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L-Theanine affects intestinal mucosal immunity by regulating short-chain fatty acid metabolism under dietary fiber feeding

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Abstract: To investigate the effects of L-Theanine (LTA) on intestinal mucosal immunity and the regulation of short-chain fatty acid (SCFA) metabolism under dietary fiber feeding, a 28-day feeding experiment was performed in Sprague-Dawley rats. The results show that LTA increased the proportion of *Prevotella, Lachnospira,* and *Ruminococcus* while increasing the total SCFA, acetic acid, propionic acid, and butyric acid contents in the feces. LTA also increased IgA, IgE, and IgG levels in the ileum, and increased villi height and crypt depth. Moreover, LTA upregulated the mRNA and protein expression of acetyl-CoA carboxylase 1, sterol element-binding protein 1c, fatty acid synthase, and 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver, while downregulating the expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 1 in the colon. Our study suggests that LTA can affect intestinal mucosal immunity by regulating SCFA metabolism under dietary fiber feeding.

Introduction

With increasing global living standards, high-fat and high-protein diets, which can cause metabolic disorders or metabolic syndrome, have become more common.[1] Meanwhile, dietary fiber intake is an important factor in human health that functions to

prevent cardiovascular and cerebrovascular diseases, such as hyperlipidemia, hyperglycemia, and atherosclerosis.[2] Due to the difficulty associated with digesting and absorbing fiber in the small intestine, it can be fermented by gut microbiota, such as *Prevotella, Lachnospira,* and *Ruminococcus*, as well as other microbiota in the colon, to produce short-chain fatty acids (SCFAs).[3, 4]

SCFAs are volatile fatty acids that primarily include acetic acid, propionic acid, and butyric acid.[5] Acetic acid is absorbed by colonic epithelial cells and is transported to the liver as a metabolic substrate involved in the synthesis of cholesterol (TC).[6] Acetic acid reacts with acetyl-CoA carboxylase 1 (ACC1) to form acetyl-CoA (CoA), and participates in the synthesis of fatty acids through the catalysis of fatty acid synthase (FASN).[7] Fatty acids then synthesize triglycerides (TG) via the catalysis of mitochondrial glycerol-3-phosphate acyltransferase and diacylglycerol acyltransferase for storage in lipid droplets or processing into low-density lipoproteins.[8] This process is regulated by sterol element-binding protein 1c (SREBP-1C), sterol element-binding protein 2 (SREBP-2), and low-density lipoprotein receptor (LDLR).[9] Moreover, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is a key rate-limiting enzyme in the first steps of TC synthesis from acetyl-CoA, which regulates the process of mevalonate synthesis from acetyl-CoA.[10] Alternatively, propionic acid is absorbed by the colonic epithelium and is formed by the action of propionyl-CoA carboxylase and methylmalonyl-CoA isomerase to produce succinyl-CoA. Succinyl-CoA enters the tricarboxylic acid cycle to form oxaloacetate, which participates in gluconeogenesis.[11] Propionic acid can also inhibit cholesterol synthesis by regulating the expression of HMGCR.[12] Meanwhile, oxaloacetate forms phosphoenolpyruvate under the catalysis of phosphoenolpyruvate carboxylase, which reacts to produce glucose-6-phosphate.[13] Glucose-6-phosphate is hydrolyzed by glucose-6-phosphatase (G6PC) to form glucose.[14] Glucose can enter the liver under the action of glucose transporter 2 (GLUT2) and can be phosphorylated by glucokinase to produce glucose 6-phosphate, which enters the glycolysis or glycogen synthesis pathways. Phosphofructokinase (PFK) and glycogen synthase kinase 3 (GSK-3β) are key enzymes regulating glycolysis and glycogen synthesis.[15] Finally, butyric acid and propionic acid participate in gluconeogenesis after being absorbed by colonic epithelial cells in the same form.

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Moreover, butyric acid participates in colonic epithelial energy metabolism.[16]

SCFAs are regarded as mediators of communication between the intestinal microbiome and the immune system. The signals they produce are transferred to various targets, including immune cells via free fatty acid receptors, which belong to the family of G protein-coupled receptors.[17] A previous study reported that SCFAs that transmit information between the gut microbiota and the immune system could improve intestinal immunity.[18] Moreover, the intestinal mucosal epithelium can enable coagulation and adherence of bacteria by regulating immunoglobulin A (IgA) and immunoglobulin G (IgG) levels.[19, 20] This prevents bacteria from contacting the surface of intestinal epithelial cells and invading the host.[21] SCFA supplementation can promote the secretion of IgA and IgG in the intestinal mucosal. Hence, SCFAs can simultaneously provide energy to colonic epithelial cells, promote intestinal development, improve the mucosal barrier, maintain the integrity of the intestinal mucosal barrier, and protect the intestinal morphology.[22] Indeed, SCFA metabolism can also improve intestinal mucosal immunity. ACC1 is crucial in SCFA metabolism. Meanwhile, a previous study has shown that T-cell-specific targeting of the ACC1, a major checkpoint controlling fatty acid synthesis, impaired intestinal T helper type 1 and T helper type 17 responses by limiting CD4+ T-cell expansion and infiltration into the lamina propria in murine models of colitis and infection-associated intestinal inflammation.[23] It suggests that ACC1 is an important protein for the metabolic immune modulation of T-cell-driven intestinal inflammatory responses.

As a new food ingredient, L-theanine (LTA), a characteristic non-protein amino acid, has demonstrated favorable effects for improving intestinal mucosal immunity. In fact, LTA has been shown to improve the intestinal structure of ducks by elevating jejunum villus height and villus height to crypt depth ratio, as well as improving non-specific immunity in the intestinal mucosal.[24] Meanwhile, LTA, which is metabolized to glutamic acid, may act as a glutamic acid donor *in vivo*, thereby increasing the synthesis of glutathione and enhancing IgG levels.[25] Further, ethylamine, another metabolite of LTA, significantly reduces the ability of gluten proteins to stimulate a T-cell-mediated immune response in the intestines.[26] Additionally, LTA is essential for regulating glycolipid metabolism,[27] which relates to SCFA metabolism. However, the

mechanism by which LTA regulates SCFA metabolism and intestinal mucosal immune functions via SCFA metabolism has not yet been examined. In this study, we, therefore, sought to examine the effects of LTA on intestinal mucosal immunity by regulating SCFA metabolism under dietary fiber feeding in rats. The results from this investigation provide a basis for improving the intestinal mucosal immune function, promoting the use of diversified food nutrition intervention, and supporting the utilization of LTA in functional food.

Experimental

Chemicals and reagents

Resistant starch II (Brand name: Fibersol-2) was purchased from Shanghai Youshan Biotech Co., Ltd. (Shanghai, China). LTA (purity ≥ 98%) was purchased from Hunan Sunfull Biotech Co., Ltd. (Changsha, China). LTA is extracted from an aqueous solution of green tea via flocculation and precipitation to remove protein. Extra tea pigment is then adsorbed by macroporous resin and purified by cation exchange resin 732 to obtain high purity LTA.

Kits for assessing IgA, IgE, and IgG, total SCFAs, acetic acid, propionic acid, and butyric acid contents were purchased from Shanghai Zcibio Technology Co., Ltd. (Shanghai, China). Assay kits for evaluating TC, TG, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were purchased from the Institute of Jiancheng Bioengineering (Nanjing, China).

The E.Z.N.A.® total RNA kit (R6688-01) was purchased from Omega Bio-tek, Inc. (Norcross, GA, USA). FastKing gDNA dispelling RT SuperMix (KR118) and SYBR green (FP209) were purchased from Beijing Tiangen Biotech Co., Ltd. (Beijing, China). The total protein extraction radioimmunoprecipitation assay kit and bicinchoninic acid kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies against β-actin (66009-1-Ig), FASN (10624-2-AP), HMGCR (13533-1-AP), GLUT2 (20436-1-AP), PCK1 (16754-1-AP), and PFKM (55028-1-AP) were obtained from Proteintech (Rosemont, IL, USA). Antibodies against ACC1 (ab20453), G6PC (ab133964), SGLT1 (ab14686), LDLR (ab52818), SREBP-1c (ab28481), and SREBP-2 (ab28482) were obtained from Abcam (Cambridge, UK).

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Animals and experimental design

All experimental procedures and animal care were performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985). Protocols were approved by the Ethics Committee of Hunan Agriculture University (registry number: 015063506, Changsha, China). Male specific pathogen-free Sprague-Dawley rats (aged 4 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China) and housed at the Tea Institution of Hunan Agricultural University under conditions of controlled temperature (25°C ± 2°C) and humidity (50–70%) and maintained under a 12-h light/dark cycle. All rats had ad libitum access to food and water. They were acclimatized for one week before the commencement of the experiments.

Fifty healthy, male, pathogen-free Sprague-Dawley rats were randomly categorized into five groups according to their body weights (n = 10 per group): normal feeding group (Control A), dietary fiber feeding group (Control B), LTA 500, LTA middle-weight group 300, and LTA 100. The rats in the LTA500, LTA300, and LTA100 groups were administered 500, 300, and 100 mg/kg of LTA, respectively. Rats in the control groups were administered physiological saline. LTA was adjusted to specific concentrations using physiological saline. The administered volume was fixed at 1 mL/100 g body weight. Meanwhile, rats in the Control B, LTA500, LTA300, and LTA100 groups were fed with dietary fiber feed containing 2% resistant starch II, 18% protein (24.7% energy supply ratio), 58% carbohydrates (67.1% energy supply ratio), 4.8% lipids (8.2% energy supply ratio) and 3% minerals. All rats were administered these agents by gavage continuously for 28 days, fasted for 12 h after the last gavage, and then sacrificed by sodium pentobarbital injection. The liver, ileum, colon, and feces were dissected from each rat and stored at -80 °C until use.

Biochemical analysis

Blood was collected from the abdominal aorta, and the serum fraction was separated by centrifugation at 3000 $r \cdot min^{-1}$ for 10 min. The contents of blood glucose, TC, TG, LDL-C, and HDL-C were measured using spectrophotometric kits in

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accordance with the manufacturer's instructions.

The feces were centrifuged at 1000 g·min^{-1} for 20 min, and the supernatant was collected. The total SCFAs, acetic acid, propionic acid, and butyric acid contents were measured using spectrophotometric kits in accordance with the manufacturer's instructions.

Evaluation of intestinal tissue section

The rat duodenum, jejunum, and ileum were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (HE). The intestinal villus height and crypt depth were measured in 100 fields of view for each group and photographed. The entire field of vision was filled to ensure that the background light in each photo was consistent between images. Villus height (mm) and crypt depth (mm) were measured in the images using Image-Pro Plus 6.0 software.

Determination of gut microbiota

The V3-V4 variable region of bacteria 16S rDNA in rat intestinal feces was amplified by polymerase chain reaction (PCR). The primers used for amplification were 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Total DNA from feces was isolated using the FastDNA® spin kit for soil according to the manufacturer's instructions. To quantify gene expression, the extracted DNA was subjected to qRT-PCR in a total reaction volume of 20 μL containing 4 μL FastPfu buffer, 2 μL dNTPs, 0.8 μL of forward and reverse primers, 0.4 μL FastPfu polymerase, 0.2 μL BSA, 10 ng template DNA, and 8.8 μL ddH_2O. Reactions were performed on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The protocol was as follows: denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, and amplification at 72 °C for 45 s. For the melting curve analysis, the temperature was increased from 60 °C to 72 °C. The PCR products were purified using an AxyPrep DNA gel extraction kit, according to the manufacturer's instructions. The fragments were sequenced using the Illumina's Miseq PE300 platform.

Regarding the Trimmomatic software for quality control of the original

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sequencing and FLASH software for splicing, (1) a 50 bp window was set. If the average quality value in the window was less than 20, all sequences at the back of the base from the front of the window were cut off, and after removing the quality control, the length of the sequence was less than 50 bp; (2) according to the overlapping base overlap, the two ends of the sequence were spliced, the maximum mismatch rate between overlaps during splicing was 0.2, and the length had to be greater than 10 bp; (3) according to the beginning and end of the sequence, the barcodes and primers at both ends split the sequence into each sample. The barcodes had to be matched exactly. The primers allowed 2 base mismatches and removed sequences with ambiguous bases.

The UPARSE software (version 7.1) was used to perform OTU clustering of sequences based on 97% similarity and remove single sequences and chimeras in the clustering process, using the RDP classifier to classify and annotate each sequence, compare the Silva database (SSU123), and set the comparison threshold to 70%.

Real-time quantitative PCR

Total RNA from liver and colon tissues were isolated using the E.Z.N.A.® Total RNA kit I according to the manufacturer's instructions. cDNA was synthesized using FastKing gDNA dispelling RT SuperMix according to the manufacturer's instructions and stored at -20 °C until quantitative reverse transcription (qRT)-PCR was performed.

Rat β-actin endogenous reference gene primer and oligonucleotide primers were synthesized by Tsingke Bioengineering Co., Ltd. (Beijing, China). The details of these primers are listed in Table 1. To quantify gene expression, the synthesized cDNA was subjected to qRT-PCR in a total reaction volume of 20 μL according to SYBR green manufacturer's instructions (Beijing Tiangen Biotech Co.). Briefly, the qRT-PCR reaction mix was comprised of 10 μL SYBR green, 2 μL cDNA template, 0.4 μL each of forward and reverse primers, 0.4 μL ROX Reference Dye II (50×), and 6.8 μL sterilized water. Reactions were performed on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The protocol included denaturation at 95 °C for 30 min, followed by 40 cycles at 95 °C for 5 s, and amplification at 60 °C for 15 s. For melting curve analysis, the temperature was increased from 60 °C to 95 °C. β-Actin was used as a housekeeping gene to normalize the transcript levels of target genes.

The threshold cycle (CT) value of each reaction was determined at the end of the qRT-PCR run. The change in transcriptional level of each target gene, normalized to the expression level of β-actin, was calculated using the following formula: relative mRNA level of target gene = 2-△△CT, where ΔΔCT = (CT Target – CT β-actin) treatment – (CT Target – CT β-actin) control.

Western blotting

Total proteins from the liver and colon tissues were isolated using lysis buffer; protein concentration was estimated using a BCA protein assay kit (Beyotime Institute of Biotechnology Inc.), and western blotting was performed as reported by Gong et al.[28]

Statistical analysis

All statistical analyses were performed using the SPSS software (version 24.0; SPSS, Inc., Chicago, IL, USA). Differences among the treated groups were compared by one-way analysis of variance with the least significant difference post hoc test. The results are expressed as the mean ± standard error (SE). $P < 0.05$ was considered to be statistically significant.

Results

Effects of L-theanine on total SCFA, acetic acid, propionic acid, butyric acid, and intestinal gut microbiota content in rat feces under dietary fiber feeding

Total SCFAs, propionic acid, and butyric acid contents were higher in the Control B group compared to the Control A group ($P < 0.05$; Table 2). The values were also significantly higher in the LTA300 and LTA500 groups than in the Control B group ($P < 0.01$). The proportions of *Prevotella* and *Ruminococcus* were higher in the Control B group than in the Control A group ($P < 0.05$), as was the proportion of *Lachnospira* ($P < 0.01$; Table 2). Meanwhile, the proportion of *Ruminococcus* was higher in the LTA100 group ($P < 0.05$) compared to the Control B group, and the proportions of *Prevotella*, *Lachnospira*, and *Ruminococcus* were significantly higher in the LTA300 and LTA500 groups ($P < 0.01$) compared to the Control B group. Alternatively, the proportion of *Helicobacter* was lower in Control B rats compared to Control A ($P < 0.05$), while it was significantly lower in the LTA100, LTA300 and LTA500 groups

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compared to Control B ($P < 0.01$).

Effects of LTA on the expression of key factors involved in acetic acid metabolism in the liver under dietary fiber feeding

The mRNA expression of ACC1, SREBP-1c, SREBP-2, FASN, HMGCR, and LDLR was significantly lower in Control B rats compared to that in Control A rats ($P < 0.01$; Table 3). However, the expression of SREBP-1c and HMGCR was higher in the LTA100 group than that in the Control B group ($P < 0.05$). Similarly, the mRNA expression of LDLR was higher in the LTA300 group than that in the Control B ($P < 0.05$), and that of SREBP-1c, FASN, and HMGCR was significantly higher in the LTA300 group than that in Control B group ($P < 0.01$). Additionally, the mRNA expression of HMGCR was higher in the LTA500 group than that in Control B ($P < 0.05$), and that of ACC1, SREBP-1c, FASN, and LDLR was significantly higher in the LTA500 group than that in Control B ($P < 0.01$).

Similar to the mRNA expression, the expression of ACC1, SREBP-1c, SREBP-2, FASN, HMGCR, and LDLR proteins was significantly lower in Control B than in Control A ($P < 0.05$) (Fig. 1). Meanwhile, the expression of ACC1, LDLR, and SREBP-2 was higher in the LTA100 group than that in Control B ($P < 0.05$). The expression of SREBP-1c and HMGCR proteins was significantly higher in the LTA100 group than that in Control B. The expression of FASN, LDLR, SREBP-1c, and SREBP-2 proteins was higher in the LTA300 group than that in Control B ($P < 0.05$). The expression of ACC1 and HMGCR was significantly higher in the LTA300 group than that in Control B ($P < 0.01$). Finally, the expression of ACC1, SREBP-1c, SREBP-2, FASN, HMGCR, and LDLR proteins was significantly higher in the LTA500 group than that in Control B ($P < 0.01$).

Effects of LTA on the expression of key factors involved in propionate and butyrate acid metabolism in the colon under dietary fiber feeding

The protein and mRNA expression of GLUT2, PCK1, G6PC, PFKM, GSK-3β, and SGLT1 was significantly higher in Control B than that in Control A ($P < 0.01$) (Figure 1, Table 3). Meanwhile, the mRNA expression of GLUT2, PCK1, G6PC,

PFKM, GSK-3β, and SGLT1 was significantly lower in the LTA100 and LTA300 groups than that in Control B ($P < 0.01$). The mRNA expression of GLUT2, PCK1, G6PC, PFKM, and GSK-3β was lower in the LTA500 group than that in Control B ($P < 0.05$), and that of SGLT1 was significantly lower in the LTA500 group than that in Control B ($P < 0.01$).

Additionally, the protein expression of GLUT2, PCK1, G6PC, PFKM, GSK-3β, and SGLT1 was lower in the LTA100 group compared to that in Control B ($P < 0.05$), while, the protein expression of GLUT2, PCK1, G6PC, PFKM, GSK-3β, and SGLT1 was significantly lower in the LTA300 group than that in Control B ($P < 0.01$), and the expression of PCK1, G6PC, PFKM, GSK-3β, and SGLT1 was lower in the LTA500 group than that in Control B ($P < 0.05$).

Effect of LTA on serum glucose, TC, TG, HDL-C, and LDL-C contents under dietary fiber feeding

Blood glucose did not differ between the LTA and control groups (Table 4). However, the LDL-C ($P < 0.05$), TC, and TG ($P < 0.01$) contents were lower in Control B than those in Control A. Moreover, TC and TG were lower in the LTA groups than those in Control B ($P < 0.05$), while HDL-C was higher in the LTA100 and LTA500 groups than that in Control B ($P < 0.05$).

Effect of LTA on ileum immunoglobulin content and intestinal morphology of rats under dietary fiber feeding

IgA, IgE, and IgG contents were higher in Control B rats than in the Control A group ($P < 0.05$) (Table 4). However, IgE ($P < 0.05$), IgA, and IgG ($P < 0.01$) contents were higher in the LTA100 group compared to those in Control B. Similarly, IgA, IgE, and IgG contents were significantly higher in the LTA300 group than those in the Control B group ($P < 0.01$), while only IgA and IgG levels were significantly higher in the LTA500 group compared to those in Control B ($P < 0.01$).

The villi height and villi height/crypt depth (V/C) in the jejunum, as well as the crypt depth in the duodenum and ileum, were higher in Control B rats compared to the Control A group ($P < 0.05$) (Fig. 2, Table 5). Crypt depth in the jejunum and the ileum

villi height were significantly higher in Control B than in Control A ($P < 0.01$). Meanwhile, the villi height, crypt depth, and V/C in the ileum and V/C in the jejunum were higher in the LTA100 group than those in Control B ($P < 0.05$). Similarly, the villi height, crypt depth, and V/C in the ileum, as well as the villi height in the jejunum, were higher in the LTA300 group than those in Control B rats ($P < 0.05$). Finally, the villi height and V/C in the jejunum, as well as the V/C in the ileum were higher in LTA500 rats compared to those in the Control B group ($P < 0.05$)

Discussion

Dietary fiber is the main source of SCFAs, which are produced by gut microbiota fermentation, with *Prevotella*, *Lachnospira*, and *Ruminococcus* serving as the primary microbiota that produces SCFAs. Hence, the type and number of intestinal microbiota members play an important role in SCFA production. A previous study has shown that LTA improves gut microbiota by maintaining the intestinal cell structure and barrier function.[29] Our results further indicate that LTA increases the proportion of *Prevotella*, *Lachnospira*, and *Ruminococcus,* which led to the increased levels of SCFAs. Specifically, increases in acetic acid, propionic acid, and butyric acid were observed. Meanwhile, LTA decreased the proportion of *Helicobacter*, which is harmful to intestinal immunity.[30]

Acetic acid is metabolized in the liver, whereas propionic acid and butyric acid are metabolized in the colon.[31] Acetic acid is used as a raw material to form acetyl-CoA through the action of ACC1. Fatty acids, which form TGs, are synthesized by acetyl-CoA under FASN catalysis; next, TGs are used to synthesize TC under the regulation of SREBP-1C, SREBP-2, and LDLR.[32] In our study, LTA upregulated the expression of ACC1 and FASN at the mRNA and protein levels and promoted the formation of fatty acids. As fatty acid levels increased, SREBP-1C, SREBP-2, and LDLR mRNA and protein expressions were upregulated, ultimately leading to increased TC and TG contents. However, Wan et al. showed that adding dietary fiber to feed can significantly reduce the contents of serum TC and TG.[33] This discrepancy may be attributed to the fact that SCFAs are used as energy sources by the body. Our results are in agreement with those of Tan et al., who suggested that the contents of TC and TG are regulated by acetic acid, which affects the expression of SREBP-1C and ACC1.[34] Interestingly,

Eftychia et al. showed that the normal cholesterol level is 2.84–5.17 mmol/L.[35] Moreover, low TC levels make it difficult for the body to perform physiological activities. After feeding rats with LTA, endogenous SCFA synthesis was greatly increased, which promoted its energy storage and increased the synthesis of TG and TC; this partially agrees with the results of a previous study showing that increasing dietary fiber effectively increases the TC content.[36] Meanwhile, Peng et al. showed that low levels of cholesterol caused upper gastrointestinal bleeding. Then, they further demonstrated that cholesterol not only mediates many important lipid metabolism functions but also maintains normal TC levels, which are essential for numerous physiological functions.[37]

After being absorbed by colonic epithelial cells, propionic acid and butyric acid produce oxaloacetate, which involves a series of enzymes. Oxaloacetate initiates the gluconeogenic pathway to form glucose via reactions catalyzed by PCK1 and G6PC.[38] Glucose can then be transported to various organs for metabolism via the action of GLUT2, followed by glycolysis or glycogen synthesis. In our study, LTA downregulated the expression of G6PC and PCK1 mRNA and protein. Propionic acid and butyric acid can synthesize oxaloacetate or inhibit colon gluconeogenesis. Moreover, glucose can be used in glycogen synthesis and glycolysis via the action of GSK-3β and PFKM.

In our study, LTA increased the levels of TG, TC, HDL-C, and LDL-C, while maintaining normal blood glucose levels by promoting the synthesis of cholesterol through acetic acid metabolism and inhibiting gluconeogenesis through propionic acid and butyric acid metabolism. Cholesterol synthesis and gluconeogenesis are closely related to intestinal mucosal immunity. Wahbi et al. showed that excessive cholesterol synthesis and gluconeogenesis disorders could cause glucose metabolism disorders, while cardiovascular diseases caused by disorders in glucose metabolism are closely related to intestinal mucosal immunity.[39] In these disorders, intestinal mucosal immunity and the gut microbiota structure are destroyed, leading to intestinal immune dysfunction and mucosal inflammation. This allows bacteria metabolites to enter the blood circulation, which can cause systemic inflammation as well as cardiovascular and cerebrovascular diseases, resulting in cholesterol and glucose metabolism disorders.[40]

Therefore, LTA plays an important role in improving intestinal mucosal immunity by maintaining normal TC and blood glucose levels under conditions of dietary fiber feeding.

A previous study revealed that increasing the SCFA concentration can increase IgA and IgG levels.[41] This suggests that SCFAs are an important component of the immune cell membrane. Duan et al. showed that SCFAs are preferentially used by intestinal epithelial cells for energy metabolism and are the primary regulators of cell proliferation and differentiation, thereby providing energy to promote intestinal crypt epithelial cell differentiation and improve the morphology of the duodenum, jejunum, and ileum.[42] Our results showed that LTA significantly increased the contents of IgA, IgE, and IgG, increased the villi height, crypt depth, and V/C in the duodenum, jejunum, and ileum of rats, and improved intestinal morphology. SCFAs can affect intestinal immune function by regulating inflammatory mediator production and signal transduction, thereby playing an important role in maintaining the intestinal mucosal barrier.[43] In this study, LTA can regulate SCFA metabolism; thus, improving intestinal mucosal immunity. However, SCFA metabolism is not the only way to affect intestinal mucosal immunity. Further studies using metabonomics and proteomics are needed to investigate the pathways and mechanisms associated with LTA-induced effects to improve intestinal mucosal immunity by regulating SCFA metabolism.

Conclusions

Our results show that LTA regulates the proportion of SCFA-producing gut microbiota, thereby increasing the total SCFA content as well as that of acetic acid, propionic acid, and butyric acid. Moreover, LTA regulates SCFA metabolism and improves intestinal mucosal immune function by improving cholesterol synthesis in the liver and inhibiting gluconeogenesis in the colon under dietary fiber feeding.

Conflicts of interest

There are no conflicts to declare.

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Abbreviations

LTA, L-theanine; SCFAs, short-chain fatty acids; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FASN, fatty acid synthase; ACC1, acetyl-CoA carboxylase; SREBP-1c, sterol regulatory element-binding protein-1c; SREBP-2, sterol regulatory element-binding protein-2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, low-density lipoprotein receptor; GSK-3β, glycogen synthase kinase 3β; PFKM, phosphofructokinase, muscle type; PCK1, phosphoenolpyruvate carboxykinase 1; G6PC, glucose-6-phosphatase catalytic subunit; SGLT1, sodium glucose transporter 1; GLUT2, glucose transporter-2; IgA, Immunoglobulin A; IgE, Immunoglobulin E; IgG, Immunoglobulin G; V/C, height of villi/crypt depth.

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Genes	Forward Nucleotide sequence primers (5'- 3')	Reverse Nucleotide sequence primers (5'- 3')	Product Size (bp)
β-actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA	165
GLUT2	CACCAGCACATACGACACCAGAC	TGGACACAGACAGAGACCAGAGC	90
PCK1	GCGTGCTGGAGTGGATGTTCG	GTCGATCTCCTCCACCTCCTTCTC	176
G6PC	GAGAAGGCCAAGAGATGGTGTGAG	TTGCGGTACATGCTGGAGTTGAG	131
PFKM	TCGACAATGACTTCTGTGGCACTG	TCTGAGCGGTGGTGGTGATGG	153
GSK-3β	GACAGTGGTGTGGATCAGTTGGTG	GCGATTGCCTCTGGTGGAGTTC	167
SGLT1	TCCCGTATGATCACTGAGTTTGC	GGCAGTTGCTGGGTTCCAT	82
ACC1	CAGCGACCACCTGAAGACACTTG	GCTTCCAGCACCGGCATCAG	84
SREBP-1C	TCTCCTGGAGCGAGCATTGA	CAGTGGTGGTAGCCATGCTG	173
HMGCR	ATGTTGACAAGGCTCTGCTGAAGG	AGCCTGAGCCGTAGGAGAATGC	133
FAS	GTTGCTGCTGCTGTGGACCTC	AGGATCACATTGCCGTGGTACTTG	146
LDLR	ACCTGCCGACCTGACGAA	CCAAGCTGATGCACTCCC	83
SREBP-2	TGGGCGATGAGCTGACTCT	CAAATAGGGAACTCTCCCAC	195

Table 1 Primers of genes for RT-PCR

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Table 2 Effects of L-theanine on the content total SCFAs, acetic acid, propionic acid, butyric acid and intestinal microorganisms in rat feces under dietary fiber feeding（n=10）

Group	Total SCFAs (PG/ml)	Acetic acid (PG/ml)	Propionic acid (PG/ml)	Butyric acid (PG/ml)	*Prevotella* (%)	*Lachnospira* (%)	*Ruminococcus* (%)	*Helicobacter* (%)
Control A	312.17±4.20	150.23±0.56	91.98±1.23	54.36±2.25	35.52	7.17	1.75	4.21
Control B	333.23±4.00*	150.14±1.11	102.33±0.98*	58.45±1.02*	25.98*	13.75**	2.35*	3.22
LTA100	361.54±9.16$^{\#\#}$	166.64±1.56$^{\#}$	117.52±2.11$^{\#}$	65.10±0.81$^{\#}$	42.58$^{\#\#}$	16.37$^{\#\#}$	2.87$^{\#}$	2.95$^{\#}$
LTA300	377.77±5.79$^{\#\#}$	177.54±1.63$^{\#\#}$	129.21±1.44$^{\#}$	69.09±1.11$^{\#\#}$	49.52$^{\#\#}$	18.33$^{\#\#}$	3.56$^{\#\#}$	2.22$^{\#\#}$
LTA500	442.87±5.76$^{\#\#}$	210.14±1.87$^{\#\#}$	141.12±1.36$^{\#\#}$	85.63±2.34$^{\#\#}$	50.14$^{\#\#}$	20.32$^{\#\#}$	4.67$^{\#\#}$	1.76$^{\#\#}$

Note: Compared with Control A, *: $P<0.05$,**: $P<0.01$; Compared with Control B, #: $P<0.05$,##:$P<0.01$； the same as follows

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Table 3 Effects of LTA on expression of key mRNA involved in acetic acid metabolism in liver and propionate and butyrate acid metabolism in colon under dietary fiber feeding in rats（n=10）

Group	ACC1	SREBP-1c	SREBP-2	LDLR	FASN	HMGCR	GLUT2	PCK1	G6PC	PFKM	GSK-3 β	SGLT1
Control A	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00
Control B	0.65 ±0.12**	0.18 ±0.03**	0.18 ±0.02**	0.35 ±0.09**	0.04 ±0.01**	0.18 ±0.01**	1.92 ±0.05**	1.78 ±0.08**	1.95 ±0.03**	2.15 ±0.03**	2.08 ±0.02**	2.23 ±0.03**
LTA 100	0.74 ±0.03	0.27 ±0.03#	0.17 ±0.01	0.36 ±0.11	0.27 ±0.01##	0.55 ±0.14#	1.42 ±0.02##	1.47 ±0.02##	1.48 ±0.03##	1.66 ±0.09##	1.63 ±0.03##	1.51 ±0.01##
LTA 300	0.75 ±0.09	0.40 ±0.06##	0.16 ±0.02	0.62 ±0.03#	0.67 ±0.04##	0.57 ±0.10##	1.31 ±0.03##	1.32 ±0.05##	1.39 ±0.02##	1.49 ±0.01##	1.52 ±0.02##	1.48 ±0.01##
LTA 500	0.92 ±0.08##	0.34 ±0.02##	0.14 ±0.02	0.78 ±0.06##	0.44 ±0.05##	0.44 ±0.07#	1.61 ±0.01#	1.62 ±0.01#	1.76 ±0.03#	1.83 ±0.03#	1.80 ±0.02#	1.62 ±0.03##

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627 Table 4 Effects of L-theanine on immunoglobulin content in ileum and blood glucose, TC, TG,
628 HDL-C, and LDL-C content in serum under dietary fiber feeding in rats（n=10）

Group	IgA (ug/g)	IgE (ug/g)	IgG (mg/g)	Blood glucose (mmol·L^{-1})	TG (mmol·L^{-1})	TC (mmol·L^{-1})	HDL-C (mmol·L^{-1})	LDL-C (mmol·L^{-1})
Control A	2582.97±89.18	165.52±7.88	80.75±3.51	4.68±0.12	0.87±0.06	2.97±0.02	0.34±0.03	0.24±0.02
Control B	3079.97±48.31*	199.95±2.91*	83.33±1.47*	4.46±0.05	0.38±0.10**	2.34±0.03**	0.57±0.05**	0.18±0.02*
LTA100	3471.60±83.81$^{\#\#}$	233.70±13.17$^{\#}$	103.38±4.23$^{\#\#}$	4.36±0.09	0.49±0.13$^{\#}$	2.54±0.05$^{\#}$	0.49±0.06$^{\#}$	0.17±0.01
LTA300	3611.36±64.92$^{\#\#}$	267.59±12.95$^{\#\#}$	115.12±2.07$^{\#\#}$	4.07±0.12	0.51±0.08$^{\#}$	2.59±0.06$^{\#}$	0.51±0.02	0.16±0.02
LTA500	3537.76±48.31$^{\#\#}$	215.49±2.32$^{\#}$	112.44±0.91$^{\#\#}$	4.29±0.08	0.45±0.05$^{\#}$	2.56±0.01$^{\#}$	0.46±0.01$^{\#}$	0.14±0.02

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635 Table 5 Effects of L-theanine on the intestinal morphology of rats under dietary fiber feeding（n=10）
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Group	Duodenum			Jejunum			Ileum		
	height of villi(μm)	crypt depth (μm)	V/C	height of villi(μm)	crypt depth (μm)	V/C	height of villi(μm)	crypt depth (μm)	V/C
Control A	0.35±0.02	0.10±0.01	3.39±0.37	0.33±0.01	0.11±0.01	2.53±0.49	0.48±0.01	0.13±0.00	3.49±0.17
Control B	0.37±0.01	0.12±0.02	3.23±0.26	0.43±0.01**	0.14±0.02	3.19±0.39	0.55±0.01	0.14±0.01	3.92±0.39
LTA100	0.41±0.03	0.13±0.02	3.08±0.44	0.44±0.01	0.15±0.01	4.08±0.76	0.57±0.04	0.16±0.01	3.57±0.52
LTA300	0.43±0.01	0.14±0.01	3.14±0.18	0.49±0.07	0.14±0.01	3.56±0.39	0.60±0.02	0.17±0.00$^{\#}$	3.51±0.03
LTA500	0.53±0.03$^{\#\#}$	0.15±0.01$^{\#}$	3.52±0.27	0.53±0.03$^{\#}$	0.13±0.02	4.19±0.55	0.63±0.03	0.18±0.00$^{\#\#}$	3.58±0.38

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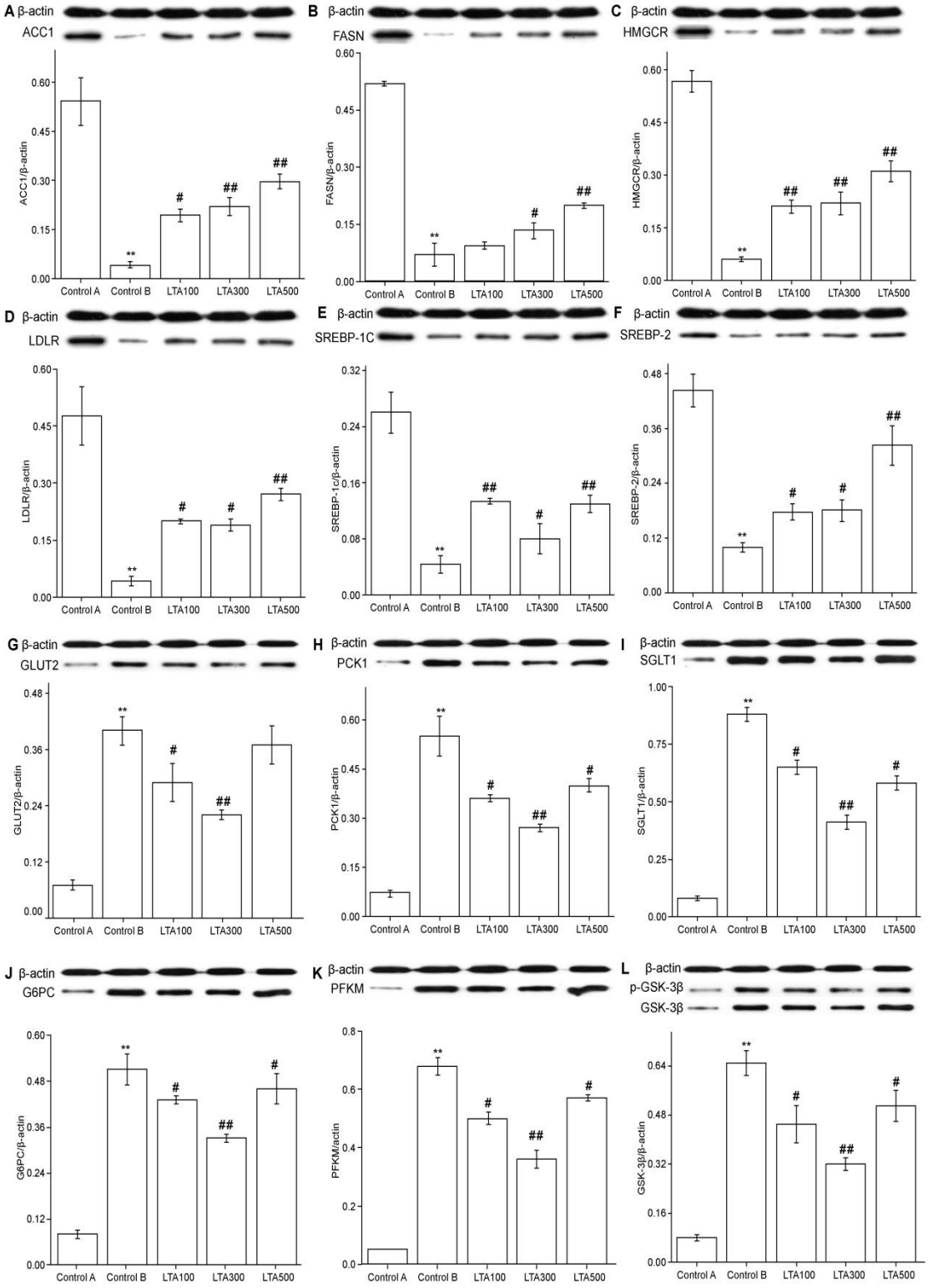

Fig. 1 Effects of LTA on the protein expression of ACC1 (A), FASN (B), HMGCR (C), LDLR (D), SREBP-1C (E) and SREBP-2 (F) in the liver, as well as the effect on the protein expression of GLUT2 (G), PCK1 (H), SGLT1 (I), G6PC (J), PFKM (K) and p-GSK-3β (L) in colon under dietary fiber feeding in rats.

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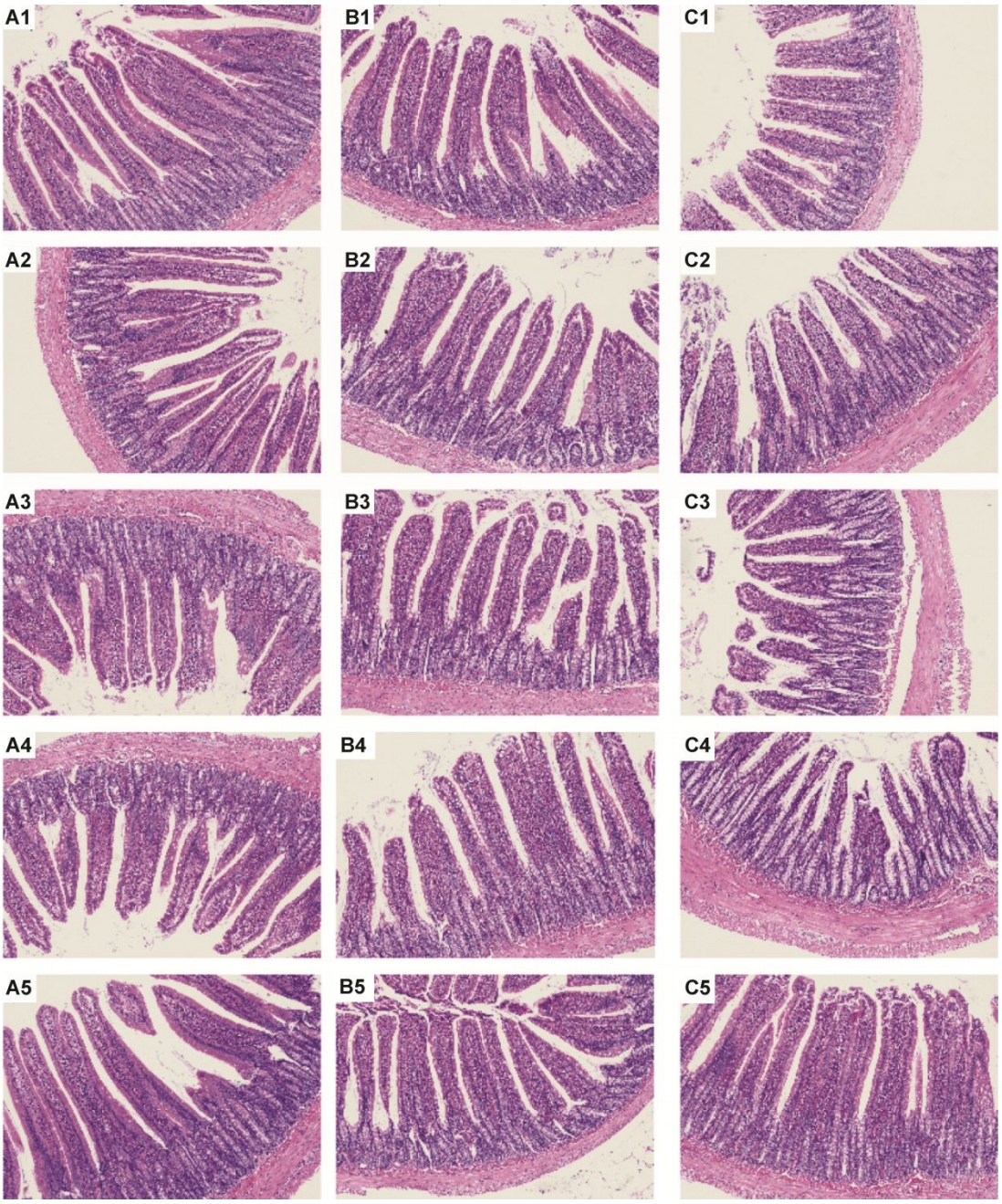

Fig. 2 Effects of LTA on duodenum, jejunum and ileum intestinal morphology sections of rats. A) duodenum; B) jejunum; C) ileum; 1) Control A; 2) Control B; 3) LTA100 ; 4) LTA300; 5) LTA500.

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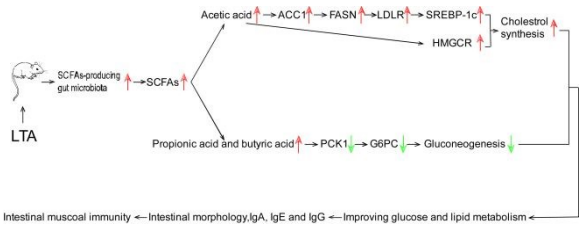

LTA regulates SCFA metabolism and improves intestinal mucosal immunity by improving cholesterol synthesis in liver and inhibiting gluconeogenesis in colon.