that paracellular permeability could be modified by chitosan, independent of changes to tight junction proteins [21]. In this study, we detected changes to the mRNA expression of ZO-1 and occludin in the group of mice that had been fed a COS-supplemented diet. In the COS group, jejunal mRNA expression of occludin was lower and expression of ZO-1 was reduced. This observation is consistent with the raised concentration of serum D-lactate and decreased level of jejunal diamine oxidase. However, mRNA expression of claudin-1 was unaffected. These findings imply that in mice the integrity of the intestinal barrier is compromised by low doses of dietary chitosan supplementation.

To conclude, in the mouse model, 30 mg/kg dose of chitosan supplements did not influence growth performance but compromised intestinal barrier integrity. The findings from this research will contribute to the guidance on low dose chitosan supplements.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

## Acknowledgments

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*Research Article*

# Identification of Dietetically Absorbed Rapeseed (*Brassica campestris* L.) Bee Pollen MicroRNAs in Serum of Mice

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MicroRNAs (miRNAs) are a class of small noncoding RNA that, through mediating posttranscriptional gene regulation, play a critical role in nearly all biological processes. Over the last decade it has become apparent that plant miRNAs may serve as a novel functional component of food with therapeutic effects including anti-influenza and antitumor. Rapeseed bee pollen has good properties in enhancing immune function as well as preventing and treating disease. In this study, we identified the exogenous miRNAs from rapeseed bee pollen in mice blood using RNA-seq technology. We found that miR-166a was the most highly enriched exogenous plant miRNAs in the blood of mice fed with rapeseed bee pollen, followed by miR-159. Subsequently, RT-qPCR results confirmed that these two miRNAs also can be detected in rapeseed bee pollen. Our results suggested that food-derived exogenous miRNAs from rapeseed bee pollen could be absorbed in mice and the abundance of exogenous miRNAs in mouse blood is dependent on their original levels in the rapeseed bee pollen.

## 1. Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNA that mediate posttranscriptional gene regulation by promoting cleavage or inhibiting translation of the target mRNA in plants or animals and play a critical role in nearly all biological processes, including metabolism and immune functions [1–3].

Recent studies suggest that plant miRNA may serve as a novel functional component of food which makes a critical contribution to maintaining and shaping animal body structure and function [4]. In 2012, a pilot study found that plant miRNAs from the diet will be absorbed by cells of the mammalian digestive tract and then packaged into microvesicles [4]. The microvesicles protect plant miRNAs from degradation and deliver them via the bloodstream to a variety of tissues (liver, kidney, heart, and brain), in which they will regulate cell gene expression [4]. Using the next-generation sequencing technology, Wang et al. demonstrated that human plasma contains a wide range of RNA from many exogenous species, including bacteria, fungi, and foods such as corn, rice, soybean, tomato, and grape [5]. Lukasik and Zielenkiewicz performed a bioinformatics analysis of publicly available raw data from studies on miRNAs composition in human and porcine breast milk exosomes to identify the fraction of food-derived miRNAs, and 35 and 17 miRNA species were identified, respectively [6]. miR-172 is the most highly enriched miRNA in *Brassica oleracea*, and after feeding mice with *Brassica oleracea*, miR-172 was found in the stomach, intestine, serum/blood, spleen, liver, kidney, and feces of mice [7].

Studies have shown that food-derived plant miRNAs have immunomodulating effects such as anti-influenza virus and antitumor [8, 9]. Zhou et al. found the first active miRNA in traditional Chinese medicine named miR-2911, a honeysuckle- (HS-) encoded atypical miRNA, that directly targets various influenza A viruses (IAVs) [8]. Subsequently, Yang et al. reported that miR-2911 levels fluctuated among various herbs. Feeding these different herb-based diets to the mice leads to different miR-2911 levels in the sera and urine which is associated with dietary intake levels [10]. Mlotshwa

and others synthesized 3 tumor suppressor miRNAs (*miR-34a, miR-143, and miR-145*) with a characteristic of plant miRNA [11], and they reported that oral administration of the cocktail reduced tumor burden in well-established Apc*Min*/+ mouse model of colon cancer [12]. Furthermore, Western donor sera contained the plant miRNA miR159, whose abundance in the serum was inversely correlated with breast cancer incidence and progression in patients, and they demonstrated for the first time that a plant miRNA can inhibit cancer growth in mammals *in vivo* and *in vitro* [9].

Rapeseed (*Brassica campestris* L.) pollen is microgametophytes of rape. Bee-collected rapeseed pollen is widely used in food and healthy products [13]. *In vivo* and *in vitro* experiments demonstrated that the immune modulating effects by bee pollen might be attributed to its prevention and treatment for diseases [13–15]. Besides, RNA is rich in rapeseed bee pollen [16]. Nevertheless, whether miRNAs in rapeseed bee pollen could be absorbed by animals remains unclear.

In this study, ICR mice were fed with rapeseed bee pollen, and then plant miRNAs including rapeseed miRNAs in mice blood were detected using next-generation sequencing technology.

## 2. Materials and Methods

*2.1. Rapeseed Bee Pollen.* The rapeseed bee pollen was bought from Bee Research Institute of Anhui Agriculture University. The implementation of the standard is GB/T11758-89-bee pollen. Single pollen rates are over 95%, and the production date was November 10, 2015.

*2.2. Animal Studies.* All animal experiments were performed using male ICR strain mice on a 12 h light/dark cycle in a pathogen-free animal feeding facility at Zhejiang Academy of Traditional Chinese Medicine. The animal study protocols were approved by the Animal Care and Use Committee of Zhejiang Academy of Traditional Chinese Medicine. At 6 weeks of age (weighted 26.37 ± 2.7 g), each mouse was fed rapeseed bee pollen (10 g/kg) by gavage. After a fixed time interval (3 h or 6 h on d1, d4, or d8), serum about 200 $\mu$L was collected from each mouse, and then total RNA was extracted using mirVana™ PARIS™ Kit (AM1556, Ambion™).

*2.3. Illumina Hiseq2500 Sequencing.* The sequencing procedure was conducted according to standard steps provided by Illumina company, Inc. Briefly, a pair of adaptors were ligated to the 3′ and 5′ ends of total RNA. Reverse transcription followed by PCR is used to create cDNA constructs based on the small RNA ligated with 3′ and 5′ adapters. This process selectively enriches those fragments that have adapter molecules on both ends. Then the fragments of around 147–157 bp (22–30 nt length small RNA + adaptors) were purified by PAGE. The purified DNA was directly used for the cluster generation and sequencing using Illumina Hiseq2500 according to the manufacturer's instructions. The image files generated by the sequencer were then processed to produce digital data. The subsequent procedures included removing adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, and snoRNA), and repeats. Subsequently, unique sequences with length in 18–26 nucleotides were mapped onto all plant miRNA precursors in miRBase 20.0 by BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. Length variation at both 3′ and 5′ ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping onto specific species mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping onto the other arm of known specific species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p-derived miRNA candidates.

*2.4. Analysis of Level of miRNAs in Rapeseed Bee Pollen by RT-qPCR.* Total RNA was extracted from 80 mg rapeseed bee pollen using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative RT-PCR was performed using Taqman miRNA probes (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. To calculate the absolute expression levels of target miRNAs, a series of synthetic miRNA oligonucleotides at known concentrations were reverse transcribed and amplified. The absolute amount of each miRNA was then calculated with reference to the standard curve. Quantitative PCR was performed using an ABI-StepOnePlus machine (Applied Biosystems).

*2.5. Statistical Analysis.* Differences are considered statistically significant at $P < 0.05$, using Student's $t$-test.

## 3. Results

*3.1. Raw Data Filtering.* We sequenced a small RNA library from blood RNA of mouse fed with rapeseed bee pollen using the Illumina Hiseq2500 system. We acquired a total of 11,089,480 raw sequences. Overview of these reads from raw data to cleaned sequences is shown in Table 1.

We illustrated small RNA reads with Rfam dataset; to remove rRNA, scRNA, snoRNA, snRNA, and tRNA, the pie charts were drawn for total reads and unique reads (Figure 1).

*3.2. Plant miRNAs Spectrum in Serum of Mice Fed with Rapeseed Bee Pollen.* After removing the junk reads, the clean reads yield 34 plant miRNAs (Table 2). Plant miRNAs are 2′-O-methyl modified on their terminal nucleotide; in contrast, mammalian miRNAs with free 2′ and 3′ hydroxyls render plant miRNAs more difficult to be ligated to the cloning adapter compared with mammalian miRNAs. As a result, in the 11,089,480 raw reads, there were only 132 reads of plant miRNAs. However, some plant miRNAs can be detected even though the mammalian miRNAs caused a strong disturbance; in turn this proves that the content of plant miRNAs in mouse blood was not low. Among the plant miRNAs, miR-166a and miR-159 were with the highest levels in mouse blood; besides, these two miRNAs were both mapped onto rapeseed genome.

*3.3. Comparison of Abundance Levels of miR-166a and miR-159 in Rapeseed Bee Pollen.* Based on the predominant two

Table 1: Overview of reads.

| Lib | Type | Total | % of total | Unique | % of unique |
|---|---|---|---|---|---|
| Raw reads | Nuclear acid | 11,089,480 | 100.000 | 209,873 | 100.000 |
| 3ADT & length filter | | 320,992 | 2.895 | 145,808 | 69.474 |
| Junk reads | | 858 | 0.008 | 610 | 0.291 |
| Rfam | RNA | 47,589 | 0.429 | 6,477 | 3.086 |
| mRNA | RNA | 3,694 | 0.033 | 905 | 0.431 |
| Repeats | RNA | 369 | 0.003 | 74 | 0.035 |
| rRNA | RNA | 19,901 | 0.179 | 2,026 | 0.965 |
| tRNA | RNA | 15,176 | 0.137 | 2,527 | 1.204 |
| snoRNA | RNA | 5,507 | 0.050 | 750 | 0.357 |
| snRNA | RNA | 527 | 0.005 | 243 | 0.116 |
| Plant miRNA | RNA | 221 | 0.002 | 33 | 0.016 |
| Another Rfam RNA | RNA | 6,478 | 0.058 | 931 | 0.444 |
| Clean reads | | 10,716,785 | 96.639 | 56,136 | 26.748 |

Table 2: Plant miRNAs in mice fed with rapeseed bee pollen.

| miRNA ID | miRNA sequence | Length (nt) | Frequency |
|---|---|---|---|
| bna-miR-166a | TCGGACCAGGCTTCATTCCCC | 21 | 35 |
| bna-miR-159 | TTTGGATTGAAGGGAGCTCTA | 21 | 22 |
| gma-miR6300 | GTCGTTGTAGTATAGTGGT | 19 | 8 |
| nta-miR6145e | ATTGTTACATGTAGCACTGGCT | 22 | 7 |
| nta-miR6146b | TTTGTCCAATGAAATACTTATC | 22 | 6 |
| nta-miR6020b | AAATGTTCTTCGAGTATCTTC | 21 | 5 |
| nta-miR6149a | TTGATACGCACCTGAATCGGC | 21 | 5 |
| ath-miR-166a | TTCGGACCAGGCTTCATTCCCC | 22 | 3 |
| osa-miR530 | TGCATTTGCACCTGCACCTCC | 21 | 3 |
| ahy-miR408 | TGCACTGCCTCTTCCCTGGCT | 21 | 3 |
| mdm-miR408a | TGCACTGCCTCTTCCCTGGCT | 21 | 3 |
| bna-miR397a | ATTGAGTGCAGCGTTGATG | 19 | 2 |
| peu-MIR2916 | CAACCATAAACGATGCCGACCAGG | 24 | 2 |
| nta-miR168a | TCGCTTGGTGCAGGTCGGGAC | 21 | 2 |
| gma-miR482b | TCTTCCCTACACCTCCCATACC | 22 | 2 |
| nta-miR482a | TTTCCAATTCCACCCATTCCTA | 22 | 2 |
| nta-miR827 | TTAGATGAACATCAACAAACA | 21 | 2 |
| ppt-miR894 | TTCACGTCGGGTTCACCA | 18 | 2 |
| gma-miR3522 | TGAGACCAAATGAGCAGCTGA | 21 | 2 |
| gma-miR4996 | TAGAAGCTCCCCATGTTCTCA | 21 | 2 |
| bna-miR403 | TTAGATTCACGCACAAACTCG | 21 | 1 |
| peu-MIR2916 | ACCGTCCTAGTCTCAACCATA | 21 | 1 |
| aau-miR162 | TCGATAAACCTCTGCATCCAG | 21 | 1 |
| bdi-miR398a | TATGTTCTCAGGTCGCCCCTGT | 22 | 1 |
| gma-miR403a | TTAGATTCACGCACAAACTT | 20 | 1 |
| gma-miR1507a | TCTCATTCCATACATCGTCTGA | 22 | 1 |
| nta-miR6159 | TAGCATAGAATTCTCGCACCTA | 22 | 1 |
| hbr-miR6173 | GCTGTAAACGATGGATACT | 19 | 1 |
| ptc-miR6478 | CCGACCTTAGCTCAGTTGGT | 20 | 1 |
| stu-miR7997c | TTGCTCGGATTCTTCAAAAAT | 21 | 1 |
| bna-miR156b | TTGACAGAAGATAGAGAGCAC | 21 | 1 |
| gma-miR166m | GCGGACCAGGCTTCATTCCCC | 21 | 1 |
| stu-miR399a | GGGCTACTCTCTATTGGCATG | 21 | 1 |
| bna-miR156a | TGACAGAAGAGAGTGAGCAC | 20 | 1 |

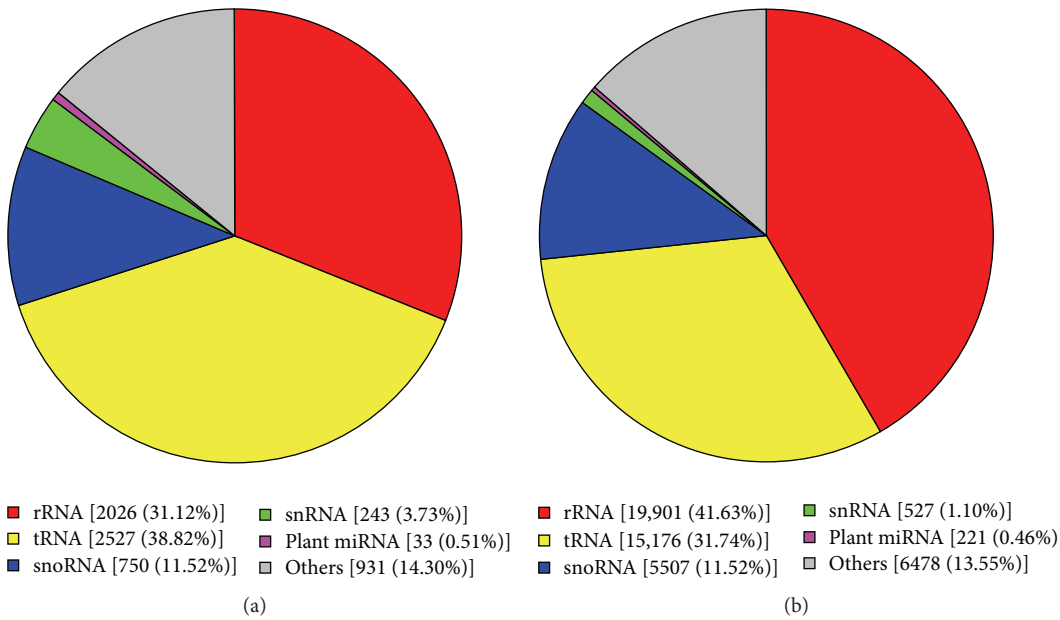


Figure 1: Pie chart of sequence category. (a) Pie chart of sequence category of total reads. (b) Pie chart of sequence category of unique reads.

miRNAs (miR-166a and miR-159) in the blood, we assumed that miR-166a and miR-159 can be found in rapeseed bee pollen, and the content of miRNAs in the rapeseed pollen also will follow the trend in the serum. To confirm this, the levels of miR-166a and miR-159 in rapeseed bee pollen were assessed by stem-loop quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay. As a result, miR-166a and miR-159 can be detected in RNA of rapeseed bee pollen (Additional Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/5413849). Moreover, the CT values of miR-166a and miR-159 were $23.8 \pm 0.23$ and $31.22 \pm 0.33$, respectively, suggesting that the abundance level of miR-166a was higher than miR-159. These results suggested that food-derived exogenous miRNA from rapeseed bee pollen could be absorbed by mouse, and the abundance of specific miRNAs is dependent on their origins from the rapeseed bee pollen.

*3.4. Comparison of Abundance Levels of miR-166a between Mice Fed with Rapeseed Bee Pollen and Control.* Given that miR-166a is the highest abundance rape-encoded miRNA in mice fed with rapeseed bee pollen, and it is rich in rapeseed bee pollen, we speculate that the miR-166a in mouse serum are mainly absorbed from rapeseed bee pollen. To test this speculation, we compared the abundance level of miR-166a in serum of mice fed with rapeseed bee pollen and control. As it is reported that the levels of plant-based miRNAs were elevated in serum of mice for 6 h [4], we compared the levels of miR-166a in serum of mice fed with rapeseed bee pollen after 6 h and control. As shown in Figure 2 and additional Figure 2, the levels of miR-166a were elevated in serum of mice fed with rapeseed bee pollen for 6 h compared with control by RT-qPCR.

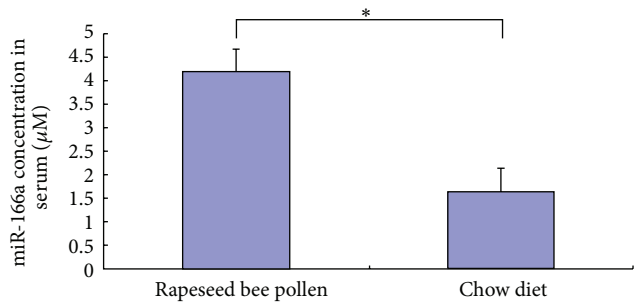


Figure 2: The abundance levels of miR-166a in mouse serum after feeding with rapeseed bee pollen or chow diet for 6 h ($n = 5$). $^{*}p < 0.05$.

## 4. Discussion

An estimated 60% of all protein-coding genes are targeted by miRNAs in human [17]. In addition, many miRNAs are deregulated in immune system, inducing diseases like autoimmune diseases, inflammation, and tumors [3]. Food-derived miRNAs have the potential to restore the downregulated miRNAs in diseases. For example, immune-related miRNAs are abundant in breast milk, and they might play a critical role in the development of the infant immune system [18]. Furthermore, Western donor sera contained the plant miRNA miR159, whose abundance in the serum was inversely correlated with breast cancer incidence and progression in patients [9]. These studies raise the intriguing prospect of using edible plants miRNAs to prevent and treat mammal diseases.

Bee pollen is rich in nutrition and medicinal composition, which ensued a wide use of bee pollen in food, health

products, medicine, cosmetics, and other fields [13, 19, 20]. In the field of medicals, bee pollen is used for prevention and treatment of prostate diseases [14], cardiovascular and cerebrovascular diseases, immune diseases, and so forth [15]. Besides, bee pollen is rich in RNA with a range of 0.6%–1% (w/w) [16].

In this study, we confirmed that miRNAs from rapeseed bee pollen can be absorbed by mice, and the abundance of exogenous miRNAs in mouse blood is dependent on their original levels in pollen. Moreover, the detailed functions of these exogenous miRNAs in mammals should be investigated to help clarify the immune function or medical efficacy of bee pollen. Nevertheless, the present study provided first hand evidence for the potential usages of rapeseed bee pollen as a supplement of plant miRNAs.

## Competing Interests

The authors declare that they have no competing interests.

## Acknowledgments

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*Research Article*

# *Macleaya cordata* Extract Decreased Diarrhea Score and Enhanced Intestinal Barrier Function in Growing Piglets

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*Macleaya cordata* extract is of great scientific and practical interest to researchers, due to its antimicrobial and anti-inflammatory responses within experimental animals. This study was designed to determine the diarrhea score and innate immunity of growing piglets after they had received *Macleaya cordata* extract supplements. A total of 240 growing pigs were randomly assigned to one of three dietary treatments, with 8 replicates per treatment and 10 piglets per replicate. All pigs received a basal diet containing similar amounts of nutrients. The three treatments were a control (no additive), an antibiotic (200 mg/kg colistin), and the *Macleaya cordata* extract supplement group (40 mg/kg *Macleaya cordata* extract). The diarrhea score was calculated after D 28. The jejunal samples were obtained from five piglets selected randomly from each treatment on D 28. In comparison with the control group, the dietary *Macleaya cordata* extract and colistin group demonstrated a substantially decreased diarrhea score. The introduction of *Macleaya cordata* extract supplements to the diet significantly increased volumes of ZO-1 and claudin-1, particularly in comparison with the pigs in the control group ($P < 0.05$). The findings indicate that *Macleaya cordata* extract does enhance intestinal barrier function in growing piglets and that it could be used as a viable substitute for antibiotics.

## 1. Introduction

In recent years, there has been an increased focus on reducing or eliminating the use subtherapeutic antibiotics within livestock diets. For this reason, there has been a concerted effort to identify alternative effective therapies that could serve as a substitute for antibiotics in pig diets. The aim is to maintain acceptable performance levels and ensure food safety for consumers [1]. The medicinal plant extracts used as an alternative for antibiotics in swine diets are effective when it comes to increasing BW, intestinal health, nutrient digestibility, and antioxidative potential, and immunity. They also decrease the incidence and vulnerability to diarrhea [1, 2].

*Macleaya cordata* extract is believed to be an effective natural appetiser in swine, bovine, poultry, and even fish

nutrition [3]. *Macleaya cordata* extract is blended from intact aerial parts and a fraction of quaternary benzo[c]phenanthridine alkaloids (QBAs), primarily sanguinarine (SG) and chelerythrine (CH), and standardised to 1.5% w/w SG [4].

Sanguinarine (SA), a quaternary benzo[c]phenanthridine alkaloid, is synthesised from dihydrosanguinarine, via the activities of dihydrobenzophenanthridine oxidase [5]. It has been shown to have beneficial effects. It is antimicrobial [6, 7], antifungal, adrenolytic, sympatholytic, immunomodulatory [5], and anti-inflammatory [8, 9].

Chelerythrine can be found within the greater celandine plant and a number of additional poppy *Fumaria* varieties. It is a quaternary benzo[c]phenanthridine alkaloid. According to studies, it mostly exhibits tumour resistant, microbe resistant, and inflammation resistant qualities. Plus, the substance is a powerful disruptor when it comes to PKC (or protein kinase C). As such, the prospective utilisation of CHE, as a form of inflammation resistance, has been the topic of much debate. Its qualities are linked to its capacity to engage with DNA and proteins [10]. This is an enzyme that plays an important part in the control of signal transduction, cell propagation, and cell variation [11].

For this study, we hypothesised that dietary supplementation with *Macleaya cordata* extracts might stimulate the immune system and, as a consequence, decrease the incidence of diarrhea in growing piglets. Thus, the objective of the study was to evaluate the impact of *Macleaya cordata* extract, as a dietary supplement, on diarrhea scores and the expression of tight junction proteins.

Table 1: Compositions and nutrient levels in basal diets (as-fed basis).

| Ingredients | Content (%) |
|---|---|
| Yellow corn | 74.90 |
| Soybean meal | 21.0 |
| L-Lysine HCL | 0.23 |
| DL-Methionine | 0.07 |
| L-Tryptophan | 0.04 |
| L-Threonine | 0.10 |
| Poultry fat | 1.00 |
| Vitamin premix[1] | 1.00 |
| Ground limestone | 1.00 |
| Monocalcium phosphate | 0.70 |
| Salt | 0.30 |
| Total composition | |
| DM, % | 89.70 |
| ME, Mcal/kg | 3.38 |
| CP, % | 19.40 |
| Ca, % | 0.68 |
| Total P, % | 0.49 |
| Available P, % | 0.21 |

[1]The trace minerals in the premix provided (per kg diet): 5 mg Cu as $CuSO_4 \cdot 5H_2O$; 80.5 mg Fe as $FeSO_4 \cdot 7H_2O$; 0.15 mg I as KI; 0.3 mg Se as $NaSeO_3$; 3.3 mg Mn as $MnSO_4 \cdot H_2O$; 81.3 mg Zn as $ZnSO_4 \cdot 7H_2O$; 5 mg vitamin K (menadione); 2.1 mg vitamin B1; 15.2 mg vitamin B2; 30 $\mu$g vitamin B12; 5, 110 IU vitamin D3; 400 IU vitamin A; 18 IU vitamin E; and 80 mg choline chloride.

## 2. Materials and Methods

*2.1. Experimental Design, Animals, and Housing.* All of the procedures used in this study have been approved by the Institute of Subtropical Agriculture, the Chinese Academy of Sciences Animal Care and Use Committees. A total of 240 (Yorkshire × Landrace) × Duroc (56 d old; BW 15.82 ± 1.13 kg) pigs were obtained from a commercial swine herd in Guangdong, China. 240 piglets were assigned, in completely random design (but based on their BW), to 24 pens. The pens each contained a total of ten piglets. The process was carried out according to the experimental animal allotment program created by Kim et al. [12]. All pens were equipped with a feeder, a nipple-type drinker (with modification; more details later), and plastic-covered expanding metal floors. The room temperature was set at 28.0°C and slowly increased by 0.5°C/wk.

All piglets were fed the same basal diet, with a similar amount of nutrients (Table 1). This diet was formulated to meet or exceed the NRC (2012) nutrient specifications for pigs weighing 10 to 30 kg. In total, there were 3 experimental treatments and study groups: the negative control (NC; no in-feed or in-water additive), the antibiotic group (AB; in feed, 0.2 g/kg colistin), and the *Macleaya cordata* extract group (MC; in-feed, 40 mg/kg *Macleaya cordata* extract). The *Macleaya cordata* extract was kindly provided by Phytobiotics Futterzusatzstoffe GmbH, Eltville, in Germany and Micolta Bioresource Inc., in Changsha, China. The pigs had unlimited access to feed and water throughout the entirety of the study.

*2.2. Sample Collection and Analytical Procedure.* On days 7, 21, and 28 of treatment, 10 mL of blood was collected in plastic uncoated tubes between 8:00 and 10:00 a.m. After collection, these blood samples were centrifuged at 8000 ×g for 10 min at 4°C, with sera collected and frozen at −20°C until further analyses.

*2.3. Faecal Score and Sampling.* To evaluate diarrhea incidence, faecal consistency scores (0, normal; 1, soft faeces; 2, mild diarrhea; and 3, severe diarrhea) were determined for each pen. These scores were collected by a trained individual, but who had no prior knowledge of the dietary treatments provided. The incidence of diarrhea was calculated by dividing the total number of pigs with diarrhea by the total number of all experimental pigs. The rate ratios were then calculated. On day 28 of the experiment, a number of the pigs (1 pig/pen) were killed. 1 sample of jejunal mucosa was collected in a sterile sample bag and stored at −20°C until analysis.

*2.4. Concentration of IgG, IgM, D-Lactate, DAO, and TNF-α.* The serum volumes of IgM and IgG were calculated according to readings from the 1251 Radio Immunoassay Analysing tool (Beijing North Institute of Biological Technology, Beijing, China). A $\gamma$-calculating instrument GC-300 (Zhongjia Co.,

Ltd., Beijing) was also used as part of this equipment. Recommendations from the maker were followed at all times.

The volume of serum D-lactate and DAO were calculated using Beckman Cx4 Chemistry Analyser (Beckman Coulter, Brea, CA) with a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Recommendations from the maker were followed at all times. All changes to or within the serum diamine oxidase were calculated with assay equipment.

The proportion of tumour necrosis factor-$\alpha$ (TNF-$\alpha$) within serum was calculated via the use of a Porcine TNF-$\alpha$ Colorimetric ELISA tool (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). 50 $\mu$L of regular plus dilute solution (or 100 $\mu$L of the sample) was introduced to microplate wells. The wells had already been pretreated with capture antibody, as well as biotinylated antibody reagent. Identification relied on the utilisation of horseradish peroxidase, a stop solution of 0.18 N $H_2SO_4$, and TMB substrate. The identification parameter for TNF-$\alpha$ was 5 pg/mL. Merging was detected at 450 and 540 nm, with the use of the KC4 data evaluation programme and an ELISA plate tool.

*2.5. Tight Junction Proteins Expression.* The tight junction protein displays of occludin, claudin-1, and zonula occludens-1 (ZO-1) were calculated with the use of western blotting. Western blot testing was carried out via the use of a sophisticated chemiluminescence identification tool (Amersham, Illinois). The results were captured with the ChemiScope 3400 device (Clinx Science Instruments, Shanghai, China). They were evaluated with the use of Quantity One equipment (Bio-Rad, California, USA). The $\beta$-actin functioned as an interior control because it expressed no dissimilarities across the populations. The significance of the protein display was represented by the ratio of the densitometry units of $\beta$-actin and tight junction protein. For a short time, all protein was removed, according to the advice provided by a protein removal tool (KGP2100 from KeyGen Biotech, Nanjing, Jiangsu China). The mucosa proteins within the intestines were isolated with a polyacrylamide substance (Millipore, MA, USA). They were then introduced to polyvinylidene difluoride membranes. These membranes were kept secure and protected for twelve hours, at a temperature of 4°C, after the introduction of the first antibodies. After the introduction of the secondary antibody, they were kept at 25°C, for 120 minutes. The first antibodies (goat polyclonal claudin-1 antibody, $\beta$-actin rabbit antibody, and rabbit polyclonal ZO-1 antibody) were sourced from Santa Cruz Biotechnology (California, US). The secondary antibodies used were goat anti-rabbit IgG-HRP and rabbit anti-goat IgG-HRP (again, sourced from Santa Cruz Biotechnology).

*2.6. Statistical Analysis.* To evaluate and verify the results, an analysis of variance (or ANOVA) method was used for basic classification and order of a completely randomized design. Before ANOVA, the evenness of its variance was checked using the Bartlett technique. Then, the regularity of the information was tested using the Kolmogorov-Smirnov method. At certain points, a Duncan multiple range method (in accordance with SPSS 22.00 Statistical Software) was utilised to calculate dissimilarities across mean values.

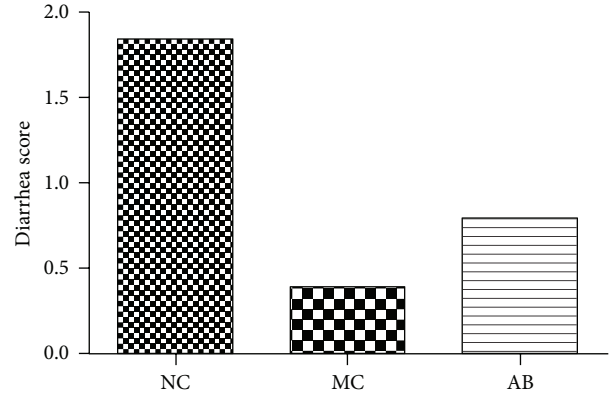


Figure 1: The diarrhea score of pigs provided with the experimental diets described. The experiment lasted for a duration of 28 days.

## 3. Results

Figure 1 demonstrates that the diarrhea score for pigs in the MC group was lower than that of the NC group and the AB group. The score for the AB group was still lower than that of the NC group.

Table 2 demonstrates the impact of dietary supplementation with *Macleaya cordata* extract on the serum biochemical parameters and volume of antibodies in the growing pigs on days 7, 14, and 28 after the start of the study.

On day 7, the concentration of IgG was higher ($P < 0.05$) in pigs from the MC group, when compared with the other 2 groups. The serum concentrations of D-lactate, DAO, IgM, and TNF-$\alpha$ did not exhibit any substantial or notable differences ($P > 0.05$) across the treatments, on day 7 after the launch of the study.

On day 14, the serum concentration of D-lactate was the only parameter that showed a significant difference ($P < 0.05$) across the treatments. There was a lower concentration in pigs from the AB group. The other serum parameters for the pigs remained the same ($P > 0.05$), due to the impact of dietary supplementation with *Macleaya cordata* extract (Table 2). On day 28, the serum concentration of IgM decreased in the pigs from the MC group, in comparison with those from the AB group ($P < 0.05$). The other parameters exhibited no notable changes ($P < 0.05$), as a consequence of the experimental treatments.

Figure 2 demonstrates the protein expression levels of occludin, ZO-1, and claudin-1 within the jejunal mucosa. The occludin levels did not notably differ across any of the groups. The protein expression levels of ZO-1 within the jejunal mucosa increased substantially ($P < 0.05$), in comparison with those in the NC and AB groups. Also, ZO-1 protein increased significantly ($P < 0.05$), in comparison with that of the NC group. Thus, the dietary supplementation of *Macleaya cordata* extract and antibiotics substantially increased the expression of Claudin-1 ($P < 0.05$), particularly when compared with that of the NC group.

Table 2: Effect of dietary supplementation with *Macleaya cordata* extract on D-lactate, IgG, IgM, and TNF-$\alpha$ in growing pigs.

| Parameter | Dietary supplementation | | | SEM± | *P* value |
|---|---|---|---|---|---|
| | No additive | *Macleaya cordata* extract | Colistin | | |
| *D 7 after initiation of treatment* | | | | | |
| D-Lactate, mg/L | 7.95 | 9.12 | 7.06 | 1.119 | 0.477 |
| DAO, units/mL | 2.53 | 2.46 | 2.42 | 0.216 | 0.536 |
| IgG, mg/mL | 138.41$^{b}$ | 174.45$^{a}$ | 143.44$^{b}$ | 10.801 | <0.05 |
| IgM, mg/mL | 43.20 | 39.60 | 38.40 | 6.635 | 0.869 |
| TNF-$\alpha$, pg/mL | 62.34 | 58.98 | 59.36 | 7.510 | 0.478 |
| *D 14 after initiation of treatment* | | | | | |
| D-Lactate, mg/L | 10.26$^{a}$ | 8.21$^{ab}$ | 7.45$^{b}$ | 0.813 | <0.05 |
| DAO | 2.42 | 2.39 | 2.30 | 0.166 | 0.632 |
| IgG, mg/mL | 183.89 | 176.17 | 184.58 | 20.04 | 0.947 |
| IgM, mg/mL | 50.00 | 49.40 | 51.00 | 7.799 | 0.989 |
| TNF- $\alpha$ | 64.13 | 59.42 | 61.37 | 6.590 | 0.589 |
| *D 28 after initiation of treatment* | | | | | |
| D-Lactate, mg/L | 9.10 | 9.36 | 7.60 | 1.169 | 0.535 |
| DAO | 2.25 | 2.30 | 2.18 | 0.157 | 0.453 |
| IgG, mg/mL | 196.38 | 219.60 | 201.72 | 16.768 | 0.604 |
| IgM, mg/mL | 63.20$^{ab}$ | 49.60$^{b}$ | 70.00$^{a}$ | 6.224 | <0.05 |
| TNF-$\alpha$ | 60.73 | 58.98 | 60.22 | 7.586 | 0.673 |

$^{a,b}$Means within the same row with different superscript differ significantly ($P < 0.05$).
The experiment lasted 28 d; $n = 6$.

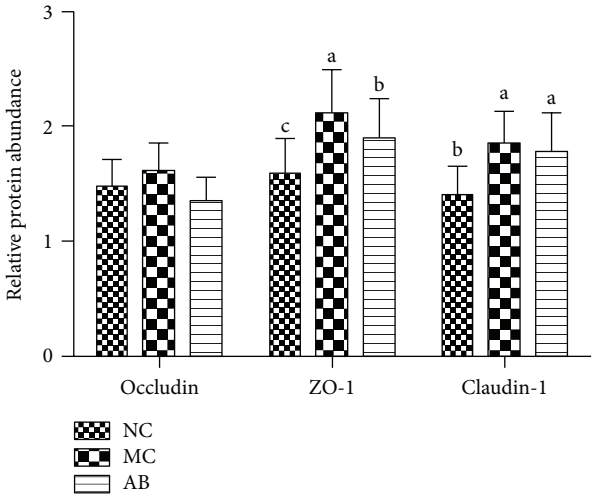


Figure 2: Tight proteins in fairly large volumes, within the jejunal mucosa of pigs. Data is equivalent to ±SEM. The a, b, and c sections, with different letters, are substantially dissimilar to one another ($P < 0.05$). The significance of protein display is the ratio of the densitometry units of tight junction protein and $\beta$-actin. The study took 28 days to complete; $n = 6$.

## 4. Discussion

Diarrheal syndrome occurs primarily because of an increase in *Escherichia coli* and others pathogenic bacteria inside the gut after weaning. This leads to a loss of water and electrolytes, via the semiliquid faeces [1]. According to Kommera et al. [13] and Kong et al. [1], the supplementation of medicinal plant extracts decreases diarrhea incidence in pigs, mainly because the extract has antimicrobial properties and it aids in the regulation of organic functions, intestinal pH, and peristalsis.

As Newton et al. [7] explain, the antimicrobial properties of sanguinarine have been clearly demonstrated. If it is added to swine diets, as a supplement, it has the potential to support the intestinal colonisation of beneficial bacteria and increase competitive exclusion in GIT. It also reduces water secretion from the intestinal epithelial cells and/or improves the absorption of water and nutrients from the intestinal lumen [1]. These results are primarily caused by an increase in the metabolism of biomolecules and by antioxidant activities within the small-intestinal mucosa.

There is a relationship between intestinal pH, microflora population, and diarrhea incidence in pigs [1]. This is clear because both stabilization of the intestinal pH value at a reasonably low level and the maintenance of an optimal balance of intestinal microbiota play a major role in the reduction of diarrhea incidence [5, 14]. It is noteworthy then that although these key results relating to the the antidiarrheal effect of medicinal plant extracts have been seen in piglets, we also found a similar response in growing pigs.

Crucially, *Macleaya cordata* extract also has other important characteristics, such as its anti-inflammatory effect and immunomodulatory properties [5, 8, 9], which improve intestinal immune function. This reduces inflammation within the small-intestinal mucosa that usually develops as a result of stress. Although the precise mechanisms are not clear, our results demonstrate that *Macleaya cordata* extract, provided as a natural supplement to growing pigs, could be a viable substitute for feed antibiotics.

Serum antibody volume is another useful indicator of humoral immunity. Actually, IgG and IgM are key components and signifiers of humoral immunity in all mammals because they are the major serum immunoglobulins. They protect the extravascular compartment against pathogenic bacteria and microorganisms [15]. Furthermore, IgG also has antibacterial and antitoxin effects [16]. These findings suggest that a higher concentration ($P < 0.05$) of IgG in pigs of MC group (Table 2) leads to an increase in the production of antibodies by B lymphocytes. This is beneficial for immune status and, consequently, animal response [15, 17].

It should be noted that this serum antibody (IgG) did not increase ($P < 0.05$ = on subsequent days (d 14 and 28)), perhaps because of a higher immunological stability in the pigs. However, other studies on medicinal plant additives have shown a variability in the serum concentration of IgG in pigs [15, 17]. Interestingly, the serum concentration of IgM did decrease ($P < 0.05$ = in pigs from MC group). IgM is the first to be synthesised, as a response to infection [17]. Despite variations in the serum concentration of immunoglobulins, after supplementation with *Macleaya cordata* extract, we observed a healthy response in growing pigs (Table 2). According to the work of Tewari et al. [18], the underlying mechanisms that regulate immunological function are likely to be multifactorial. As such, they recommended that future studies use molecular biology and proteomics technologies.

When it comes to sustaining and protecting the translocation of bacteria in the intestines, an undamaged intestinal barrier is very important. It stops allergenic and toxic substances from making their way inside the gut and becoming a danger [19, 20]. D-Lactate and diamine oxidase can usually be found in modest amounts, within the circulation [21]. Yet, if the protective capacity of the intestine is decreased, mucosal permeability begins to rise. This enables a larger volume of D-lactate and DAO to get inside the peripheral circulation. For this reason, D-lactic acid and plasma DAO can potentially be used as indicators of damage to the intestinal barrier system [22, 23]. This paper has discussed, in depth, the relationship between MC extracts and this degree of protection within the intestine. For example, there is much evidence to support the idea that if baby pigs are taken from their mothers and weaned too early, it leads to diminished intestinal barrier capacities [24, 25]. On the other hand, according to the data collected, MC extract supplements actually boost jejunal mucosa DAO. However, it lowers the volume of plasma DAO and D-lactate. This suggests that intestinal mucosal development and barrier strength can be enhanced by introducing MC supplements.

The intestinal barrier is tightly controlled by a carefully arranged piece of the epithelial junctional complex. It is usually called "the tight junction" [26]. The porousness and protective capacities of the intestine have, for a long time, been treated as signifiers of intestinal epithelial barrier strength. It consists of various distinct proteins, like the transmembrane protein occludin [27], some members of the claudin family [28], junctional adhesion molecule [29], various linker proteins such as ZO-1, and more. The most valuable and essential are occludin, ZO-1, and claudin-1 because they are linked to the functional and operational management of the tight junctions [30]. ZO-1 is a valuable linker protein for the tight junction because it joins the C-terminal selections of $\beta$-actin and occludin [30]. It also functions as a connection between cytoskeleton proteins and the plasma membrane. Claudin-1 seems to be mostly linked to the display of ZO-1 inside the small intestine. Finally, for the epithelial tight junction, occludin is a vital membrane protein. It plays a big part when it comes to sustaining the strength and barrier quality of the tight junction [27]. For this study, we calculated fluctuations within the display of occludin, ZO-1, and claudin-1 at the mRNA level. This allowed us to more accurately judge the molecular catalyst for the decrease in intestinal strength within growing pigs after they had been given artificially increased levels of Zn. The outcomes show that adding *Macleaya cordata* to the diet, as an additional supplement, boosts volumes of ZO-1 and Claudin-1 proteins.

The experiment shows that introducing *Macleaya cordata* extracts to the diet, as an additional supplement, can increase resistance to intestinal damage, to some degree. This is achieved, throughout the growing stage, via increases in the volume and generation of claudin-1 and ZO-1. A number of earlier studies, on animals, had already made it clear that intestinal strength is closely linked to claudin-1 and ZO-1 volumes [31]. According to Kansagra et al. [32], fluctuations within intestinal strength, throughout the weaning stage, can be explained by the deterioration of tight junction reliability and quality. These studies discussed the fact that baby pigs in the weaning phase suffer various pressures (e.g., swapping from a liquid diet to a solid one). This makes them vulnerable to infections created by enterotoxigenic *E. coli* [33]. It happens because tight junction proteins have become disarrayed or their volumes have been lowered [21]. For all of these reasons, epithelial strength is compromised. In turn, this unusually high degree of intestinal porousness leads to increased antigenic vulnerability, a weaker immune system, and persistent inflammation. This greatly decreases barrier strength [34] and, eventually, leads to dangerous levels of after-weaning sickness.

To summarise, boosting the diet with *Macleaya cordata* extract supplements caused a reduction in faecal dysfunction. This result was underscored by larger volumes of claudin-1 and ZO-1. Consequently, the fortifying impact of *Macleaya cordata* supplements may be, to some degree, linked with the enhancement of intestinal barrier strength, particularly when it comes to treating conditions like diarrhea.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contributions

Gang Liu and Guiping Guan contributed equally to this paper.

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*Review Article*

# Crosstalk between Vitamin D Metabolism, VDR Signalling, and Innate Immunity

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The primary function of vitamin D is to regulate calcium homeostasis, which is essential for bone formation and resorption. Although diet is a source of vitamin D, most foods are naturally lacking vitamin D. Vitamin D is also manufactured in the skin through a photolysis process, leading to a process called the "sunshine vitamin." The active form of vitamin D, 1,25-dihydroxyvitamin D (calcitriol), is biosynthesised in the kidney through the hydroxylation of 25-hydroxycholecalciferol by the CYP27B1 enzyme. It has been found that several immune cells express the vitamin D receptor (VDR) and CYP27B1; of the latter, synthesis is determined by several immune-specific signals. The realisation that vitamin D employs several molecular mechanisms to regulate innate immune responses is more recent. Furthermore, evidence collected from intervention studies indicates that vitamin D supplements may boost clinical responses to infections. This review considers the current knowledge of how immune signals regulate vitamin D metabolism and how innate immune system function is modulated by ligand-bound VDR.

## 1. Background

The actions of vitamin D are familiar in its classic capacity of mineral metabolism and bone health. Vitamin D promotes the intestinal absorption of phosphate and calcium, it stimulates the differentiation of progenitor cells to osteoclasts, and it recovers calcium from bones and encourages bone matrix mineralisation. Research into osteomalacia and rickets provided early evidence of the important role of vitamin D [1]. These diseases represent vitamin D deficiency and present symptoms of hypocalcaemia (low serum calcium levels) and skeletal deformity due to poor mineralisation of the bones [2]. Patients with these diseases usually have serum vitamin D levels below 20 nmol/L. To help reduce the incidence of rickets, infants in USA and in other countries typically receive daily vitamin D supplements of at least 200 IU (5 $\mu$g). Although this strategy has reduced the incidence of rickets, it has not eliminated rickets, and rickets still persist [3]. Vitamin D deficiency is not limited to bone-related diseases but is also implicated in cardiovascular disease, autoimmune maladies such as type 1 diabetes mellitus, several cancers, inflammatory bowel disease, and multiple sclerosis [4].

The role of vitamin D, as it applies to human health, has undergone reevaluation following the discovery that VDR and CYP27B1 are expressed in cells such as the intestine, pancreas, prostate, and some immune cells, none of which are implicated in bone and mineral metabolism [4]. The biosynthesis of calcitriol by immune cells and peripheral tissue is of particular interest to immunology studies. This molecule is thought to modulate immune function in a manner similar to active cytokines [5]. This review outlines the role of vitamin D and its effects on the innate immune system.

## 2. Vitamin D Sources

There are three sources of vitamin D: UVB radiation-dependent endogenous production, dietary supplements, and nutritional sources. By far the most significant of vitamin D is UVB exposure and dietary supplementation is the least. There are several analogues of vitamin D; the two forms that are most relevant to human health are ergocalciferol ($D_2$) and cholecalciferol ($D_3$). The numbers of nonfortified foods that contain relevant quantities of either form of vitamin D are limited, fatty fish (mackerel, salmon, and sardines), cod liver

oil, and some types of mushrooms, such as sundried shiitake [4, 6].

The conversion of 7-dehydrocholesterol to vitamin $D_3$ occurs in the epidermal layer of skin. 7-Dehydrocholesterol maximally absorbs UVB radiation from the sun at wavelengths of ~300–325 nm, the presence of which is influenced by latitude, altitude, season, and cloud cover. For approximately six months of each year, at sea-level locations at latitudes of 45°, the UVB intensity is inadequate for vitamin D synthesis. This "vitamin D winter" extends at distances further away from the equator [7].

Dietary vitamin D intake is dependent upon a country's fortification policy and on an individual's dietary habits; by fortifying staple foods, such as dairy produce, some countries such as Canada and USA help to reduce vitamin D deficiency. In spite of these efforts, a global perspective review revealed that dietary supplements contribute 6–47% of vitamin D intake [8]. This suggests that where endogenous vitamin D production is low because UVB is insufficient, maintaining healthy levels of vitamin D is heavily dependent on supplements. As well as the factors identified earlier that determine UVB adequacy, an individual's endogenous vitamin D production is influenced by their genes, skin pigmentation, clothing, lifestyle, and use of sunscreen [9].

## 3. Vitamin D Biosynthesis and Sufficiency

To become bioactive, cholecalciferol ($D_3$) undergoes two modifications. In the first one, hepatic hydroxylating enzymes CYP2R1 and CYP27A1, and possibly others, catalyse $D_3$ to produce prohormone 25-hydroxycholecalciferol (25D) [4]. This is the predominant circulating metabolite and it has a half-life of several weeks; it is used to determine vitamin D status. The prohormone undergoes 1$\alpha$-hydroxylation by CYP27B1, resulting in its active form, 1,25-dihydroxyvitamin D (1,25D). This second modification occurs in the kidney, where regulated production is induced by parathyroid hormone (PTH) (Figure 1). It was originally thought that circulating levels of 1,25D were primarily derived from the kidney. Recent research has found that CYP27B1 expression is not limited to the kidney [10] and that other several tissues locally biosynthesise 1,25D, which acts intracellularly to regulate events within the cell or in a paracrine fashion. Through a negative feedback loop, 1,25D initiates the robust expression of *CYP24A1*, which codes for the CYP24 enzyme. This enzyme degrades 25D and 1,25D by hydroxylation of carbon 24, to generate biologically inactive metabolites.

Vitamin D deficiency is defined as circulating 25D levels below 20 ng/mL (50 nM) and insufficiency as 20–30 ng/mL (50–75 nM) [4]. Many researchers define sufficiency as levels of 30–32 ng/mL or higher. These levels were determined by the inverse relationship present between the circulating levels of 25D and PTH, the latter of which becomes deficient when 25D levels rise above 30 ng/mL. Yet a report from the Institute of Medicine defines sufficiency as ranging between 20 and 50 ng/mL (75–125 nM), with levels greater than 50 ng/mL (125 nM) considered to be excessive [11]. The report attracted controversy by concluding that few in the population suffered from vitamin D insufficiency or deficiency [12]. Furthermore,

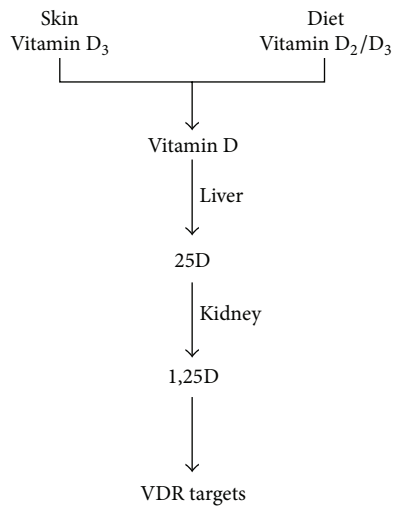


Figure 1: Vitamin D from skin and diet is converted to 25D in the liver and then converted to 1,25D in the kidney. 1,25D modulates the innate immunity system.

the report argued that there was a paucity of experimental evidence of vitamin D having important physiological functions beyond bone health. Without adequate randomised, placebo-controlled clinical trials investigating nonbone events, guidelines to establish recommended dietary intake could not be determined. Yet within recent years, there have been studies demonstrating that, in response to infection, immune system cells synthesise and respond to 1,25D. There is a growing body of clinical evidence collected from intervention trials showing that vitamin D sufficiency has prophylactic capability. Lastly, although vitamin D intoxication may occur, it is still not common and requires circulating levels to be in excess of 125 ng/mL [4]. Intoxication is typified by elevated blood levels of calcium.

## 4. The Vitamin D Receptors

The VDR is a member of the nuclear receptor superfamily. The VDR is activated by the binding of 1,25D [13]. Microarray expression profile studies have provided an enhanced understanding of vitamin D physiology and much can be explained by the VDR functioning as a gene transcription regulator [14]. The structure of the VDR incorporates an $\alpha$-helical ligand-binding domain and a highly conserved DNA-binding domain [13]. 1,25D-liganded VDR forms a heterodimer with the retinoid-X receptor (RXR), which binds to vitamin D response elements (VDREs) in the regulatory area of the gene controlled by 1,25D.

VDREs are comprised of 5′-PuG(G/T)TCA-3′ (where Pu is any purine) repeat motifs separated by 3 bp (DR3) or everted repeats separated by 6 bp (ER6) or 8 bp (ER8) [13]. ER8 motifs recognised by VDR-RXR heterodimers encode the cytokine, interleukin-10 [15]. Transcription is initiated through the DNA-bound VDR/RXR complexes' recruitment of coregulatory proteins that instigate the necessary histone modification, chromatin remodelling, and RNA polymerase

II binding [16]. Although several VDREs have been identified in locations close to the promoter regions [14], evidence from recent research indicates that DNA-bound VDR can operate over distances of 75 kbp to regulate target gene transcription [17]. Meanwhile, the VDR is able to suppress transcription, such as repressing cytokine gene expression in activated T-cells. In the presence of 1,25D, VDR/RXR heterodimers can dislodge DNA-bound nuclear factor (NF-AT), inhibiting cytokine expression. It has also been recently discovered that 1,25D-bound VDR interacts with FoxO transcription factors to suppress the cyclin D2 gene expression [18].

## 5. Vitamin D and Innate Immunity System

Reports of treating tuberculosis with cod liver oil presented the first evidence that vitamin D is a potential innate immune system stimulant [19]. Contemporary studies describe how calcitriol promotes the antimicrobial activity of macrophages and monocytes, which play a critical role in combatting pathogens such as *Mycobacterium tuberculosis*. The 1,25/VDR/RXR complex not only boosts the innate immune cells chemotactic and phagocytic capabilities, but also directly activates transcription of cathelicidin (hCAP18) and defensin $\beta$2 (DEFB) [20]. Following recognition of *M. tuberculosis*, through toll-like receptor signalling, monocytes induce CYP27B1 and VDR activity and directly modulate gene expression that favours cathelicidin production [21]. Other cytokines like interferon-$\gamma$ or interleukin-4 also influence CYP27B1 expression [22]. Production of human cathelicidin (hCAP18), derived from LL-37, is upregulated in response to infection; it destroys microbial lipoprotein membranes [23].

Where infections are severe, there is an upsurge of neutrophils, which led to the original proposal of neutrophils being the primary source of cathelicidin [24]. This opinion has since been revised, as although neutrophils express VDR, they appear to lack the CYP27B1 capability required to convert 25D to 1,25D; this is essential to stimulate cathelicidin gene expression [22]. However, critically ill, septic patients have been found to have significantly lower serum 25D levels. This finding, derived from a cross-section analysis, showed a correlation between low serum 25D levels and reduced concentrations of cathelicidin [25]. This observation lends support to the hypothesised role of vitamin D regulating antimicrobial protein levels in a concentration-dependent manner and may be fundamental to infection control.

Vitamin D not only modulates monocytes, but also is important to other antigen presenting cells (APCs), especially dendritic cells (DCs). Calcitriol has also been credited with inhibiting the T cell cytokines, interleukin-2, and interleukin-17, as well as monocyte toll-like receptors [26]. A study of calcitriol supplements in healthy humans found that a high dose (1 $\mu$g, twice a day for 7 days) led to the significant suppression of interleukin-6, a proinflammatory cytokine [27].

Dendritic cells express CYP27B1 which enables calcitriol and cholecalciferol to induce tolerogenic behaviour in these cells. The presence of the enzyme allows DCs to generate a high local concentration of calcitriol, which is needed to modulate immune responses. Data from *in vitro* studies using VDR and CYP27B1 knockout mice showed abnormal DC chemotaxis and a considerable increase in numbers of mature DCs [28]. In a placebo-controlled clinical trial of 95 tuberculosis patients, the inflammatory responses resolved quicker in patients that received a high dose of vitamin D and adjunctive therapy [29].

The gastrointestinal (GI) tract is a selectively permeable barrier that permits water and nutrient transport whilst inhibiting systemic pathogenic infection. Evidence from VDR knockout mice suggests that vitamin D has a role in regulating the GI tract barrier. The knockout mice showed a heightened vulnerability to lipopolysaccharides and chemically induced GI inflammation (DSS colitis) [30]. The integrity of the epithelial barrier was lost in the mice that had been exposed to DSS [30]. Compared to wild-type (WT) mice, VDR mice treated with DSS displayed a reduction in expression of E-cadherin, claudin-1, ZO-1, and occluding proteins [31]. In GI epithelial cells 1,25D stimulated transcription of E-cadherin [32]. The permeability of the gut increased in line with the loss of tight junction proteins in VDR knockout mice and vitamin D deficient mice [31]. Furthermore, elevated levels of inflammatory cytokines, such as TNF-$\alpha$, were found to contribute to the loss of GI barrier integrity in vitamin D deficient and VDR knockout mice [30]. Together, the evidence from these studies indicates that vitamin D has an important regulatory role in maintaining the GI epithelium and its barrier function.

## 6. Molecular Mechanisms Underlying Vitamin D Regulate Innate Immunity

Investigating the immunomodulatory capability of vitamin D signalling is at the forefront of current research on developing the understanding of the mechanisms of vitamin D metabolism and 1,25D signalling as they apply to innate immune responses. It is known that CD14 expression is vigorously stimulated by 1,25D. CD14 is a TLR4 coreceptor that is required to recognise lipopolysaccharide.

With the completion of the Human Genome Project in 2003, the position of promoter-proximal consensus VDREs became known. The *in silico* screen pinpointed VDREs adjoining the transcription start-sites of genes encoding the antimicrobial peptides $\beta$-defensin 2 and cathelicidin [33]. Together with various cytokines and chemokines, antimicrobial peptides are amongst the first defensive mechanisms of the innate immune system to respond [34]. Immune responses may be enhanced by cathelicidin and some $\beta$-defensins that not only act against microbes, but also have chemoattractant capabilities, recruiting neutrophils, monocytes, and other immune cell molecules to the site of infection [34]. In the cell types investigated, expression of cathelicidin was robustly invigorated by 1,25D. A particularly interesting *in vivo* study looked at the regulation of cathelicidin peptide in the bile duct, which typically is microbe-free. The researchers noted that expression of the cathelicidin gene in the epithelial cells was regulated by the concentration of bile acids [35]. The probable contribution of VDR in the signalling

is in accord with earlier studies, which indicate that VDRs have a bile acid sensing ability. Selecting animal models for research into cathelicidin and $\beta$-defensins needs to be made with care. The *CAMP* and *HBD2* are not conserved in mice, and, in humans and primates, the *CAMP* VDRE is embedded in an Alu repeat transposable element [20]. This particular VDRE-containing Alu repeat in the *CAMP* gene has only been found in the branch of primates that includes Old and New World monkeys, apes, and humans [36].

Cell-based investigations found that, in contrast to its effect on *CAMP* expression, the induction of *HBD2* expression by 1,25D alone was limited or absent [21]. Yet the robust expression of *HBD2* by interleukin-1$\beta$ was doubled in the presence of 1,25D. It was later shown that signalling through TLR1/2 pattern receptors initiated interleukin-1$\beta$ expression. For strong *HBD2* expression, both 1,25D and interleukin-1$\beta$ were necessary [21]. It is probable that interleukin-1$\beta$ signalling is mediated by NF-$\kappa$B transcription factor binding to the *HBD2* proximal promoter [37].

The importance of NF-$\kappa$B binding sites to innate immune signalling should not be underestimated. In a recent study, researchers discovered that ligand-bound VDR instigates expression of the genes for the nucleotide oligomerisation domain protein/caspase recruitment domain-containing protein (NOD2/CARD15), which is an encoding pattern recognition receptor [38]. NOD2 is an intracellular pattern recognition receptor, though structurally distinct from the archetypal TLRs. NOD2 is activated in response to muramyl dipeptide, which is a product of lysosome degradation of bacterial peptidoglycan. *NOD2* expression in epithelial and myeloid cells is vigorously induced by 1,25D through the VDR complex binding to distal high-affinity VDREs. NOD2 signalling also prompts NF-$\kappa$B activity and promotes *HBD2* expression [39]. Inducing NOD2 with 1,25D followed by muramyl dipeptide stimulated *HBD2* expression [38].

## 7. Conclusion and Future Research

Research over the past few years has confirmed that vitamin D has a role that extends beyond bone health and it is important for effective innate immune system responses. A wide range of tissue and cell types has been shown to express enzymes that metabolise vitamin D, which offers a reasonable mechanism for the autocrine, intracrine, and paracrine metabolism of cholecalciferol to the active calcitriol. As the understanding of the two-way interaction between vitamin D in its various forms and the immune system has grown, it has become apparent that vitamin D is fundamental in the innate immune system's response to microbial infections. Immune system dysregulation appears to be linked to vitamin D deficiency or adequacy. It is almost certain that there is more to discover about this interaction. However, it is important to bear in mind that species-specific differences can complicate research findings and many vitamin D-dependent mechanisms observed in humans may not be repeated in many animal models.

To date there is an absence of consensus as to what the recommended target serum level of vitamin D should be. It is also debated as to which vitamin D analogue is most beneficial as a dietary supplement, as different forms offer particular advantages to different immunomodulatory responses. To establish the effect of vitamin D supplements on the pathophysiology of various diseases requires a greater number of clinical trials involving more participants. Such trials may provide information on the effect of vitamin D upon the efficacy of other treatments as well as determining the optimal dosages and form. For now, the current evidence points to vitamin D being a relatively safe nutrient that offers promise in disease prevention and as an adjunctive therapy for immune-homeostasis impairment diseases.

## Competing Interests

The author declares that there are no competing interests regarding the publication of this paper.

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*Research Article*

# Effect of High Dietary Tryptophan on Intestinal Morphology and Tight Junction Protein of Weaned Pig

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Tryptophan (Trp) plays an essential role in pig behavior and growth performances. However, little is known about Trp's effects on tight junction barrier and intestinal health in weaned pigs. In the present study, twenty-four (24) weaned pigs were randomly assigned to one of the three treatments with 8 piglets/treatments. The piglets were fed different amounts of L-tryptophan (L-Trp) as follows: 0.0%, 0.15, and 0.75%, respectively, named zero Trp (ZTS), low Trp (LTS), and high Trp (HTS), respectively. No significant differences were observed in average daily gain (ADG), average daily feed intake (ADFI), and gain: feed (G/F) ratio between the groups. After 21 days of the feeding trial, results showed that dietary Trp significantly increased ($P < 0.05$) crypt depth and significantly decreased ($P < 0.05$) villus height to crypt depth ratio (VH/CD) in the jejunum of pig fed HTS. In addition, pig fed HTS had higher ($P < 0.05$) serum diamine oxidase (DAO) and D-lactate. Furthermore, pig fed HTS significantly decreased mRNA expression of tight junction proteins occludin and ZO-1 but not claudin-1 in the jejunum. The number of intraepithelial lymphocytes and goblet cells were not significantly different ($P > 0.05$) between the groups. Collectively, these data suggest that dietary Trp supplementation at a certain level (0.75%) may negatively affect the small intestinal structure in weaned pig.

## 1. Introduction

The postweaning period represents a delicate transitional phase in pig's life. The numerous stresses to their endocrinology, metabolism, and physiology that piglets experience following weaning are reflected in homeostatic changes to their bodies. The gastrointestinal tract is particularly responsive to stressors. Weaning is known to compromise the digestive, absorptive, and secretory capacity of the small intestine which can cause morphological and histological changes of the small intestine [1–4]. In addition, weaning induces a deleterious effect on intestinal barrier function [5, 6].

Tryptophan has a potential role to facilitate stress adaptation of animals and human through increasing hypothalamic serotonin (5-hydroxytryptamine, 5-HT) level [7, 8]. Several studies have shown that dietary Trp may reduce

stress hormone [7, 9, 10], reduce aggressive behaviors [11], and improve growth performance [8] in weaned pigs. As the precursor of serotonin, tryptophan is also known to play an essential role in the regulating several physiological function, such as motility (in duodenal and ileal), secretion and sensitivity [12], and intestinal permeability [13, 14] in the gastrointestinal tract. However, little is known about the effect of dietary Trp on intestinal epithelial cells growth and health, as well as intestinal epithelial tight junction proteins in weaned piglets [7, 15] (Table 5). This study was designed to evaluate the effects of dietary Trp on intestinal epithelial morphology and mRNA level of tight junction proteins in weaned pig.

## 2. Materiel and Methods

*2.1. Experimental Animals and Diets.* The study was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences and performed according to the Chinese Guidelines for Animal Welfare. This animal experiment was conducted at technology innovation platform for national research institutions located in Hunan province "Experimental Station for Healthy Production of Livestock and Poultry & Environmental Control." Thirty-six healthy piglets of similar body weight (8.26 ± 0.15 kg) (landrace × large white) from different litters were obtained from a local farm and transported to the experimental site of the Institute of Subtropical Agriculture. Piglets were weighed and allocated to individual pens in the same room. The piglets were fed mash feed. Diets were formulated to approximately meet or exceed the nutrient requirements of growing pigs as suggested by NRC 2012 [16]. The isonitrogenous diets were formulated and supplemented with different amounts of L-tryptophan (L-Trp) as follows: 0.0%, 0.15, and 0.75%, respectively, named zero Trp (ZTS), low Trp (LTS), and high Trp (HTS). All of the diets were based on corn and soybean meal. Proteins from whey powder and fishmeal were kept constant. The diets were balanced for the same level of crude protein. The compositions of the diets are shown in Table 1.

The piglets were housed individually in an environmentally controlled nursery with hard-plastic slatted flooring. The piglets were exposed to a 3-day adaptation period before being allocated to one of three dietary treatments. For 21 days, the piglets were given ad lib access to water and their respective diet. Each day, surplus feed and waste were collected and weighed. This was then dried at 100°C for several hours and weighed again to determine the initial dry matter content and calculate the feed intake. Each piglet's body weight was monitored weekly. The feed conversion rate was calculated from the body weight and feed intake data.

*2.2. Sample Collection.* At day 22, after pigs were feed-deprived overnight, approximately 10 mL of blood was collected from jugular vein and serum samples were obtained by centrifugation at 2,000 ×g for 10 min at 4°C. These samples were immediately stored at −80°C for amino acids, D-lactase, and diamine oxidase determination. After blood sample collection, pigs were euthanized by electrical stunning and exsanguination. The gastrointestinal tract was removed and immediately dissected, with the small intestine being divided at the duodenum, jejunum, and ileum. Using sterile instruments, approximately 20 cm of intestinal tissue was removed from the center of each of these sections. Tissue samples were flushed with ice-cold saline to recover mucosa, yielding approximately 5 g from each tissue. The mucosa samples were straightaway frozen in liquid nitrogen and stored at −80°C until RNA was ready to be collected. To determine the morphology of the small intestine mucosa and to evaluate the proliferation of crypt cells, a 2 cm segment from each small intestine section was cut and fixed in 4% formaldehyde.

*2.3. Intestinal Morphology and Crypt Cell Proliferation.* Samples of the duodenum, jejunum, and ileum were fixed in formalin and then embedded in paraffin. Using a microtome, cross sections of the samples were cut to an approximate thickness of 5 $\mu$m and stained with haematoxylin and eosin.

Table 1: Ingredient and chemical composition of experimental diets.

| Item | Dietary Trp supplementation% | | |
|---|---|---|---|
| | ZTS | LTS | HTS |
| *Ingredients*% | | | |
| Corn | 64.61 | 64.96 | 65.37 |
| Soybean meal | 19.50 | 19.00 | 17.30 |
| Whey powder | 4.50 | 4.50 | 4.50 |
| Fish meal | 5.50 | 5.50 | 5.50 |
| Soybean oil | 2.40 | 2.40 | 3.00 |
| Lysine | 0.55 | 0.55 | 0.60 |
| Methionine | 0.18 | 0.18 | 0.20 |
| Threonine | 0.18 | 0.18 | 0.20 |
| Tryptophan | 0.00 | 0.15 | 0.75 |
| DCP | 0.76 | 0.76 | 0.76 |
| Limestone powder | 0.52 | 0.52 | 0.52 |
| Salt | 0.30 | 0.30 | 0.30 |
| [2]Premix | 1.00 | 1.00 | 1.00 |
| Total | 100.00 | 100.00 | 100.00 |
| *Nutrient levels*% | | | |
| DE (MJ/kg) | 14.65 | 14.63 | 14.62 |
| CP | 18.08 | 18.05 | 18.05 |
| Lysine | 1.23 | 1.23 | 1.23 |
| Methionine + cysteine | 0.68 | 0.68 | 0.68 |
| Threonine | 0.73 | 0.73 | 0.73 |
| Tryptophan | 0.15 | 0.30 | 0.90 |
| Leucine | 1.25 | 1.25 | 1.25 |

[1]ZTS: zero Trp supplementation (0% Trp); LTS: low Trp supplementation (0.15% Trp); HTS: high Trp supplementation (0.75% Trp).
[2]The following minerals and vitamins per kilogram were provided in the premix (as-fed basis): Zn (ZnO), 50 mg; Cu ($CuSO_4$), 20 mg; Mn (MnO), 55 mg; Fe ($FeSO_4$), 100 mg; I (KI), 1 mg; Co ($CoSO_4$), 2 mg; Se ($Na_2SeO_3$), 0.3 mg; vitamin A, 8,255 IU; vitamin D3, 2,000 IU; vitamin E, 40 IU; vitamin $B_1$, 2 mg; vitamin $B_2$, 4 mg; pantothenic acid, 15 mg; vitamin $B_6$, 10 mg; vitamin $B_{12}$, 0.05 mg; vitamin PP, 30 mg; folic acid, 2 mg; vitamin $K_3$, 1.5 mg; biotin, 0.2 mg; choline chloride, 800 mg; and vitamin C, 100 mg.

Table 2

| Gene | Accession number | Primer sequence 5′-3′ |
|---|---|---|
| Occludin | NM_001163647.1 | F: TCCTGGGTGTGATGGTGTTC |
| | | R: CGTAGAGTCCAGTCACCGCA |
| Zonula occludens-1 | XM_003353439.2 | F: AAGCCCTAAGTTCAATCACAATCT |
| | | R: ATCAAACTCAGGAGGCGGC |
| Claudin-1 | NM_001244539.1 | F: AGAAGATGCGGATGGCTGTC |
| | | R: CCCAGAAGGCAGAGAGAAGC |

For each section, the density of goblet cells and lymphocytes together with the height of the villus (VH) and depth of crypts (CD) was calculated using computer-assisted microscopy (Nikon, ECLIPSE E200, Tokyo, Japan). To assess VH and CD consistently, measurements were made of the distance between the tip of the villus to the mouth of the crypt (VH) and the crypt mouth to the crypt base (CD). The ratio of VH to CD (VH/CD) was determined.

*2.4. Serum Large Neutral Amino Acids Analysis.* The serum obtained from the blood samples was used for large neutral amino acids determination. Isotope dilution liquid chromatography-tandem mass spectrometry was used to analyze serum amino acids. Analysis was conducted by Beijing Amino Medical Research Co., Ltd., Beijing, China.

*2.5. Serum D-Lactate and Diamine Oxidase Levels in the Plasma.* Serum D-lactate content was measured by a commercial kit (Sino-German Beijing Leadman Biotech Ltd., Beijing, China) and Beckman CX4 Chemistry Analyzer (Beckman Coulter, Brea, CA). The activities of the serum diamine oxidase were determined using kits according to the user's manual (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

*2.6. Relative Quantification of mRNA Expression.* The abundance of occludin, zonula occludens-1 (ZO-1), and mRNA in the ileal mucosa was determined by RT-PCR.

TRIzol reagent (Invitrogen, Carlsbad, CA, US) was used in accordance with the manufacturer's instructions to isolate RNA from the sample of liquid nitrogen-pulverised ileal mucosa. To ensure the integrity of the RNA, it was checked using 1% agarose gel electrophoresis and 10 $\mu$g/mL ethidium bromide stain. The quantity and quality of the RNA were analyzed by UV/Vis spectroscopy using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Germany).

Complementary DNA (cDNA) was synthesised using 5x PrimeScript Buffer 2 and PrimeScript reverse transcriptase Enzyme Mix 1 (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China). To assess gene expression, the ensuing cDNA was diluted and used as a PCR template. The reaction was carried out in 10 $\mu$L of PCR solution (ABI Prism 7700 Sequence Detection System); quantitative PCR (qPCR) analyses were conducted (ABI 7900HT Fast Real-Time PCR System; Applied Biosystems, Carlsbad, CA, USA) using 5 $\mu$L SYBR Green mix, 1 $\mu$L cDNA (diluted ×4), 0.2 $\mu$L each of forward and reverse primers (details available in Table 2), 3.6 $\mu$L $H_2O$, and 0.2 $\mu$L ROX Reference Dye (50x). Samples were exposed to predenaturation of 10 s at 95°C followed by 40 cycles of amplification; each cycle consisted of 5 s at 95°C and 20 s at 60°C. This was followed by a melting curve stage of a heating rate of 0.1°C/$s^{-1}$ and melt temperature of 60°C to 90°C. GADPH was used as the reference gene. The relative expression of mRNA was calculated for the treatment and control groups as a ratio of target genes to GAPDH using $R = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference between $Ct^{(\text{gene of interest})} - Ct^{(GAPDH)}$ of the treatment condition and $Ct^{(\text{gene of interest})} - Ct^{(GAPDH)}$ of the control condition.

*2.7. Statistical Analysis.* All statistical analysis was performed using the Analysis of Variance (ANOVA) with the comparison of means by Duncan's Multiple Comparison Test using IBM SPSS statistics version 22. A $P$ value less than 0.05 was considered to be significant.

## 3. Results

*3.1. Growth Performance.* The growth performance is presented in Table 3. No significant differences ($P > 0.05$) were observed among treatments in daily gain, feed intake, and gain/feed. However, ADG was numerically improved by 8% and 11% during d7–d14 and 16% and 36% during d14–d21, respectively, by low Trp supplementation (LTS) and high Trp supplementation Trp (HTS) compared to zero Trp supplementation (ZTS).

*3.2. Plasma Large Neutral Amino Acids.* Table 4 showed that dietary Trp supplementation significantly increased ($P < 0.05$) Trp and Trp/LNAA (large neutral amino acids) concentration in the plasma from the pig fed LTS and HTS compared with those fed ZTS. The concentration of valine (Val) and isoleucine (Ile) significantly decreased ($P < 0.05$) in plasma from pig fed dietary Trp but Leu (leucine), Tyr (tyrosine), and Phe (phenylalanine) were the same between control and treatment groups.

*3.3. Effect of Tryptophan on Pig Intestinal Morphology.* The morphology of the duodenum, jejunum, and ileum at 21 days of the experiment is shown in Table 4.

The results showed that pig fed HTS significantly increased ($P < 0.05$) CD and decreased VH/CD ($P < 0.05$) in the jejunum compared to ZTS and LTS. No significant differences ($P > 0.05$) were observed for villi height between groups.

Table 3: Effect of dietary tryptophan on pigs growth performance.

| Item | Dietary L-tryptophan | | | | |
|---|---|---|---|---|---|
| | ZTS | LTS | HTS | SEM | *P* value |
| *Initial BW, Kg* | 8.26 | 8.25 | 8.26 | 0.15 | 0.62 |
| *Final BW, Kg* | 14.54 | 14.89 | 15.53 | 0.49 | 0.46 |
| *ADG, g* | | | | | |
| d0– d7 | 321.43 | 298.21 | 285.71 | 15.36 | 0.40 |
| d7– d14 | 244.64 | 264.29 | 273.81 | 22.00 | 0.63 |
| d14–d21 | 330.36 | 385.71 | 450.00 | 28.46 | 0.12 |
| d1–d21 | 298.81 | 316.07 | 336.51 | 18.61 | 0.46 |
| *AFI, g* | | | | | |
| d0–d7 | 464.29 | 419.64 | 405.36 | 00.00 | 00.00 |
| d7–d14 | 496.43 | 500.00 | 497.62 | 25.59 | 0.96 |
| d14–d21 | 671.43 | 703.57 | 726.19 | 31.25 | 0.53 |
| d1–d21 | 544.05 | 541.07 | 562.36 | 18.16 | 0.67 |
| *Gain: feed* | | | | | |
| d0–d7 | 0.69 | 0.71 | 0.62 | 0.04 | 0.33 |
| d7–d14 | 0.49 | 0.54 | 0.53 | 0.02 | 0.48 |
| d14–d21 | 0.49 | 0.52 | 0.62 | 0.03 | 0.16 |
| d1–d21 | 0.54 | 0.58 | 0.59 | 0.02 | 0.55 |

ZTS: zero Trp supplementation (0.00% Trp); LTS: low Trp supplementation (0.15% Trp); HTS: high Trp supplementation (0.75% Trp).

Table 4: Large neutral amino acids in the plasma.

| Item | Dietary L-tryptophan (%) | | | | |
|---|---|---|---|---|---|
| | ZTS | LTS | HTS | SEM | *P* value |
| Trp | 2.16[b] | 5.48[a] | 7.22[a] | 0.59 | 0.006 |
| Val | 17.06[a] | 10.67[b] | 11.13[b] | 0.90 | 0.006 |
| Ile | 10.91[a] | 8.29[b] | 7.37[b] | 0.54 | 0.02 |
| Leu | 18.12 | 16.16 | 16.14 | 0.60 | 0.33 |
| Tyr | 6.77 | 7.16 | 5.93 | 0.44 | 0.54 |
| Phe | 15.46 | 14.23 | 16.2 | 0.48 | 0.25 |
| LNAA | 68.33 | 56.50 | 56.76 | 2.52 | 0.08 |
| Trp/LNAA | 3.18[b] | 9.70[a] | 11.12[a] | 0.01 | 0.002 |

[a,b]Values with different letters within the same row are different ($P < 0.05$).
SEM: standard error mean; ZTS = 0.00%; LTS = 0.15%; HTS = 0.75%.

*3.4. Effect of Trp on Intestinal Barrier Function.* The effect of dietary Trp on serum DAO activity and D-lactate content is shown in Table 6. There was no significant increase in serum DAO and D-lactate levels between the control and LTS group. Pig fed HTS had significantly increased DAO and D-lactate level compared to ZTS and LTS.

As shown in Figure 1, HTS significantly decreased mRNA level of occludin and ZO-1 in the jejunum of pig fed HTS compared to LTS and ZTS. mRNA expression of claudin-1 was the same between the groups.

## 4. Discussion

In this study, we found that plasma Trp was significantly increased in the plasma of pigs fed dietary Trp. This could be an indicator for Trp uptake in the small intestine. Similar results were found by Koopmans et al.; they reported that

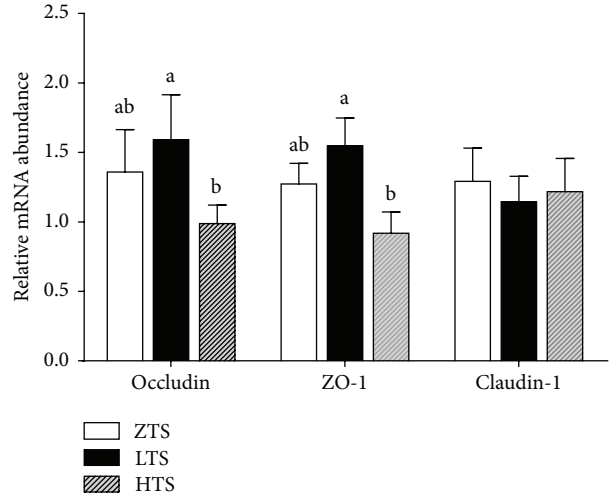


Figure 1: mRNA abundance in the jejunum. [a,b]Values with different letters within the same row are different ($P < 0.05$).

dietary Trp supplementation increased plasma Trp concentration by two times [17]. The result of this study is in accordance with Martínez-Trejo et al. and Li et al. who reported that Trp supplementation affects behavior but did not have any effect on productive performance (FI, DG, and FCR) [18, 19]. The small intestinal histomorphology including villus height, crypt depth, and their ratio is one of important indications of gut health in pigs. A healthy gut has a high villus height to crypt depth ratio. In the present study, pig fed HTS had deeper crypt and lower villus height to crypt depth ratio in the jejunum. These results parallel the findings of Koopmans et al., who suggested that dietary (0.5 g/kg Trp) in nursery pig tends to increase villus height at certain sites along the small intestine [17]. Reduced villus height and increased crypt depth (reduced ratio) lead to increased endogenous secretion and reduced nutrient absorption, disease resistance, and performance [18]. Deep crypts are associated with a high cell turnover. Villi are continually renewed as vulnerable to ordinary sloughing as well as pathogenic assault and pathogen-initiated inflammation [19]. Equally, the high level of activity associated with deeper crypts is costly in energy and nutrients; to obtain adequate nutrients needed for higher mucosal growth may divert energy that is needed for performance. This could be one of the reasons why pig fed HTS had no significant improvement on growth performance in this study.

D-Lactic acid is the final end-product of bacterial fermentation in the gut. Increased plasma D-lactic acid levels reflect changes in intestinal permeability [20]. DAO is an enzyme that serves as an indicator of intestinal epithelial integrity. The intestinal mucosa damaged leads to an increase in serum DAO level [21]. In the present study, HTS increased D-lactate content and DAO activity in the serum of pig fed HTS at d21 after weaning which may indicate impaired intestinal integrity. Tight junction proteins are the principal determinants of epithelial and endothelial paracellular barrier functions [22, 23]. To maintain the integrity of the intestinal barrier, epithelial cells are joined by tight junction proteins,

Table 5: Effect of dietary tryptophan supplementation on intestinal morphology.

| Item | Dietary L-tryptophan (%) | | | | |
|---|---|---|---|---|---|
| | ZTS | LTS | HTS | SEM | *P* value |
| *Duodenum* | | | | | |
| Villus height ($\mu$m) | 384.79 | 386.67 | 428.00 | 9.90 | 0.15 |
| Crypt depth ($\mu$m) | 126.09 | 134.39 | 152.00 | 7.98 | 0.44 |
| VH/CD | 3.09 | 3.08 | 2.95 | 0.14 | 0.90 |
| Goblet cell (unit) | 20.00 | 24.57 | 19.33 | 1.57 | 0.22 |
| Lymphocyte count (unit) | 259.88 | 243.29 | 241.17 | 9.15 | 0.46 |
| *Jejunum* | | | | | |
| Villus height ($\mu$m) | 350.99 | 299.48 | 336.06 | 12.34 | 0.22 |
| Crypt depth ($\mu$m) | 116.20$^{b}$ | 103.29$^{b}$ | 152.44$^{a}$ | 7.80 | 0.02 |
| VH/CD | 3.11$^{a}$ | 2.98$^{a}$ | 2.27$^{b}$ | 0.12 | 0.004 |
| Goblet cell (unit) | 10.75 | 14.75 | 11.13 | 1.04 | 0.142 |
| Lymphocyte count (unit) | 281.13 | 302.13 | 296.38 | 8.20 | 0.34 |
| *Ileum* | | | | | |
| Villus height ($\mu$m) | 266.96 | 309.95 | 300.11 | 8.72 | 0.1 |
| Crypt depth ($\mu$m) | 106.77 | 104.78 | 138.73 | 7.48 | 0.12 |
| VH/CD | 2.701$^{ab}$ | 3.12$^{a}$ | 2.25$^{b}$ | 0.14 | 0.09 |
| Goblet cell (unit) | 23.71 | 20.13 | 17.29 | 2.03 | 0.24 |
| Lymphocyte count (unit) | 257.57 | 304.25 | 299 | 11.07 | 0.1 |

$^{a,b}$Values with different letters within the same row are different ($P < 0.05$).
ZTS: zero Trp supplementation (0% Trp); LTS: low Trp supplementation (0.15% Trp); HTS: high Trp supplementation (0.75% Trp).

Table 6: Plasma DAO and diamine oxidase levels.

| Item | Dietary Trp supplementation | | | | |
|---|---|---|---|---|---|
| | ZTS | LTS | HTS | SEM | *P* value |
| DAO | 144.61$^{b}$ | 138.55$^{b}$ | 176.60$^{a}$ | 1.81 | 0.006 |
| D-lactate | 14.50$^{b}$ | 14.34$^{b}$ | 15.68$^{a}$ | 0.09 | 0.003 |

$^{a,b}$Values with different letters within the same row are different ($P < 0.05$).
ZTS: zero Trp supplementation; LTS: low Trp supplementation; HTS: high Trp supplementation.

such as claudin, occludin, and ZO-1 [24]. By allowing ions, small molecules, nutrients, and water to cross the cell, whilst restricting pathogens, tight junction proteins mediate the epithelial barrier. The results of this study showed that HTS significantly decreased mRNA expression of tight junction proteins ZO-1 and occludin which was consistent with the increased concentration of serum D-lactate and DAO in the plasma. This suggests that high dietary supplementation of Trp may compromise the intestinal barrier integrity in weaned pig. According to Jiang et al., Trp deficiency or excess could cause antioxidant system disruption and change tight junction protein transcription abundances in the fish gill [25]. One of the most important factors in the growth of intestinal microbiota is gut motility [26]. Intestinal motility, together with mucus secretions, provides another epithelium defense mechanism by forcing pathogens and toxins along the gut lumen to be eventually voided. However, increase in intestinal motility and secretion in the small intestine may lead to intestinal permeability [27]. According to Kojima et al., 5-HT, released from damaged enterochromaffin cells resulting from an allergic reaction, increases the strength and rate of intestinal peristalsis and then contributes to diarrhoea [28]. Overall, our results showed that, at moderate supplementation (0.15%), dietary Trp did not affect pig performance. However, at a much higher level (0.75%), dietary Trp could negatively affect intestinal morphology and tight junction proteins in weaned pigs. The change in morphological and tight junction expression in pig fed high dietary Trp (0.75%) supplementation in the present study provides new information regarding the potentials for using Trp in weaned pigs.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

## Acknowledgments

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*Research Article*

# Oregano Essential Oil Improves Intestinal Morphology and Expression of Tight Junction Proteins Associated with Modulation of Selected Intestinal Bacteria and Immune Status in a Pig Model

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Oregano essential oil (OEO) has long been used to improve the health of animals, particularly the health of intestine, which is generally attributed to its antimicrobial and anti-inflammatory effects. However, how OEO acts in the intestine of pig is still unclear. This study was aimed at elucidating how OEO promotes the intestinal barrier integrity in a pig model. Pigs were fed a control diet alone or one supplemented with 25 mg/kg of OEO for 4 weeks. The OEO-treated pigs showed decreased ($P < 0.05$) endotoxin level in serum and increased ($P < 0.05$) villus height and expression of occludin and zonula occludens-1 (ZO-1) in the jejunum. These results demonstrated that the integrity of intestinal barrier was improved by OEO treatment. The OEO-treated pigs had a lower ($P < 0.05$) population of *Escherichia coli* in the jejunum, ileum, and colon than the control. This is in accordance with the greater inactivation ($P < 0.05$) of inflammation, which was reflected by the mitogen-activated protein kinase (MAPK), protein kinase B (Akt), and nuclear factor $\kappa$B (NF-$\kappa$B) signaling pathways and expression of inflammatory cytokines in the jejunum. Our results show that OEO promotes intestinal barrier integrity, probably through modulating intestinal bacteria and immune status in pigs.

## 1. Introduction

The ability of the intestinal epithelium to function as a barrier between the external environment and the closely regulated internal milieu is essential for human and pig health [1, 2]. Increased intestinal permeability is a potential factor of gastrointestinal dysfunction and pathology, including Crohn's disease, multiple organ dysfunction, bacterial translocation, food allergies, and acute pancreatitis [3, 4]. Currently, antibiotics are widely available and have a variety of proposed beneficial effects to promote intestinal health, but the application of these drugs is limited by their toxicity and side effects [5]. Therefore, it is urgent to find alternative treatments with fewer side effects.

Oregano (*Origanum vulgare* L.) is an aromatic plant widely distributed throughout the Mediterranean area and Asia [6]. Oregano essential oil (OEO), a volatile oil, is concentrated from natural plant products which contain the volatile aroma compounds. These mixtures of volatile compounds exert different biological actions, such as antimicrobial, anti-inflammatory, and antioxidative activities [7]. There have been evidences showing the therapeutic effects of OEO supplementation on barrier defects in the gut of mammals, including mouse, rat, and broiler models [8–10]. However, there has been no report about the use of OEO supplementation to improve the intestinal barrier integrity of pigs.

Several studies have indicated that intestinal microbiota and immune status are important factors that influence the function of the intestinal barrier [11]. Alteration of the microbial composition results in increased immune stimulation, epithelial dysfunction, or enhanced mucosal

permeability [12]. In the present study, we hypothesized that dietary OEO supplementation promotes intestinal barrier integrity by regulating intestinal bacteria and inflammation. We thus would use the pig model to test this hypothesis and study the morphology and permeability of the intestine, the composition of the intestinal microbiota, the activation of innate immunity, and the expression of proinflammatory cytokines after OEO supplementation.

## 2. Materials and Methods

All animal handling protocols were approved by the Huazhong Agricultural University Animal Care and Use Committee guidelines.

*2.1. Animals, Diets, and Treatments.* A total of 170 pigs (Large White × Landrace) with an initial body weight (BW) of 72 kg (±4.0 kg) were obtained from the same farm (Wuhan China Pork Co. Ltd., Wuhan, China). The pens were located in a building at a temperature maintained between 15 and 25°C. For the experiment, the 170 pigs were split into two groups according to the diet (each group of 85 pigs was further split into 5 replicate pens each holding 17 pigs): (1) control treatment without supplementation and (2) supplementation of OEO (25 mg/kg of feed for 28 d, as-fed basis). The composition of the control diet is shown in Table 1. The OEO was in the form of a powder called Phytogen (Meritech Bioengineering Co. Ltd., Guangzhou, China). The components of OEO are shown in Supplementary Table S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/5436738). Pigs were allowed feed and water ad libitum over a period of 4 weeks.

*2.2. Sample Collection.* On the day of slaughter, between 9:00 and 14:00, a total of 12 pigs (100 kg BW), with 6 pigs from each dietary treatment, were transported in an open truck to the slaughterhouse. Blood samples were collected after electrical stunning and then quickly separated into five tubes. A 10 mL sample was placed on ice immediately, which was subsequently centrifuged at 1300 ×g at 4°C for 15 min to obtain serum. The serum samples were stored at −80°C for subsequent analysis. The digesta samples were immediately removed from the jejunum, ileum, and colon of each pig and stored at −80°C until further analysis. Samples of the jejunum itself were removed from the middle jejunum segment and then rinsed with ice-cold physiological saline. One section was snap-frozen in liquid nitrogen and then stored at −80°C until further analysis. Other sections of jejunum (3 cm) were kept in 4% neutral buffered formalin for gut morphological analysis.

*2.3. Gut Morphological Analysis.* The digestive tract was removed from the jejunum and fixed in 10% phosphate-buffered formalin. The samples were sectioned at 5 mm thickness and stained with hematoxylin and eosin. Villus height, villus width, and villus crypt depth were measured on the stained sections using a light microscope fitted with an image analyzer (Image Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA). Twenty villi and crypts were measured for each segment.

Table 1: Composition and analysis of the basal diet.

| | Basal diet* |
|---|---|
| *Composition (g/kg)* | |
| Wheat | 380.00 |
| Corn, grains | 464.10 |
| Soybean meal (46%) | 89.00 |
| Monocalcium phosphate | 14.00 |
| Limestone | 7.00 |
| Mycetes adsorbent | 1.50 |
| Antimildew agent | 0.50 |
| Salt | 3.50 |
| Soybean oil | 20.00 |
| Ethoxyquin | 0.25 |
| Probiotics | 0.20 |
| Y402 premix† | 20.00 |
| *Analysis*‡ | |
| Dry matter, DM (%) | 86.80 |
| Metabolism energy (MJ/kg) | 13.20 |
| Crude protein, CP (%) | 13.90 |
| Crude fiber (%) | 2.80 |
| Ash (%) | 3.60 |
| Fat (%) | 4.30 |
| Calcium (%) | 0.60 |
| Phosphorus (%) | 0.60 |

*Control group (C) was fed with the above basal diet, whereas the oregano essential oil (OEO) group consumed the basal diet supplemented with 25 mg/kg OEO.
†Premix contained per kg 10.5 g Fe, 1.4 g Cu, 8.5 g Zn, 4 g Mn, 7.5 mg Se, 30 mg I, 350 kIU of vitamin A, 40 kIU of vitamin D3, 1.5 kIU of vitamin E, 50 mg of vitamin K3, 50 mg of vitamin B1, 150 mg of vitamin B2, 100 mg of vitamin B6, 0.1 mg of vitamin B12, 86.4 g lysine, 17.5 g methionine, 25 g threonine, 4 g phytase, and 15 g choline (kIU: 1000 international units).
‡Metabolism energy was calculated from data provide by Feed Database in China (1999).

*2.4. Measurement of Serum Endotoxin Level.* Serum endotoxin level was measured by a quantitative chromogenic end point tachypleus amebocyte lysate endotoxin detection kit following the manufacturer's instructions (Xiamen TAL Experimental Plant Co., Ltd., China). Briefly, serum samples were diluted to 1 : 10 with water/Tris-HCl buffer. After centrifugation at 1270 ×g for 10 min, the supernatant was removed and incubated with limulus amebocyte lysate at 37°C for 10 min, followed by incubation with the provided chromogenic substance for 6 min. The absorbance at 545 nm was measured after the addition of appropriate reagents.

*2.5. Extraction of Microbial DNA from the Gastrointestinal Tract Digesta.* Total DNA of jejunum, ileum, and colon *digesta* was extracted and purified from gastrointestinal tract digesta using a QIAamp DNA Stool Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry (NanoDrop).

Table 2: Species and genus specific primers used for real-time PCR.

| Gene | Primers (sense/antisense 5′-3′) | Size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| Total bacteria | F: ACTCCTACGGGAGGCAGCAG<br>R: ATTACCGCGGCTGCTGG | 175 | 60 |
| *Lactobacillus* spp. | F: CACCGCTACACATGGAG<br>R: TGGAAGATTCCCTACTGCT | 341 | 58 |
| *Escherichia coli* | F: CATGCCGCGTGTATGAAGAA<br>R: TTTGCTCATTGACGTTACCCG | 96 | 60 |
| *Enterococcus faecalis* | F: CCCTTATTGTTAGTTGCCATCATT<br>R: ACAATGGGAAGTACAACGAGT | 144 | 61 |
| *TNF-α* | F: CACCACGCTCTTCTGCCTACTG<br>R: TTGAGACGATGATCTGAGTCCTTGG | 115 | 63 |
| *MCP-1* | F: GTCCTTGCCCAGCCAGATG<br>R: CGATGGTCTTGAAGATCACTGCT | 148 | 60 |
| *IL-1β* | F: AAAGGGGACTTGAAGAGAG<br>R: CTGCTTGAGAGGTGCTGATGT | 286 | 58 |
| *IL-6* | F: AAGGTGATGCCACCTCAGAC<br>R: TCTGCCAGTACCTCCTTGCT | 151 | 60 |
| *INF-γ* | F: GAGCCAAATTGTCTCCTTCTAC<br>R: CGAAGTCATTCAGTTTCCCAG | 140 | 61 |
| *ZO-1* | F: GGCGCACGGCGAAGGTAATT<br>R: CTATCAAACTCAGGAGGCGGCACT | 405 | 60 |
| *Occludin* | F: GGAGTGATTCGGATTCTGTCTATGCT<br>R: CGCCTGGGCTGTTGGGTTGA | 423 | 60 |
| *β-actin* | F: CCAGGTCATCACCATCGG<br>R: CCGTGTTGGCGTAGAGGT | 158 | 60 |

*2.6. Protein Immunoblot Analysis.* Briefly, 100 mg of frozen tissue of jejunum was homogenized in 1 mL RIPA lysis buffer (with 1 mM PMSF and 10 $\mu$L/mL phosphatase inhibitors). For nuclear p65 measurement, the nuclear fractions were isolated using the Nuclear/Cytosol Fractionation Kit (BestBio, China). Next, they were centrifuged at 12000 ×g at 4°C for 10 min and the supernatants were collected for assay. After the protein concentration was determined by a standard BCA protein assay, protein sample was loaded per lane and separated on SDS-PAGE. The target protein was then electrophoretically transferred to nitrocellulose membranes, which were blocked in TBST (5% nonfat milk, 10 mM Tris, 150 mM NaCl, and 0.05% Tween-20) for 2 h. Next, they were incubated with first antibodies, anti-Phospho-p38 MAPK (1 : 1000, Cell Signaling, USA), anti-Phospho-JNK (1 : 1000, Cell Signaling, USA), anti-Phospho-ERK1/2 (1 : 1000, Cell Signaling, USA), anti-Phospho-Akt (1 : 1000, Cell Signaling, USA), anti-p38 MAPK (1 : 1000, Affbiotech, USA), anti-JNK (1 : 1000, Affbiotech, USA), anti-ERK1/2 (1 : 1000, Affbiotech, USA), anti-Akt (1 : 1000, Affbiotech, USA), anti-ZO-1 (1 : 1000, Affbiotech, USA), anti-occludin (1 : 1000, Affbiotech, USA), anti-NF-$\kappa$B p65 (1 : 1000, Cell Signaling, USA), anti-PCNA (1 : 5000, BD Transduction Laboratories, San Diego, CA), or anti-actin antibodies (1 : 1000, Cell Signaling, USA) at 4°C overnight. After three washes with Tris-buffered saline containing 0.1% Tween-20, blots were incubated with the HRP-conjugated secondary antibodies, anti-rabbit IgG (1 : 15,000, Jackson ImmunoResearch, USA) or anti-mouse IgG (1 : 15,000, Jackson ImmunoResearch, USA), for 2 h and were washed again. Chemiluminescence detection was performed using the ECL reagent (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Specific bands were detected and were analyzed and quantified by Image J Software (NIH, Bethesda, MD, USA).

*2.7. Quantitative PCR.* Total RNA was extracted from samples of jejunum using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The DNA obtained from the intestinal luminal content was used as the template to analyze intestinal bacteria. Primers (Table 2) used in this study were either synthesized according to our previous protocols or designed with Primer 5.0 according to pig gene sequences. Real-time PCR was performed according to our previous study [10]. The relative expression of genes in the treatment group was normalized based on the values of the control group.

*2.8. Statistics.* Statistical analysis was performed using Prism software (Prism 5.0, GraphPad Software, La Jolla, CA). Numbers ($n$) used for statistics are noted in the figures. All data were analyzed by $t$-test procedures of SAS (v 8.2, SAS Inst., Inc., Cary, NC). All the values were presented as means ± standard error of the mean (SEM), and those at $P < 0.05$ were considered significant.

## 3. Results

*3.1. Morphology of the Jejunum.* Morphology is a good indicator of the status of the intestine [13]. As shown in Figure 1, although no significant differences were observed in

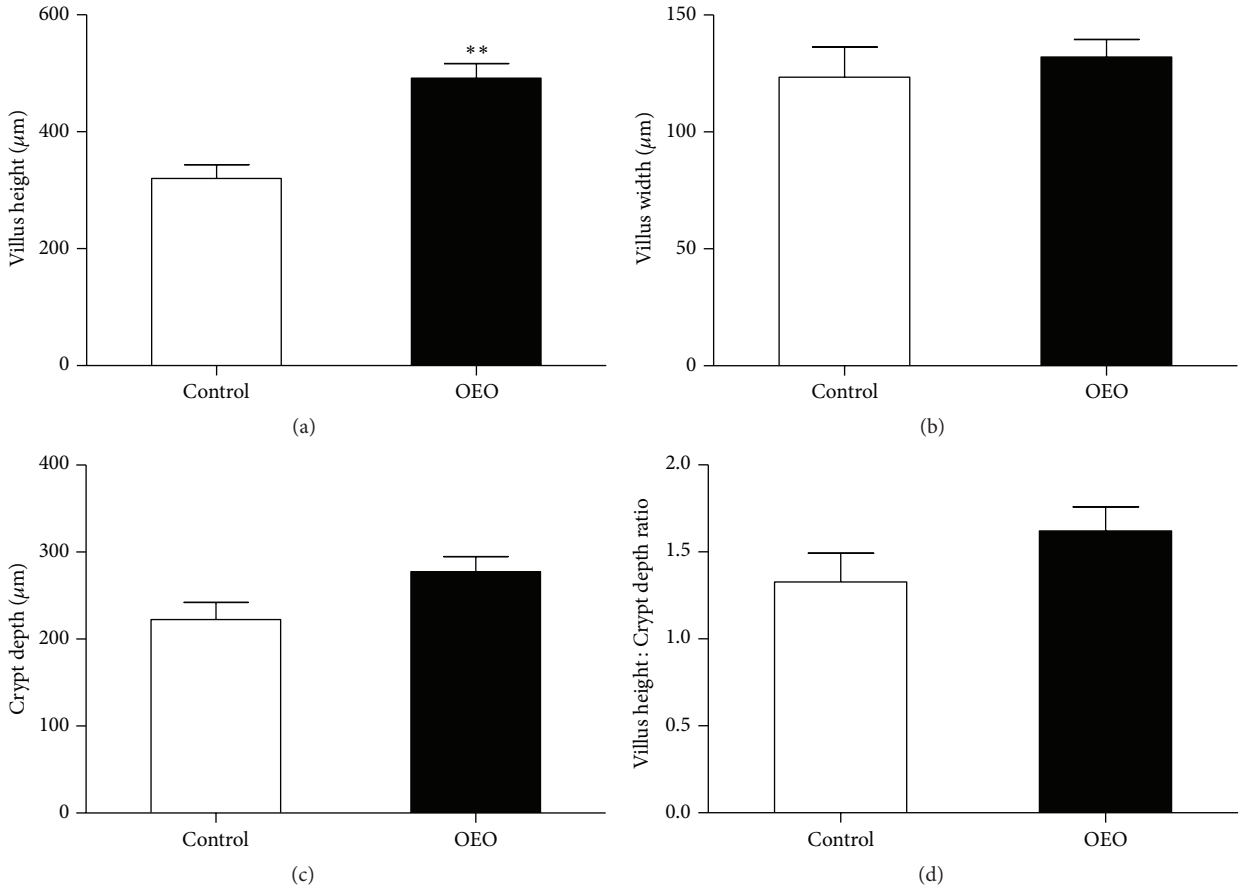


FIGURE 1: Effect of OEO on villus height, villus width, crypt depth, and the villus height : crypt depth ratio in the jejunum of pig. Values are means ± SEM, $n = 6$. $^{**}$Significantly different ($P < 0.01$) from the control group.

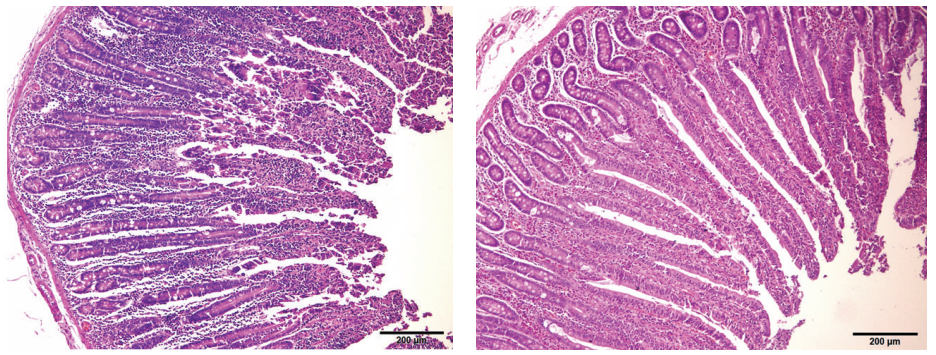


FIGURE 2: Effect of OEO on jejunum morphology in pig. The jejunum was cut off and fixed in 10% formaldehyde-phosphate buffer and then stained with hematoxylin and eosin. Hematoxylin and eosin staining with original magnification ×100. Bars represent 200 $\mu$m.

villus width, crypt depth, and the ratio of villus height to crypt depth between different treatments ($P > 0.05$), the villus height of the OEO-treated pigs was significantly higher than that of the control ($P < 0.05$). As shown in Figure 2, the villi were scattered and seriously desquamated in the jejunum of the control group, while higher and intact villi were observed in the jejunum of OEO-treated pigs.

*3.2. Endotoxin Levels in the Serum.* Endotoxin level is a useful biomarker for evaluating the integrity of the gastrointestinal tract [14]. The effects of OEO supplementation on endotoxin level in the serum of pigs are shown in Figure 3. The OEO-treated pigs showed a significantly lower ($P < 0.05$) concentration of endotoxin in the serum than the control.

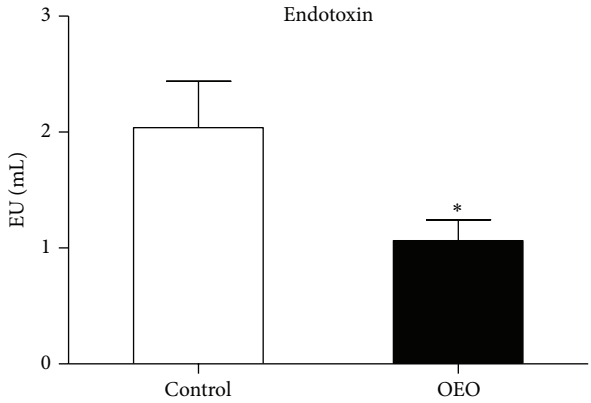


Figure 3: Effect of OEO on endotoxin levels in the serum of pig. Values are means ± SEM, $n = 6$. *Significantly different ($P < 0.05$) from the control group.

*3.3. The Expression of Barrier Tight Junction Proteins in the Jejunum.* To determine the effects of OEO supplementation on the intestinal mucosal tight junction, the expression of occludin and zonula occludens-1 (ZO-1) was measured at mRNA and protein level. As shown in Figure 4, the mRNA levels of *occludin* and *ZO-1* were significantly higher in the OEO-treated pigs than in the control group ($P < 0.01$). Similarly, OEO supplementation also increased ($P < 0.05$) the abundance of occludin and ZO-1 protein in the jejunum.

*3.4. Major Microbiota in Different Regions of the Intestinal Tract.* The effects of OEO supplementation on selected microbial populations in different intestinal tracts are shown in Figure 5. Although there were no significant differences ($P > 0.05$) in the total bacteria, *Lactobacillus* and *Enterococcus* spp. populations, the OEO-treated pigs had a significantly lower population of *Escherichia coli* (*E. coli*) in the jejunum ($P < 0.05$), ileum ($P < 0.05$), and colon ($P < 0.01$) compared with the control.

*3.5. MAPKs, Akt, and NF-κB Pathways in the Jejunum.* To investigate the mechanism responsible for the protective effect of OEO against inflammation in the jejunum of pigs, we further studied the underlying signaling pathways: the MAPKs, Akt, and NF-κB pathways, which are induced by intestinal microorganisms and have been shown to be implicated in the induction of proinflammatory genes [15]. As shown in Figure 6(a), in the jejunum, OEO supplementation inhibited ($P < 0.05$) the activation of JNK, ERK1/2, and Akt due to lower abundance of phosphorylated JNK, ERK1/2, and Akt proteins compared with the control group. Similarly, OEO supplementation decreased ($P < 0.05$) the abundance of NF-κB p65 protein in the nucleus of the jejunum (Figure 6(b)). In contrast, OEO supplementation had no effect on phosphorylated p38.

*3.6. The mRNA Levels of Proinflammatory Cytokines in the Jejunum.* We examined the gene expression levels of four major inflammatory cytokines involved in mucosal inflammation in the jejunum tissues: interleukin-1$\beta$ (IL-1$\beta$), interleukin-6 (IL-6), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), monocyte chemotactic protein-1 (MCP-1), and interferon gamma (INF-$\gamma$). The results are presented in Figure 7. The OEO-treated pigs showed significantly decreased ($P < 0.05$) levels of *TNF-α*, *IL-1β*, *IL-6*, *MCP-1*, and *INF-γ* compared with the control group.

## 4. Discussion

Defects in the intestinal barrier of animals can be induced by several types of ongoing environmental/life factors linked to physiology, psychology, antigens, and toxins [16]. Intestinal barrier defects are a serious problem in human as well as in pigs [17, 18]. Various aromatic plants and their products have been reported to have beneficial effects for the intestine of animals [19]. Furthermore, in a previous study of our laboratory, it was also observed that the intestinal barrier could be improved by OEO supplementation in rat models [10]. However, to our knowledge, there has been no report about the effect of OEO on the intestinal barrier of finishing pigs. Therefore, in the present study, we investigated whether dietary supplementation of OEO could have a protective effect on the intestinal barrier and whether OEO could be used as a feed additive for pigs.

Intestinal mucosal permeability is directly related to the integrity of the intestinal barrier [20]. The function of the intestinal barrier can be commonly assessed by many indexes, such as serum endotoxin level, intestine morphology, and intestinal tight junction proteins [14, 21, 22]. In the present study, the height of villi in the jejunum of pigs was increased after treatment with OEO, indicating that OEO may protect the intestine against villous atrophy and epithelial cell necrosis. Consistently, the endotoxin level in pig serum decreased significantly after treatment with OEO. These results were in agreement with previous findings, which demonstrated that using an OEO supplemented diet improves the ileum villus height and decreases serum endotoxin level in broilers [23]. In addition, we found that the expression of occludin and ZO-1, the two major tight junction proteins in epithelia affecting the organization and the stability of the tight junction [21], was significantly decreased by OEO treatment. Similarly, Placha et al. [9] also observed that broilers fed with OEO had improved intestinal barrier integrity. Our results indicate that OEO supplementation can be a promising approach for protecting the intestinal barrier in pigs.

The intestinal microbiota plays critical roles in the maintenance of mucosal homeostasis [11]. A greater population of *E. coli* might affect the intestinal mucosa because they release toxins, resulting in an intimate interaction between the microbiota and the host enterocytes [24, 25]. The abundance and composition of intestinal bacteria can be easily affected by various dietary factors [26]. In the present study, the dietary consumption of OEO decreased the populations of *E. coli* in the jejunum, ileum, and colon. These results are consistent with those of Tan et al. [27] and Sun et al. [23], who reported that that population of *E. coli* in intestinal is decreased in OEO-treated pigs and broilers, respectively. Thymol and carvacrol, the main active components of

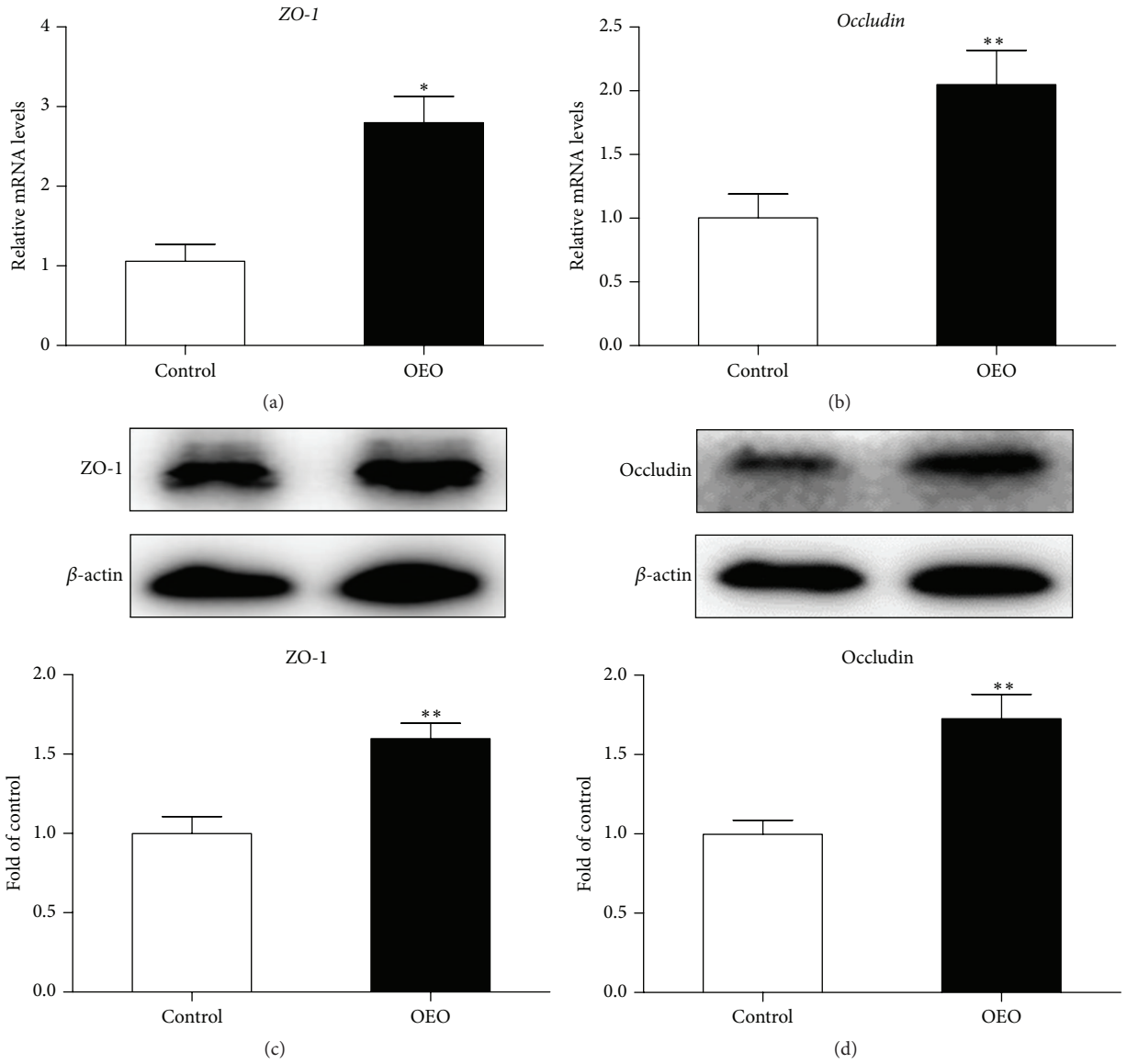


Figure 4: Effect of OEO on the ZO-1 and occludin levels in the jejunum of pig. (a) *ZO-1* mRNA level, (b) *occludin* mRNA level, (c) ZO-1 protein level, and (d) occludin protein level. Expression of the selected genes was quantified by quantitative reverse transcription-PCR. Equal loading was assessed by $\beta$-actin immunoblotting. Values are means ± SEM, $n = 6$. $^{*}$Significantly different ($P < 0.05$) from the control group. $^{**}$Significantly different ($P < 0.01$) from the control group. ZO-1, zonula occludens-1.

essential oil derived from thyme and oregano [7], were also documented to inhibit the proliferation of *E. coli in vitro* [28].

Recently, it has been recognized that the gut microbiota can influence the immune function beyond the gut [26]. The relationship between the immune system and the commensal flora is a precarious one, and perturbations in immune or epithelial homeostasis can lead to gut inflammation [29]. The initial sensing between bacteria and the host occurs through pattern recognition receptors [30]. In the intestine, the activated pattern recognition receptors trigger intestinal immune responses through various downstream signal transductions, such as MAPKs (e.g., p38 MAPK, ERK1/2, and JNK), Akt, and NF-$\kappa$B [31, 32]. The Akt and MAPKs pathways modulate intestinal innate immunity through regulating the phosphorylation of inhibitory $\kappa$B kinases to activate the NF-$\kappa$B pathway [33]. The NF-$\kappa$B pathway, which is activated by intestinal microbes, plays an important role in activating host proinflammatory responses [34]. The activation of these pathways is associated with the increased expression of TNF-$\alpha$, IL-1$\beta$, IL-6, MCP-1, and INF-$\gamma$ [35, 36]. The innate immune system is activated as a defense mechanism and is generally beneficial [37]. However, if the inflammation is uncontrolled, the migration of innate immune cells such as neutrophils, macrophages, and dendritic cells into the target mucosal tissues will result in mucosal injury [32]. Regulating intestinal inflammation is of great significance for intestinal health [15, 38, 39]. OEO has been found to possess a significant anti-inflammatory effect *in vitro* and *in vivo* [8, 40]. The present

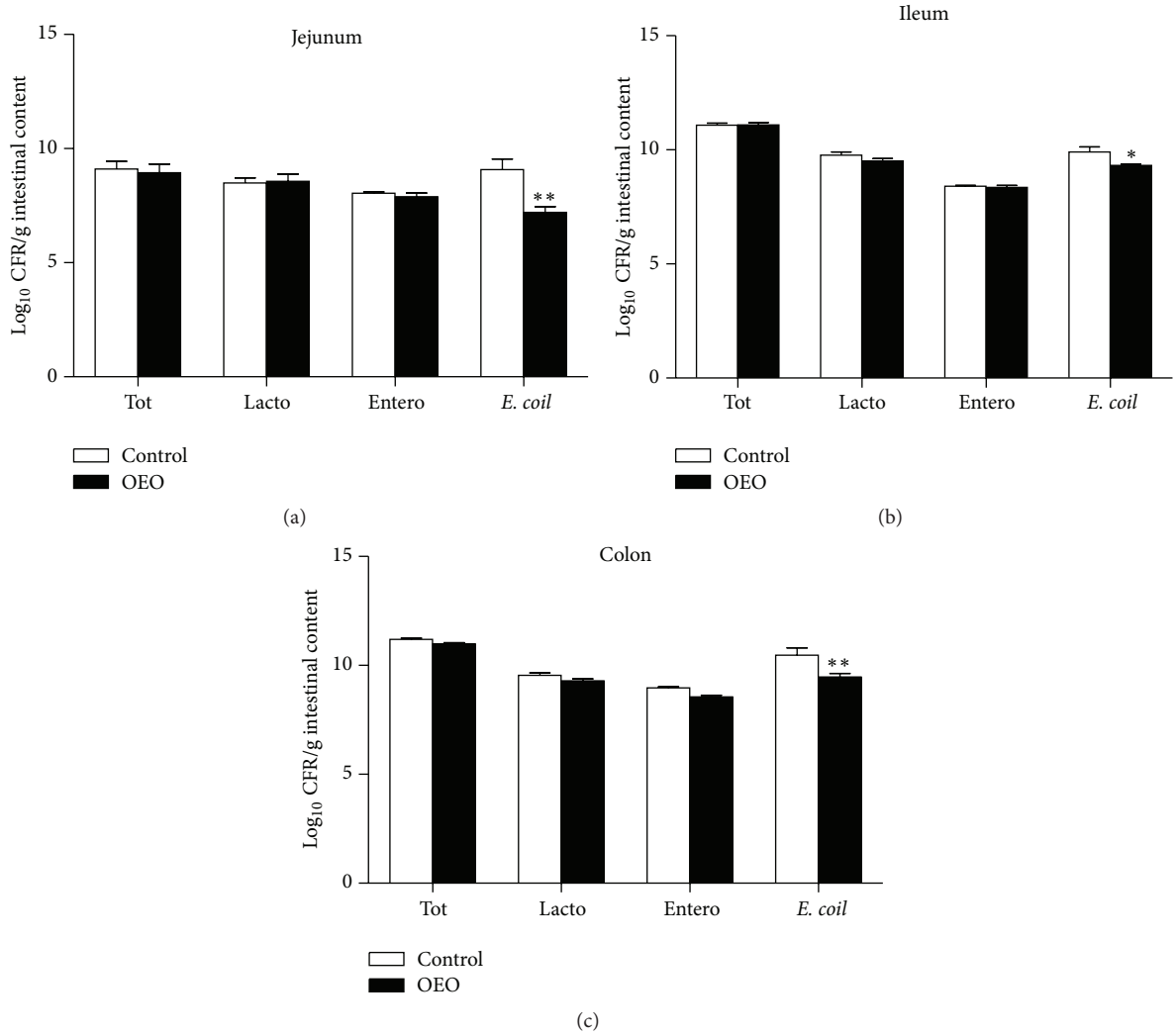


Figure 5: Effect of OEO on selected microbial populations in the jejunum, ileum, and colon tract of pig. Values are means ± SEM, $n = 6$. *Significantly different ($P < 0.05$) from the control group. **Significantly different ($P < 0.01$) from the control group. Tot, total bacteria; Lacto, *Lactobacillus* spp.; Entero, *Enterococcus faecalis*; *E. coli*, *Escherichia coli*; $\text{Log}_{10}$, 16S rRNA gene copies/g contents.

study indicates that OEO inhibits the inflammation in the intestine and downregulates the expression of *TNF-α*, *IL-1β*, *IL-6*, *INF-γ,* and *MCP-1* in the jejunum. Similarly, it has been previously shown that OEO effectively reduces the production of proinflammatory cytokine and thereby attenuates the TNBS-induced colitis in mice [41]. As the major component of OEO, carvacrol was also reported to probably influence the release and/or synthesis of inflammatory mediators [41]. As the mechanisms mediating the suppressive effects of OEO on inflammation are still unclear, we can only speculate that there might be several potential modes of action based on our results. One possibility could be the influence of OEO on NF-κB p65 and phosphorylated MAPKs (ERK1/2 and JNK) and Akt pathways, which can activate the expression of the genes involved in immune and inflammatory responses, such as *TNF-α*, *IL-1β*, *IL-6*, *INF-γ,* and *MCP-1*. On the other hand, intestinal microbial disorder (such as increased number of *E. coli*) is a potent inducer of intestinal inflammation. The present study has shown that OEO can inhibit intestinal *E. coli* in pigs.

## 5. Conclusion

In conclusion, our results indicate that dietary administration of OEO can reduce the production of proinflammatory cytokines and promote the integrity of the intestinal barrier in pigs. The protective effect of OEO on the intestine is associated with the decrease of intestinal *E. coli* population and the inactivation of the JNK, ERK1/2, Akt, and NF-κB signaling pathways. These results will contribute to a better understanding of the possible mechanisms by which OEO promotes the integrity of the intestinal barrier in pigs.

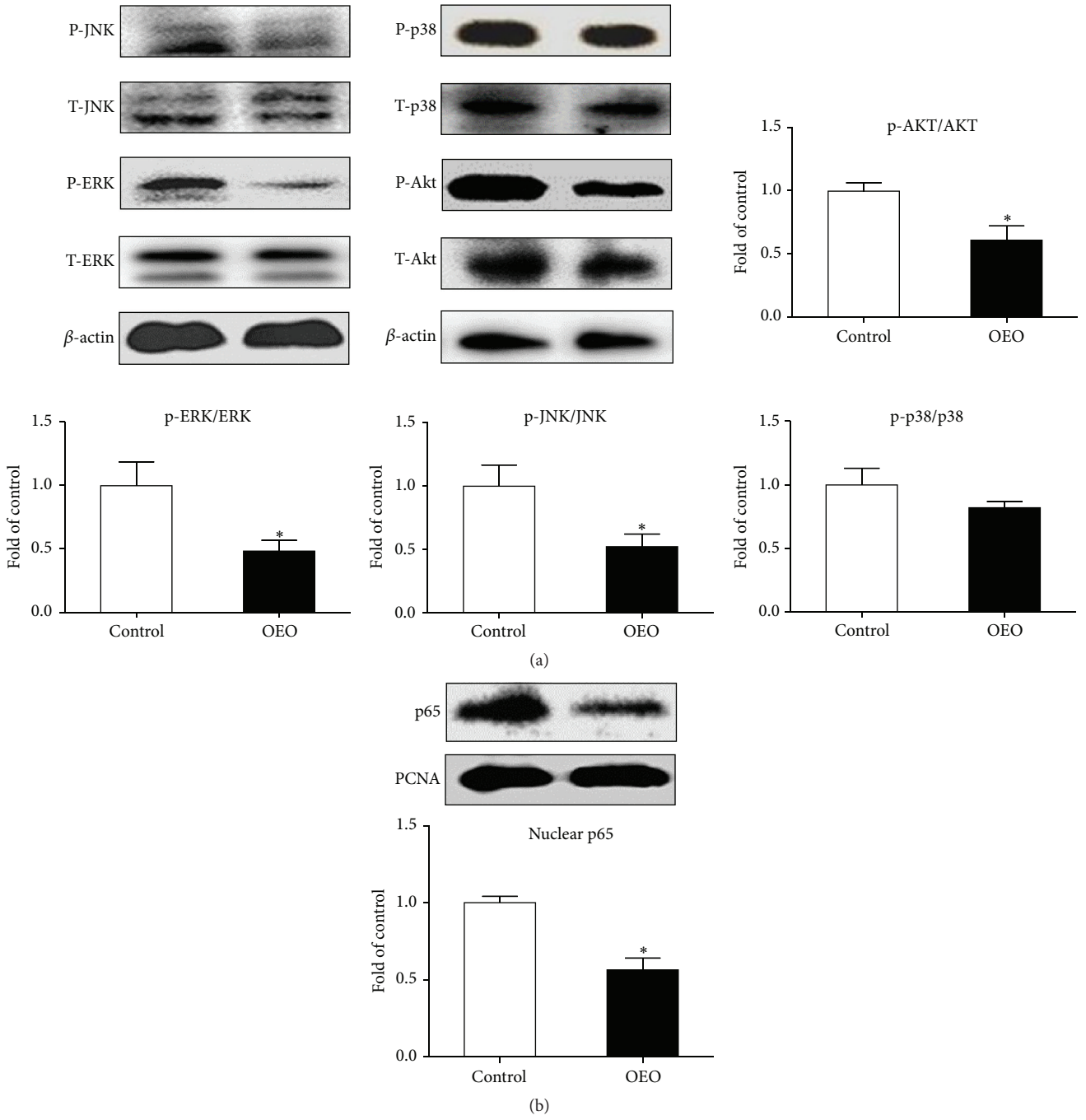


Figure 6: Effect of OEO on the levels of p-Akt, p-ERK, p-p38, p-JNK, and NF-$\kappa$B p65 proteins in the jejunum of pig. (a) Akt protein, ERK protein, p38 protein, and JNK protein levels. Equal loading was assessed by $\beta$-actin immunoblotting. (b) Nuclear p65 protein level. Equal loading was assessed by PCNA immunoblotting. Values are means ± SEM, $n = 6$. *Significantly different ($P < 0.05$) from the control group. ERK1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun N-terminal protein kinase; Akt, protein kinase B; NF-$\kappa$B, nuclear factor kappa B; P, phosphorylated; T, total.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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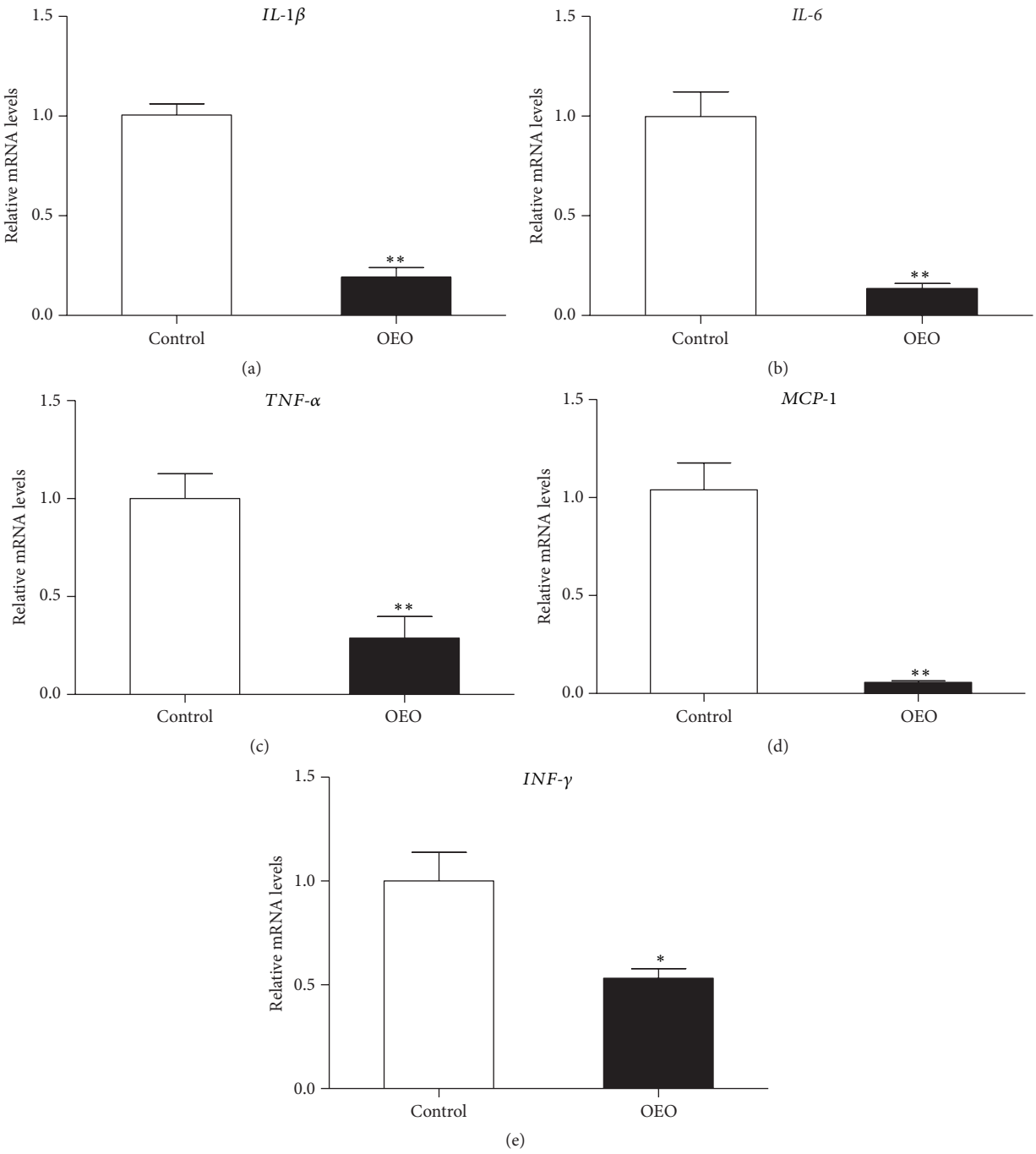


Figure 7: Effect of OEO on the mRNA levels of proinflammatory cytokines in the jejunum of pig. Expression of the selected genes was quantified by quantitative reverse transcription-PCR. (a) *IL-1β* mRNA level, (b) *IL-6* mRNA level, (c) *TNF-α* mRNA level, (d) *MCP-1* mRNA level, and (e) *INF-γ* mRNA level. Values are means ± SEM, $n = 6$. $^{*}$Significantly different ($P < 0.05$) from the control group. $^{**}$Significantly different ($P < 0.01$) from the control group. IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic protein-1; INF-γ, interferon gamma.

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