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Modulation of *Lactobacillus acidophilus* KLDS 1.0738 on intestinal SCFAs metabolism and GPR41/43 expression in β -lactoglobulin-sensitized mice

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Running title: Remission of *Lactobacillus* on allergy

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Abstract

The present research intends to investigate the correlation between the beneficial modulation of *L. acidophilus* on gut microbiota composition, metabolic activities and reducing the cow's milk protein allergy. Mice sensitized with β -lactoglobulin (β -Lg) were treated with different dose of *L. acidophilus* KLDS 1.0738 for 4 weeks, starting 1 week before allergen induction. The results showed that intake of *L. acidophilus* significantly suppressed the hypersensitivity responses, together with increased faecal microbiota diversity and SCFAs concentration (including propionate, butyrate, isobutyrate and isovalerate) when compared with the allergic group. Moreover, treatment with *L. acidophilus* induced the expression of SCFAs receptors, G protein-coupled receptor 41 and 43 (GPR41/43), in the spleen and colon of the allergic mice. Further analysis revealed that the GPR41 and GPR43 mRNA expression both positively correlated with the serum concentrations of TGF- β and IFN- γ ($P < 0.05$), but negatively correlated with the IL-17, IL-4 and IL-6 in the *L. acidophilus*-treated group compared to the allergic group ($P < 0.05$). These results suggest that *L. acidophilus* protected against the development of allergic inflammation by improving the intestinal flora, as well as up-regulating SCFAs and their receptors GPR41/43.

KEYWORDS

G-protein coupled receptor; gut microbiota; *Lactobacillus acidophilus*;

β -lactoglobulin allergy

1 INTRODUCTION

Cow's milk protein allergy (CMA), as the most common food allergy, often causes gastrointestinal dysfunction and unbalanced immune response to the babies and young children. Accumulating studies suggest that probiotics, act as potential beneficial microorganisms, may help to prevent and treat the development of inflammatory diseases through regulating intestinal microbiota environment and host gut metabolism, in particular short-chain fatty acids (SCFAs) production [1]. Shi *et al.* reported that the protective effects of *Lactobacillus* cocktails on alleviating intestinal inflammation were related to restoring the cefixime-induced gut microbiota and SCFAs production in murine model [2]. Cremon *et al.* observed that the risk of irritable bowel syndrome (IBS) in forty patients could be reduced by *Lactobacillus paracasei* through modulating the gut microbiota structure and increasing the fecal acetate and butyrate levels [3]. In addition, Simonyte *et al.* reviewed that probiotics regulating the gut microbiota-SCFA axis was accounting for inducing the anti-inflammatory response [4]. Canani *et al.* indicated that treatment with *Lactobacillus rhamnosus* GG-supplemented formula

promoted tolerance in infants with CMA by increasing the relative abundance of butyrate-producing bacterial strains and fecal butyrate levels [5]. Therefore, assessing the roles of probiotics on modulation of gut microbiota and SCFAs in CMA allows us to better understand the possible mechanism of controlling food allergy.

In addition to acting as energy sources, SCFAs have recently attracted attention as potential mediators of the effects of gut microbiota on intestinal inflammation [6]. Further evidence revealed that SCFAs were involved in regulating biological response via SCFAs receptor-dependent mechanisms [7]. G-protein-coupled receptor 41 (GPCR41) and GPR43, the primary receptors on the immune cells, were mostly activated by acetate, butyrate and propionate [8]. Maslowski *et al.* reported that stimulation of GPR43 by SCFAs was necessary for the normal resolution of inflammatory responses, because acetate had no beneficial impact on GPR43-deficient mice with DSS-induced colitis [9]. The role of SCFAs also has been studied in a clinical trial, Haines *et al.* demonstrated that intake of soluble fibre containing probiotics improved lung function in asthmatic airways by up-regulating GPR41 and GPR43 sputum gene expression [10]. These observations highlighted that the GPR43 binding of SCFAs potentially provided a molecular link between bacterial metabolites and immune responses. However,

whether the effects of probiotics on preventing from CMA are mediated by the SCFA–GPCRs signal have not been investigated so far.

Our previous experiments found that *L. acidophilus* KLDS 1.0738 effectively reduced hypersensitivity reactions in β -Lg-induced allergic murine model. Accordingly, in this study we focused on evaluating the modulations of *L. acidophilus* KLDS 1.0738 on the fecal microbiota and metabolites of the allergic mice. Further, the potential association of SCFA–GPCRs signal induced by *L. acidophilus* with inhibition of allergic inflammation was explored, which might provide a new sight to interpret the potentially anti-allergic effects of probiotics.

2 MATERIALS AND METHODS

2.1 Bacteria and culture conditions

The *L. acidophilus* KLDS 1.0738 was sustained by the Key Laboratory of Dairy Science of Northeast Agriculture University (Harbin, China). The probiotic strain was cultured in modified MRS (Huankai Microbial, China) until stationary growth phase and collected by freezing centrifugation. Then the bacteria cells were lyophilized and stored at -80°C until used. The number of viable bacteria in powder was approximately 10^{11} CFU/g.

2.2 Animals and experimental design

This study adopted a β -Lg-sensitized murine model based on our previous methods with slight modifications [11]. Briefly, Six week-old female BALB/c mice obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) were randomly divided into four groups (n=15 in each group): (1) Normal mice received sterile PBS alone; (2) Allergic group mice were intraperitoneally injected with 200 μ L 1 mg/mL β -Lg plus Freund's adjuvant on day 7, 14 and 21, respectively. (3) Sodium butyrate group: the β -Lg-sensitized mice were given intraperitoneal injections of 200 μ L sodium butyrate (50 mM) from week 1 to week 4 on a weekly basis; (4) *L. acidophilus* group: 0.096 (low), 0.96 (middle) or 9.6 (high) g/kg *L. acidophilus* KLDS 1.0738 suspended in 200 μ L PBS were oral administrated to allergic mice three times a week, starting 1 week before antigen stimulation and continuing until the time of sacrifice. On the 28 day, all sensitized mice were intragastrically challenged two times with β -Lg (30 mg/mouse) at 30 min apart. Two hours after final challenge, all the mice were sacrificed and tissue samples were collected (Fig. 1). The experiments above were conducted on compliance with animal care guidelines and were approved by the Northeast Agricultural University.

2.3 Evaluation of allergic inflammatory

The concentrations of IgE, β -Lg-specific IgE and cytokines (including IL-17, IL-4, IL-6, TNF- β , TGF- β and IFN- γ) in serum were detected by using ELISA kit (Tiangen, China) in accordance with the instruction of manufacturer.

The lung and colon tissues were embedded in paraffin after immersion-fixed for 24 h in 10% v/v formalin. Subsequently, the paraffin-embedded tissues were cut into slices and stained with hematoxylin and eosin for general examination of morphology.

2.4 Analysis of the bacterial composition of fecal samples

Total DNA was extracted from murine feces samples by using TIANamp Stool DNA Kit (Tiangen, China) following the manufacturer's instructions. The V3 region of the bacterial 16S rRNA gene was amplified using previously described universal primers [12]. PCR reactions were performed for 5 min at 94°C, with 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and then a final step for 10 min at 72°C. After the reaction, 10 μ L PCR products were analyzed on 8% polyacrylamide gels with a 35% to 55% denaturant ingredient (100% denaturant contained 7 M urea and 40% formamide). The electrophoresis was run in 1 $\times$ Tris-acetate-EDTA buffer at 60°C and 150 V for 4 h. The gels were stained with AgNO₃ and scanned by using gel system (BIO-RAD). DGGE bands

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were excised for DNA sequencing, then soaked in 100 μ L TE buffer at 4°C overnight. The DNA in elution was used as template to amplify with the previous primers without the GC-clamp using the same conditions as previously described. The PCR products were sequenced by Sangon Biotech Co., Ltd (Shanghai, China). The resulting sequences were compared with sequences revealed by the BLAST algorithm in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to get the closest known alignment identities.

2.5 Quantification of fecal lactic acid and SCFAs

The feces from mice were collected into sterile tubes, and then SCFAs (acetate, propionate, butyrate, isobutyrate, isovalerate) and lactic were measured using high-performance liquid chromatography (HPLC) according to Dostal *et al.* with some modifications [13]. Fecal samples (200 mg) were suspended in 1.2 mL of PBS and vortexed for 2 min. The suspension was centrifuged at 10000 rpm for 10 min at 4°C to remove the solid material, and the supernatants were collected and filtered through a membrane filter (pore size 0.45 μ m). 10 μ L of sample was injected into HPLC, which was equipped with an Aminex HRX-87H column (300 mm $\times$ 7.8 mm) at a flow rate of 0.6 mL/min at 30°C and 5 mM-H₂SO₄ as the eluent solution. Concentrations of lactic acid and SCFAs were calculated using the internal standard method and expressed as μ mol/g faeces.

2.6 Determination of amino acids

The 50 mg feces were placed in an oven at 60°C for drying and then crushed through an 80 mesh sieve. The 10 mL of 6 mol/L HCl was added to dissolve feces in tubes. The sample was flushed with nitrogen gas and hydrolyzed for 24 hours at 110°C. Afterwards, the 1 mL hydrolyzed samples with depicting was added to 1 mL buffer solution and later filtered with Millipore 0.22 µm syringe filters. Filtrated sample was used to determine 16 amino acids with an amino acid autoanalyzer (Hitachi L-8800, Japan). A mixture of amino acids at known concentrations (Sigma Chemical Co., Milan, Italy) was used as standard.

2.7 RNA isolation and quantification of GPRs genes

Total RNA from colon and spleen samples was extracted using the RNA sample Total RNA kit (Tiangen, China) following the manufacturer's instructions. The RNA (2 µg) was synthesized cDNA using FastQuant cDNA (Tiangen, China). mRNA expression of GPR41/43 was measured with the ABI 7500 Fast Real-Time PCR (Life technologies, USA) using RealMasterMix (SYBR Green) (Tiangen, China). The housekeeping gene β -actin was used for normalizing the real-time PCR results. The primer sequences of GPR41/43 were listed in Table 1. The conditions for RT-PCR were as follows: 95°C for 30 s, followed by 40 cycles

of 90°C for 5 s, 58°C for 30 s and 72°C for 45 s. Data was calculated using the comparative CT method (Transcript Accumulation Index, TAI= $2^{-\Delta\Delta CT}$ method).

2.8 Statistical Analysis

All results were expressed as the mean $\pm$ SD. Statistical analyses and principal component analysis (PCA) were performed using the SPSS 19.0 statistical software. All variables were compared between groups using the ANOVA-LSD post hoc test. Values of $P < 0.05$ were considered to be statistically significant.

3 RESULTS

3.1 Effects of *L. acidophilus* KLDS 1.0738 on preventing from β -Lg allergy

A β -Lg-induced allergic murine model was employed to determine the effective role of *L. acidophilus* KLDS 1.0738 on protecting against hypersensitivity. As shown in Fig. 2a and b, the levels of total IgE and specific β -Lg-IgE were inhibited by *L. acidophilus* in a dose-dependent manner, but were drastically induced in allergic murine group. Additionally, treatment with butyrate or different dose of *L. acidophilus* strain both reduced the pro-inflammatory cytokines levels (IL-4, IL-6, IL-17 and TNF- β) and increased the anti-inflammatory cytokines levels (IFN- γ and TGF- β), especially the high dosage of *L. acidophilus* group (Fig. 2c). Furthermore, reduced inflammatory infiltrate

and less tissue damage were also observed in the lung and colon of *L.*

acidophilus-treated group (Fig. 3).

3.2 Effects of *L. acidophilus* on gut flora in β -Lg-sensitized mice

Fig. 4 showed the DGGE profile of PCR amplification from the fecal microbiota. The predominant bacteria in the fecal samples were re-amplified, sequenced and identified by the BLAST algorithm in the GenBank database (Table 2). The β -Lg allergen stimulation resulted in a significant reduced microbial diversity, along with increasing the proportion of *Bacteroides* and *Clostridium*. In contrast, treatment with *L. acidophilus* and butyrate enhanced the microbial diversity, similar to that shown in the normal group, as well as increased the abundance of potentially beneficial strains (*Lactobacillus*, *Bifidobacterium* and *Lachnospiraceae*). Meanwhile, similarity dendrogram revealed the fecal microbiota of allergic group was markedly different from the others treatments. For comparison, the bacterial composition of high-dose *L. acidophilus* group was more similar to the normal group than the butyrate-treated group, suggesting the possible effects of probiotics on the recovery from β -Lg-induced allergy (Fig. 5).

3.3 Effects of *L. acidophilus* KLDS 1.0738 on intestinal metabolim in

β -Lg-sensitized mice

In agreement with fecal microbiota studies, it was found that the microbial metabolites (including fecal SCFAs, lactate and amino acids) changed in significant differences among the four experimental treated groups. Bovine β -Lg challenge led to intestinal metabolism disruption, significantly increasing the acetate levels and decreasing the propionate, butyrate, isobutyrate, isovalerate and lactate levels compared with the normal group (Fig. 6a, $P<0.05$). As expected, mice treated with *L. acidophilus* exhibited dose-dependently promoting SCFAs concentrations, but reducing the levels of acetate. Notably, there was no significant variation in the lactate concentrations between the normal group and the high dose of *L. acidophilus* or butyrate group (Fig. 6b).

Analysis of 16 amino acids metabolites in the fecal samples were performed by amino acid autoanalyzer. Differences in the relative amino acids compositions were analyzed by PCA score plots. Compared with the allergic group, remarkable increased concentrations of valine, arginine and proline were observed in the fecal samples of *L. acidophilus*-treated group (Fig. 6c). Furthermore, PCA loading plots in Fig. 6d revealed that amino acids were almost clustered together, but were clearly separated from valine. Although there was a clear separation of the PCA score plot among the normal, allergic, *L. acidophilus* and butyrate group, there

was no clear discrimination among low, middle dose of *L. acidophilus* group and butyrate group (Fig. 6e).

3.4 Effects of *L. acidophilus* on the GPR41/43 expression in β -Lg-sensitized mice

We next examined the ability of *L. acidophilus* KLDS 1.0738 on regulating the SCFAs receptors under allergic inflammation (Fig. 7). In comparison to the allergic group, oral administration of *L. acidophilus* significantly increased the mRNA levels of GPR43 and GPR41 in the colon and spleen tissue in a dose-dependent manner ($P<0.05$). Additionally, the levels of GPR43 and GPR41 mRNA were also increased in the mice treated with butyrate.

Further analysis was conducted to investigate the correlation of the SCFAs receptors and cytokines production between the high dose *L. acidophilus* group and the allergic group. As shown in Fig. 8, the mRNA expression of GPR43 was positively related to the anti-inflammatory cytokines (IFN- γ , TGF- β ; $P<0.01$), but was negatively correlated with the pro-inflammatory cytokines (IL-4, IL-6, IL-17; $P<0.01$). At the same time, the increased expression of GPR41 was also positively related to decreased the pro-inflammatory cytokines levels, which suggested that both the GPR41 and GPR43 triggered by *L. acidophilus* might be involved in inhibiting allergic responses (Fig. 9).

4 DISCUSSION

We previously demonstrated that oral administration of *L. acidophilus* KLDS 1.0738 had protective effects on β -Lg-induced hypersensitivity, characterised by inhibiting IgE production, regulating the imbalance in T helper cell (Th) subsets and inducing oral tolerance [11]. Recent reports in animal models and in clinical trials confirmed that the immunoinhibitory effects of probiotics were dependent on improving the gut microflora composition and SCFAs metabolism [14, 15]. In view of this, further studies are required to assess whether gut flora-SCFAs axis activated by *L. acidophilus* is responsible for reducing β -Lg allergy.

It is known that disturbances of the intestinal ecosystem were closely associated with the development of allergic inflammation. In the present study, bovine β -Lg-sensitized mice exhibited aggravated inflammation, together with reducing bacterial biodiversity in the feces, the relative abundance of gut beneficial bacteria was lower, whereas the counts of *Bacteroides* and *Clostridium* were higher in β -Lg-sensitized mice compared to those of the normal mice. Our observations supported previous findings, which demonstrated that allergic infants at 2 years of age had higher counts of *Bacteroides* and higher ratios of *Bacteroides* to *Bifidobacterium* than non-allergic infants [16]. And an increasing trend in *Clostridium* species, the potential pathogenic bacteria involved in gastrointestinal infection, was also documented in allergic infants [17]. On the contrary, treatment

of allergic mice with *L. acidophilus* KLDS 1.0738 led to modify the abnormal intestinal microflora towards a pattern more similar to the normal mice, which contributed to the induction of the local intestinal immune system, including enhanced epithelial barrier integrity, activated Foxp3⁺ T regulatory cells and so on [18]. However, these positive protective effects would disappear in the absence of certain probiotics [19].

The bacterial metabolites were generally recognized as an important communication tool between the probiotics, gut microbiota and the host immune system, and helped to positively control the inflammation, carcinogenesis, metabolism and allergic diseases [20]. We next found that intake of *L. acidophilus* strongly improved the functions of host metabolic, including SCFAs, lactic acids and amino acids. At First, increasing production of butyrate, propionate and lactate might be in response to an enrichment of potential SCFAs-producers, such as predominant *Lactobacillus* and *Bifidobacterium* species [21]. *Lachnospiraceae* also has been proved to increase the production of butyric acid, and in turn has promoted the proliferation of *Bifidobacteria* and *Lactobacilli* [22,23]. Secondly, Duncan *et al.* suggested that lactic acids could be converted to butyrate when incubated with *Bifidobacterium* and butyrate-producing bacteria [24]. While Kolling *et al.* indicated that the effect of probiotics on suppressing the metabolism of the *Clostridium* strain was related to a sufficient amount of lactic acid in the

cecal [25]. Moreover, the concentration of certain amino acids, such as valine, histidine and arginine, was significantly up-regulated in *L. acidophilus* groups as compared with the allergic group. Among them, Valine is one of essential amino acids and has been reported to participate in restoring intestinal barrier [26]. Histidin exhibited an anti-inflammatory effect in endothelial inflammation via inhibiting NF- κ B activation [27]. Arginine, a conditionally essential amino acid for infants, had a positive effect on gut mucosa [28]. These above data suggested that *L. acidophilus* as modulators of the gut microbiota and metabolism might be beneficial to alleviate the severity of allergic symptoms.

Considerable evidence indicated that SCFAs, the major end-products of gut microbial fermentation, played a critical role in mucosal integrity and immune response through activating SCFAs related signalling receptors, particularly GPR41 and GPR43 [29]. Chang *et al.* suggested that the GPR41/43 activated by SCFAs had an important function in the differentiation and/or activation of T lymphocytes [30]. Kim *et al.* reported that GPR43-deficient mice had exacerbated inflammation along with increased frequencies of Th17 cells in animal models of colitis [31]. Similarly, the present study found that administration of butyrate or *L. acidophilus* both enhanced the expression of GPR41 and GPR43 in the colon and lung in the β -Lg allergic mice, along with decreasing IL-4, IL-6 and IL-17 and increasing IFN- γ and TGF- β . Importantly, there was a positive correlation

between mRNA expression of GPR41/43 and anti-inflammatory cytokines, as well as the negatively correlated with the pro-inflammatory cytokines, when analyzed the correlation between the allergic group and the high dose of *L. acidophilus* group. In addition, another studies found that GPR41/43 binding of SCFAs diminished the downstream mitogen-activated protein kinases (MAPKs) signaling pathways (including MAPK/ERK, SAPK/JNK and p38 MAPK), which was correlated to inhibiting inflammation development and Th17 polarization [32,33]. For instance, Haghikia *et al.* demonstrated that dietary SCFAs could expand gut T regulatory cells by suppression of the JNK1 and p38 pathway [34]. Kim *et al.* showed that SCFAs triggered intestinal epithelial cells via GPR41 and GPR43 and led to delaying the inflammatory cytokines (IL-6, IL-17A) and chemokines (CXCL1 and CXCL2) production via mediating ERK1/2 and p38 MAPK signaling pathways [35]. Kobayashi *et al.* revealed that propionate attenuated TNF- α -stimulated monocyte chemotactic protein-1 (MCP-1) expression in human renal cortical epithelial cells by inhibiting phosphorylation of p38 and JNK in a GPR41/GPR43-dependent manner [36]. Accordingly, SCFA-GPR43 is probably one of several pathways by which the probiotics regulates inflammatory responses in the gut, and follow-up experiments are required to explore the role of GPR41/43-related downstream pathways affected by SCFAs.

Taken together, our results revealed that *L. acidophilus* KLDS 1.0738 regulating the structure of intestinal flora has a positive impact on host metabolism, nutrition and immune inactivation. In addition, activation of SCFAs-GPR41/43 interactions might be representing a potential mechanism to account for the anti-allergic effects of probiotics, and the detailed mechanism of SCFAs-mediated regulation of T cells and protection against food allergy deserves further investigation.

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DISCLOSURE

No potential conflict of interest was reported by the authors.

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Figures

Fig. 1 Mice were intraperitoneally injected with 200 μ L 1 mg/mL β -lactoglobulin (β -Lg) plus Freund's adjuvant on day 7, 14 and 21 in allergic group (n=15 in each group). 0.096 (low), 0.96 (middle) or 9.6 (high) g/kg *L. acidophilus* KLDS 1.0738 suspended in 200 μ L PBS were oral administrated to allergic mice three times a week for 4 weeks. And the β -Lg-sensitized mice were given intraperitoneal injections of 200 μ L sodium butyrate (50 mM) from week 1 to week 4; as a normal, sterile PBS was administered. On 28 day, all sensitized mice were intragastrically challenged two times with β -Lg (30 mg/mouse) at 30 min apart.

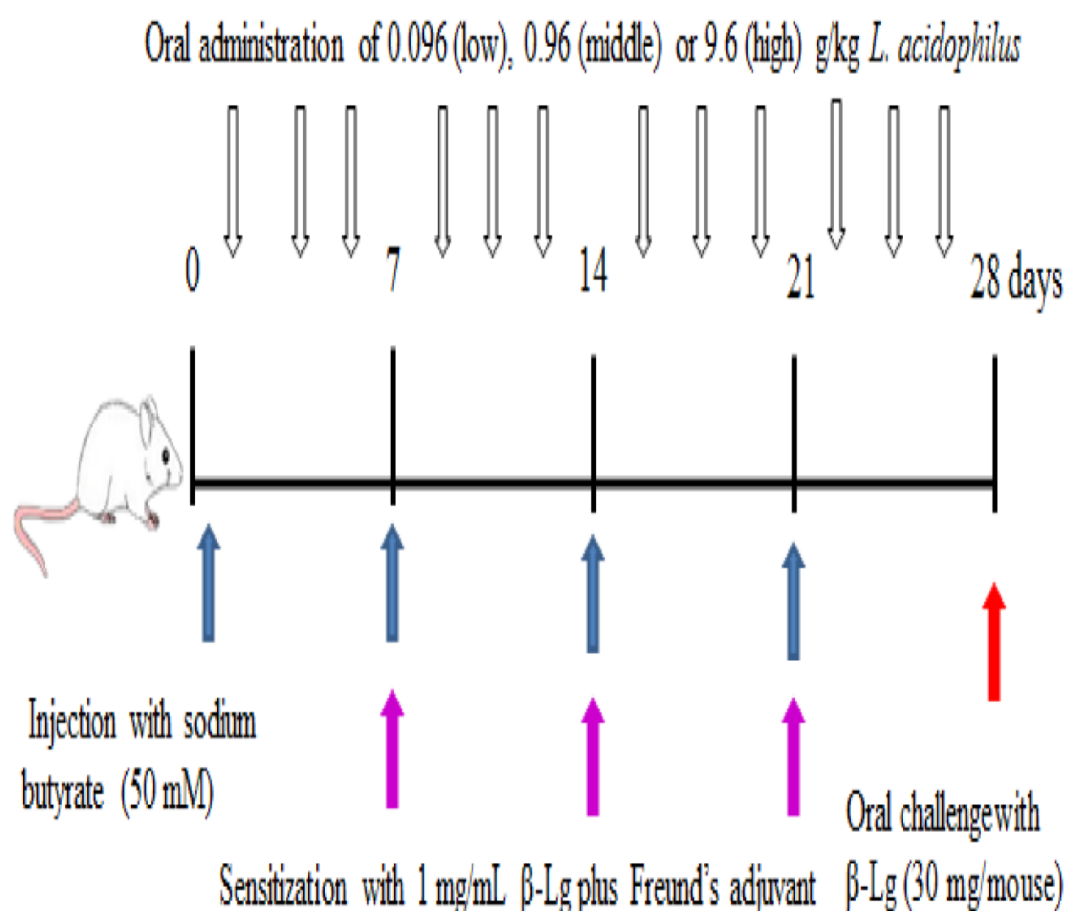


Fig. 2 Anti-inflammatory effect of oral administration of *L. acidophilus* KLDS 1.0738 in β -Lg-sensitized mice. Six-week-old BALB/c female mice were sensitized with 200 μ L 1 mg/mL β -Lg plus Freund's adjuvant (A: Allergic group), and the β -Lg-challenged mice were treated with different dose of *L. acidophilus* (L: low dose, 0.096 g/kg; M: medium dose, 0.96 g/kg; H: high dose, 9.6 g/kg) for 4 weeks. 50 mM butyrate (B: Sodium butyrate group) was intraperitoneally given to the β -Lg-challenged mice one times a week from days 1 to 28. Normal mice were treated with sterile saline (N). The mice were sacrificed on day 28 and serum

was collected to measure the production of IgE (a), β -Lg-specific IgE production (b) and cytokines (c). The results were presented as the mean $\pm$ SD (n =5) of three independent experiments. * P <0.05 versus normal group, & P <0.05 versus allergic group by the ANOVA-LSD post hoc test.

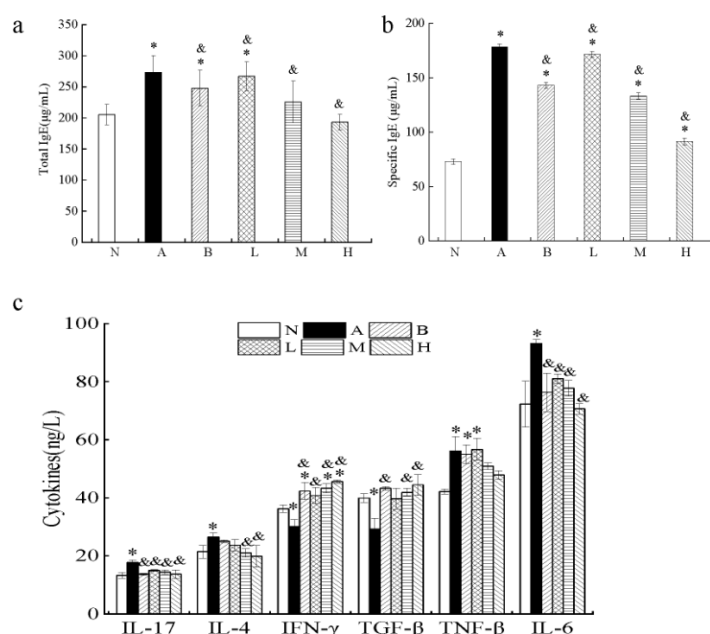


Fig. 3 Effect of oral administration of *L. acidophilus* KLDS 1.0738 on the histological of lung and colon in β -Lg allergic mice. Representative images of haematoxylin–eosin staining of lung (a) and colon (b) in mice were administrated with (N) normal diet or (A) 1 mg/mL β -Lg adsorbed in Freund's adjuvant or (B) butyrate or (L-H) *L. acidophilus* by dose of 0.096, 0.96 and 9.6 g/kg, respectively (magnification, 200 $\times$).

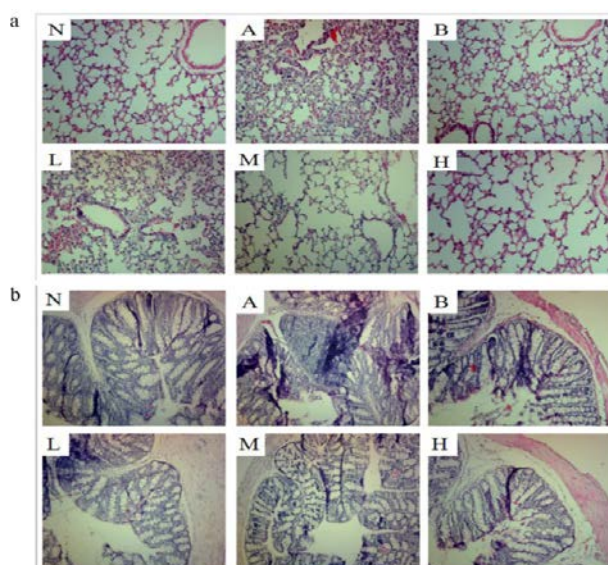


Fig. 4 DGGE fingerprints of fecal microbiota PCR products from mice. N1-N3 and A1-A3 represent the three normal and allergic mice, respectively. While, B1-B3, L1-L3 and H1-H3 represent the mice treated with butyrate (B), low (L) and high (H) dose of *L. acidophilus*. Bands indicated by numbers were excised, and, after re-amplification and sequencing and correspond to the amplicons identified in Table 2.

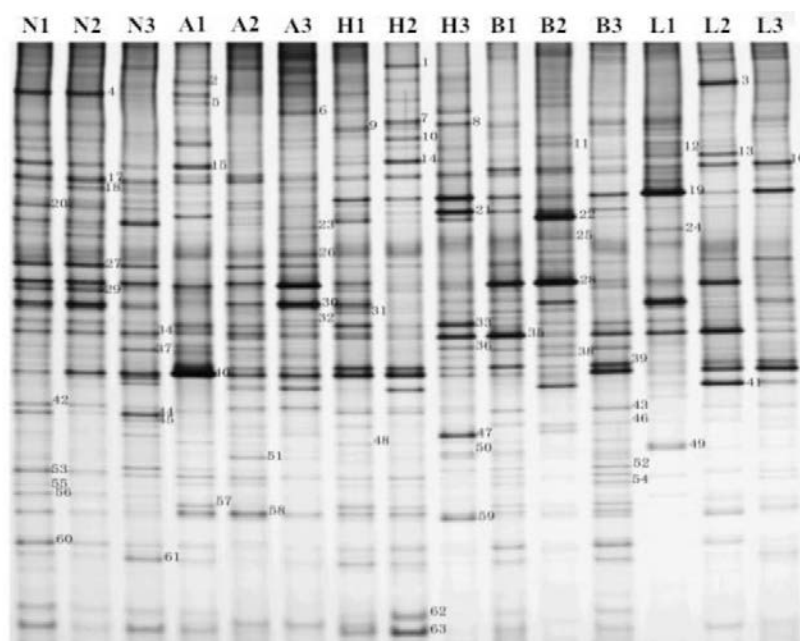


Fig. 5 UPGMA cluster analysis of DGGE profiles of from samples of the fecal bacteria in mice. N: Normal group; A: Allergic group; B: Sodium butyrate group; L and H represent the mice in groups treated with *L. acidophilus* by dose of 0.096 and 9.6 g/kg, respectively.

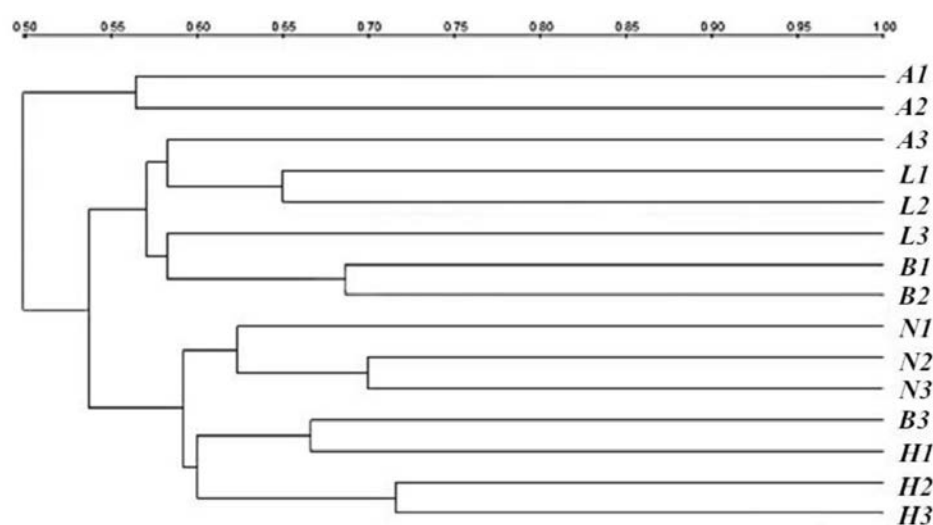


Fig. 6 Effect of *L. acidophilus* KLDS 1.0738 on short-chain fatty acids (SCFAs), lactate and amino acids in β -Lg-challenged mice. The mice were sensitized with 1 mg/mL β -Lg adsorbed in Freund's adjuvant, and mice were administrated with different dose of *L. acidophilus* or injected of 50 mM sodium butyrate for 4 weeks. The feces was harvested after the euthanasia of mice to measure the concentration of SCFAs (a), lactate (b), amino acids (c), PCA loading plots of amino acids (d) and PCA scores of amino acids (e). N: Normal group; A: Allergic group; B: Sodium butyrate group; L-H represent the groups treated with *L. acidophilus* by dose of 0.096, 0.96 and 9.6 g/kg, respectively. The results were presented as the mean $\pm$ SD (n=5) of three independent experiments. * P <0.05 versus normal group, & P <0.05 versus allergic group by the ANOVA-LSD post hoc test.

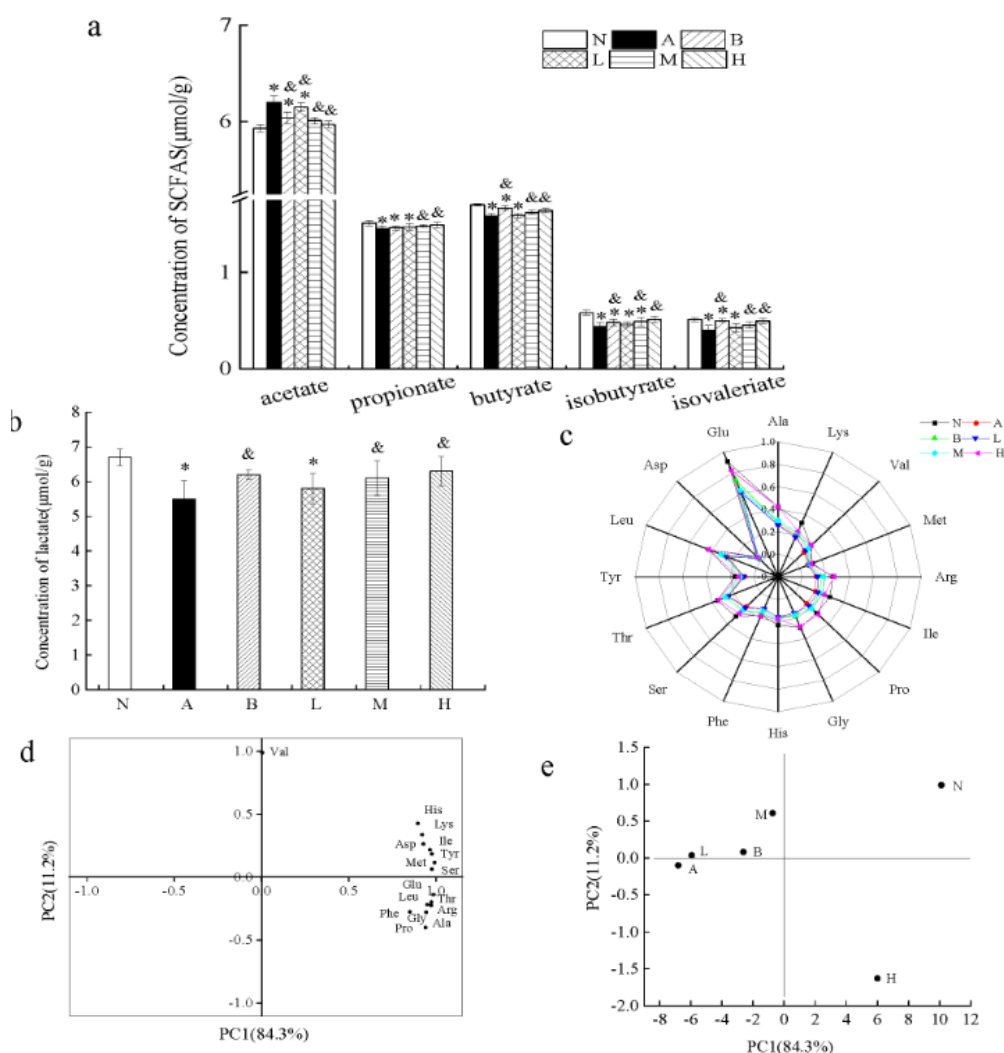


Fig. 7 Effect of *L. acidophilus* KLDS 1.0738 on G protein-coupled receptor 41 (GPR41) and G protein-coupled receptor 43 (GPR43) mRNA in (a) colon and (b) spleen tissue of β -Lg-sensitized mice. The mRNA expression of GPR41 and GPR43 was investigated in two groups of mice treated with sterile saline (N: Normal group) and intraperitoneally injected with 1 mg/mL β -Lg adsorbed in Freund's adjuvant (A: Allergic group), and in groups of β -Lg allergic mice intraperitoneally injected with (50 mM) sodium butyrate (B: Sodium butyrate group) and intragastrically administered with *L. acidophilus* by dose of 0.096 (L), 0.96 (M) and 9.6 g/kg (H), respectively. The results were presented as the mean $\pm$ SD (n=5) of three independent experiments. * $P<0.05$ versus normal group, & $P<0.05$ versus allergic group by the ANOVA-LSD post hoc test.

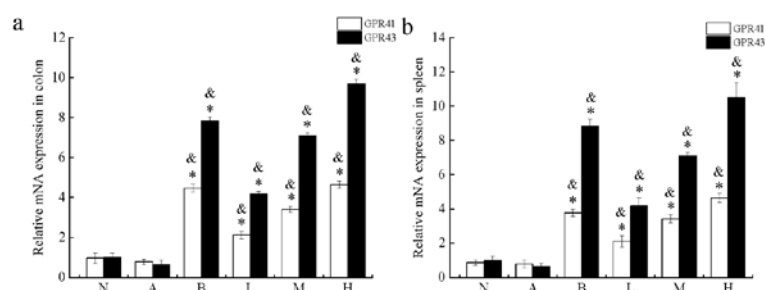


Fig. 8 Correlation analysis performed using the cytokines in serum and GPR43 mRNA expression in colon modified by β -Lg and *L. acidophilus* KLDS 1.0738 treatment. (a-c) Correlation analysis of GPR43 mRNA and pro-inflammatory cytokines (d-e) correlation between GPR43 mRNA and anti-inflammatory cytokines in the *L. acidophilus*-treated (dots on the right side) compared to the allergic group (dots on the left side).

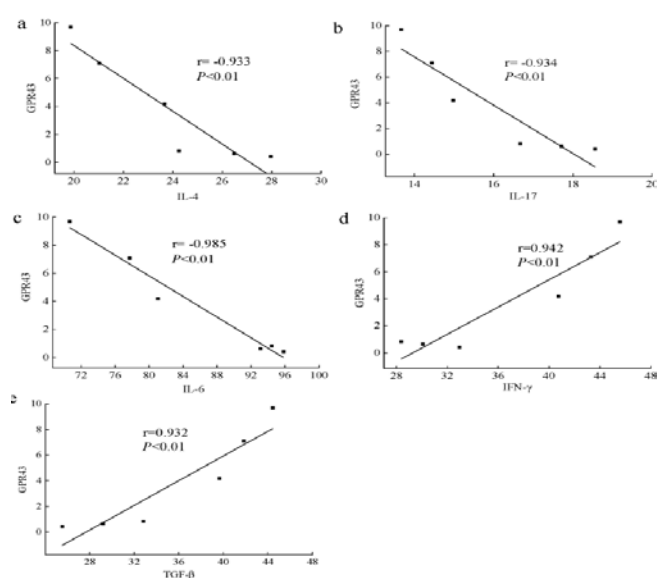
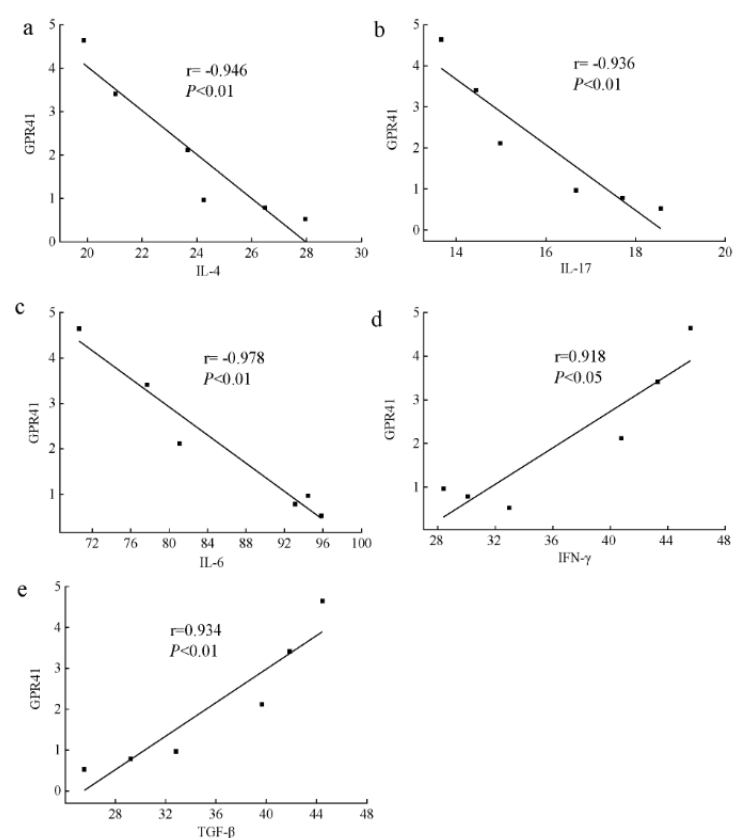


Fig. 9 Linear correlation analysis showed that the production cytokines in serum correlated to GPR41 mRNA expression in colon in allergic group responding to *L. acidophilus*-treated group. (a-c) Linear correlation between GPR41 mRNA and pro-inflammatory cytokines (d-e) correlation between GPR41 mRNA and anti-inflammatory cytokines in the *L. acidophilus*-treated (dots on the right side) compared to the allergic group (dots on the left side).



Abbreviations

CMA, Cow's milk protein allergy; GPR41/43, G protein-coupled receptor 41 and 43; HPLC, high-performance liquid chromatography; IBS, irritable bowel syndrome; *L. acidophilus*, *Lacidophilus acidophilus*; MAPKs, mitogen-activated protein kinases; PCA, principal component analysis; SCFAs, short-chain fatty acids; Th, T helper cell; β -Lg, β -lactoglobulin;

Table 1. Quantitative RT-PCR primers designed for the detection of GPR43 and GPR41

Molecule		Sequence(5'-3')
GPR43	Forward	AATCACAGGAAACGGGAAGCC
	Reverse	GTCTGGGGTCATTCTCCTTGG
GPR41	Forward	GAAAAGAGTGGACCGCAGGA
	Reverse	AGGGCAATCTGAGTGACAGC
β -actin	Forward	CGCAAAGACCTGTATGCCAAT
	Reverse	GGGCTGTGATCTCCTTCTGC

†GPR43: G protein-coupled receptor 43; GPR41: G protein-coupled receptor 41

Table 2 Identification based on blast comparison in genbank, of the bands obtained by PCR-DGGE

Band No.	Most similar sequence relative (species)	Identity(%)	group
27	<i>Firmicutes bacteria</i>	100	N
60	<i>Lactobacillus johnsonii</i>	98	N
61	<i>Lactobacillus reuteri strain</i>	100	N

26	<i>Uncultured bacterium</i>	97	A
40	<i>Bacteroides sp.</i>	98	A
58	<i>Helicobacter sp.</i>	97	A
13	<i>Bacteroides rodentium strain</i>	98	L
16	<i>Bacillus sp.</i>	100	L
41	<i>Uncultured Escherichia sp</i>	100	L
28	<i>Akkermansia muciniphila</i>	100	B
35	<i>Lactobacillus animalis strain</i>	99	B
39	<i>Lachnospiraceae</i>	97	B
59	<i>Lactobacillus acidophilus</i>	99	H
62	<i>Bifidobacterium breve</i>	100	H
63	<i>Bifidobacterium adolescentis</i>	97	H

‡ N: Normal group; A: Allergic group; B: Sodium butyrate group; L and H represent the mice in groups treated with *L. acidophilus* by dose of 0.096 and 9.6 g/kg, respectively.