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Tripartite Interaction of *Lactiplantibacillus plantarum* Y42, Tryptophan and Gut Microbiota Modulates AhR/Nrf2/NF-κB Axis for Dual Protection Against Intestinal Barrier Damage and Inflammation

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ABSTRACT: The aim of this study was to investigate the effects of the supernatant from the co-culture of intestinal flora from healthy human feces, *Lactiplantibacillus plantarum* Y42 (*L. plantarum* Y42) and tryptophan on intestinal barrier function impairment and inflammatory injury in lipopolysaccharide (LPS)-induced Caco-2/RAW264.7 co-culture model. The co-culture supernatant promoted the expression of tight junction proteins (Claudin-1, Occludin, ZO-1), regulated inflammatory factors, reduced cell permeability, and alleviated the barrier function damage and inflammation of Caco-2/RAW264.7 cells exposed to LPS. The promotion of tight junction protein expression and inhibition of nuclear factor kappa-B (NF-κB) signaling pathway by the co-culture supernatant were all dependent on the activation of aromatic hydrocarbon receptor (AhR) signaling pathway. The main metabolites in the co-culture supernatant were indole-3-lactate acid (ILA), 3-indolealdehyde (IAld) and indole-3-acetic acid (IAA), which as the ligands activate AhR pathway. These findings provide support for the development of postbiotics based on *L. plantarum* Y42 to alleviate intestinal inflammation and restore intestinal barrier function.

Keywords: *Lactiplantibacillus plantarum*, tryptophan metabolites, ILA, AhR

1. Introduction

Dysregulation of immune response and impairment of barrier function can cause chronic intestinal inflammation, which leads to many gastrointestinal diseases and systemic diseases[1, 2]. The destruction of the intestinal barrier will allow bacteria and other harmful substances to enter the intestine through the mucus channel and distribute in various organs, endangering intestinal health[3, 4]. There is an important relationship between the occurrence of intestinal inflammation and the defect of intestinal tight junction barrier[5]. Therefore, reducing intestinal permeability, protecting intestinal barrier function and reducing enteritis is essential for maintaining intestinal and overall health. More and more research has been conducted on the production of tryptophan metabolites by the gut microbiota that contribute to intestinal and systemic homeostasis[6]. Tryptophan is an essential amino acid, which is mainly absorbed in the small intestine. After the tryptophan that is not absorbed by the small intestine enters the colon, it is metabolized by intestinal flora to produce indole derivatives (ILA, IAA, IAld, indole-3-acetate (I3A) and Kynurenine),

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which activate the aromatic alkane receptor to maintain intestinal homeostasis and intestinal barrier function[7, 8]. There have been numerous reports that *Bifidobacterium* and *Lactobacillus* metabolize tryptophan to produce indole derivatives including ILA, IAA, IAld and so on, thereby maintaining intestinal barrier function and alleviating intestinal inflammation[7, 8]. Therefore, it is of great value to study the role of tryptophan metabolites of *lactic acid bacteria* in protecting intestinal barrier and alleviating inflammatory diseases. In this study, *L. plantarum* Y42, microorganisms extracted from human feces and tryptophan were co-cultured to simulate the intervention of *L. plantarum* and tryptophan in intestinal flora metabolites to metabolize tryptophan, and the effects of co-cultured supernatant on inflammatory damage and barrier function damage were investigated.

The co-culture model of Caco-2/RAW264.7 cells can better simulate the intestinal environment under the disease state[9]. In this study, the co-culture model of Caco-2/RAW264.7 was used to investigate the mechanism of alleviating effect of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan co-culture supernatant on LPS-induced intestinal epithelial cell barrier damage and inflammatory damage. In addition, whether the protective effect of the co-cultured supernatant on intestinal epithelial barrier function injury and inflammatory injury depends on AhR was also investigated. This study will help understand the synergistic effect of probiotics and tryptophan on the gut flora composition and metabolites.

2. Methods

2.1 Bacterial and Cells culture

L. plantarum Y42 were stored in Dalian Probiotics Function Research Key Laboratory, Dalian Polytechnic University and cultured in De Man, Rogosa, and Sharpe (MRS) broth (37 °C, 18 h).

Raw264.7 and Caco-2 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (10% fetal bovine serum and 1% penicillin-streptomycin solution (Meilun Biotechnology)) at 37 °C and 5% CO_2 concentration.

2.2 Metabolism of tryptophan by fecal intestinal flora in healthy human

Stool samples from healthy young adults who have not taken antibiotics for 3 months. Three volunteers were required to sign written informed consent. The mixed stool samples (10 g) from three volunteers were thoroughly mixed with sterile PBS (80 mL, pH = 6.8). The medium was prepared according to the method of Zhang et al[10]. Colon digestive fluid (27 mL) was mixed with fresh feces pulp (3 mL). The samples were charged with nitrogen for 5 min and cultured in a shaking bed at 37 °C for 72 h. The 5 mL samples were mixed with 5 mL bacterial suspension (containing 2 g/L tryptophan, G_T_Y42), PBS (containing 2 g/L tryptophan, G_T) or PBS (G) and cultured at 37 °C for 24 h. Finally, the sample 4000 r was centrifuged for 10 min and the supernatant was collected for the detection of tryptophan metabolites and cell experiments.

2.3 Cytotoxicity analysis

The supernatant was concentrated and then re-suspended in 10 times DMEM medium. Caco-2 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate, and cytotoxicity tests were conducted

according to the method of Teng et al[11]. The supernatant (100 μL) was added into the well for 24 h and 10 μL CCK-8 reagent was added. Calculate cell activity.

2.4 Caco-2 monolayer cell model

Caco-2 cells were inoculated into the 12- well transwell chambers at a concentration of 2×10^4 cells/well and cultured for 21 d to form a fully confluent monolayer characterized by tight junctions[11]. The TEER was measured every other day to assess monolayer cell integrity. When the TEER value tends to be flat, it indicates that the Caco-2 monolayer cell model has been successfully established.

2.5 Caco-2/RAW264.7 model

When the TEER of Caco-2 monolayer cells tended to be flat, RAW264.7 cells were seeded to BL chamber with 1×10^5 cell/well and cultured for 24 h to form Caco-2/RAW264.7 co-culture system. G_T_Y42, G_T, G metabolites or CH223191 (10 μM) were incubated in AP chamber for 4 h, and LPS (2 μg/mL) was added in BL chamber for 20 h. Finally, the TEER value was measured and the cell culture supernatant and the cells in the BL chamber were collected.

2.6 Cell permeability assay

After the cells were treated, 4-kDa FITC-glucan solution (5 mg /mL) was added in the AP chamber for 24 h. The fluorescence intensity (excitation of 485 nm and emission of 520 nm) was measured in the medium of BL chamber.

2.7 ELISA

The contents of IL-1β, IL-6, IL-10 and IL-22 in the cell culture supernatant were detected by ELISA kit.

2.8 RT-qPCR

The extraction of total RNA, reverse transcription of cDNA and detection of gene expression were strictly in accordance with Tintelnot et al[12]. The primer sequence is shown in Table S1. Quantification was performed by StepOne Real-time PCR system (Applied Biosystems, USA).

2.9 Western Blot

The experiment was conducted following the method of Gao et al [13]. Protein was extracted from cells with protein extraction kit, and protein concentration was detected with BCA kit. After SDS-PAGE electrophoresis, the protein was transferred to Polyvinylidene difluoride (PVDF) membrane and incubated with primary antibody. The dilution ratio of primary antibody β-actin, Claudin-1, Occludin, ZO-1, AhR, cytochrome p450 1a1 (Cyp1a1), inhibitor kappa B-α (IκB-α), Nrf2, heme oxygenase-1(HO-1) and NF-κB p65 was 1:1000. Then it was incubated with the corresponding horseradish peroxidase-labeled secondary antibodies (1:2000), and finally quantitative analysis was carried out by ImageJ software.

2.10 Tryptophan Metabolites

The 100 μL sample was added with internal standard working liquid (10 μL, 4000 ng/mL), then added with 390 μL methanol. Take the supernatant 350 μL and blow dry with nitrogen, add 70 μL 1% acetonitrile water (0.1% formic acid), vortex stir for 30 s, ultrasound at low temperature for 15 min (5 °C, 40 KHz), centrifuge at 13000 rcf at 4 °C for 15 min, then take the supernatant for testing. Liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-Qtrap) was used in this experiment, following the protocol of Wang et al[14].

2.11 Statistical analysis

All experiments were repeated at least three times. Values are shown as means ± SD. Using GraphPad Prism software (8.2.1) and analysis platform (https://cloud.majorbio.com/), statistical analysis (oneway ANOVA or t tests, Tukey multiple comparisons or Multiple test correction) and drawing. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ and (****) $P < 0.0001$.

3. Results

3.1 Effect of the supernatant from the co-culture of intestinal flora from healthy human feces, L. plantarum Y42 and tryptophan on the barrier function of Caco-2 cell monolayers damaged by LPS

The cytotoxicity of the co-culture supernatant of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan (G_T_Y42, G_T and G) on Caco-2 cells was assessed by CCK-8 assay. Previous studies found that the supernatant diluted ten times effectively relieved the inflammation of RAW264.7 cells. As shown in Fig. S1, the supernatant diluted ten times had no effect on Caco-2 cell viability. Therefore, in subsequent experiments, the supernatant will be diluted tenfold.

In this study, Caco-2/RAW264.7 co-culture model was used to evaluate the effect of the co-culture supernatant of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan pretreatment on the permeability of Caco-2 cell monolayers. As shown in Fig. 1a, TEER values gradually increased during culture and leveled off at 21 d, indicating that the 21 d cultured monolayer of Caco-2 cells has been formed. As shown in Fig. 1b and Fig. 1c, compared with the Control group, LPS significantly decreased the TEER value and increased the FITC-glucan permeability of Caco-2 cell monolayers. While the treatment by G_T_Y42 not only significantly increased TEER value, but also reduced FITC-glucan permeability of Caco-2 cell monolayers.

On the other hand, the treatments by G_T_Y42, G_T and G significantly alleviated the down-regulation of Claudin-1, Occludin and ZO-1 protein expression in LPS-induced Caco-2 cell monolayers, compared with the control group (Fig. 1).

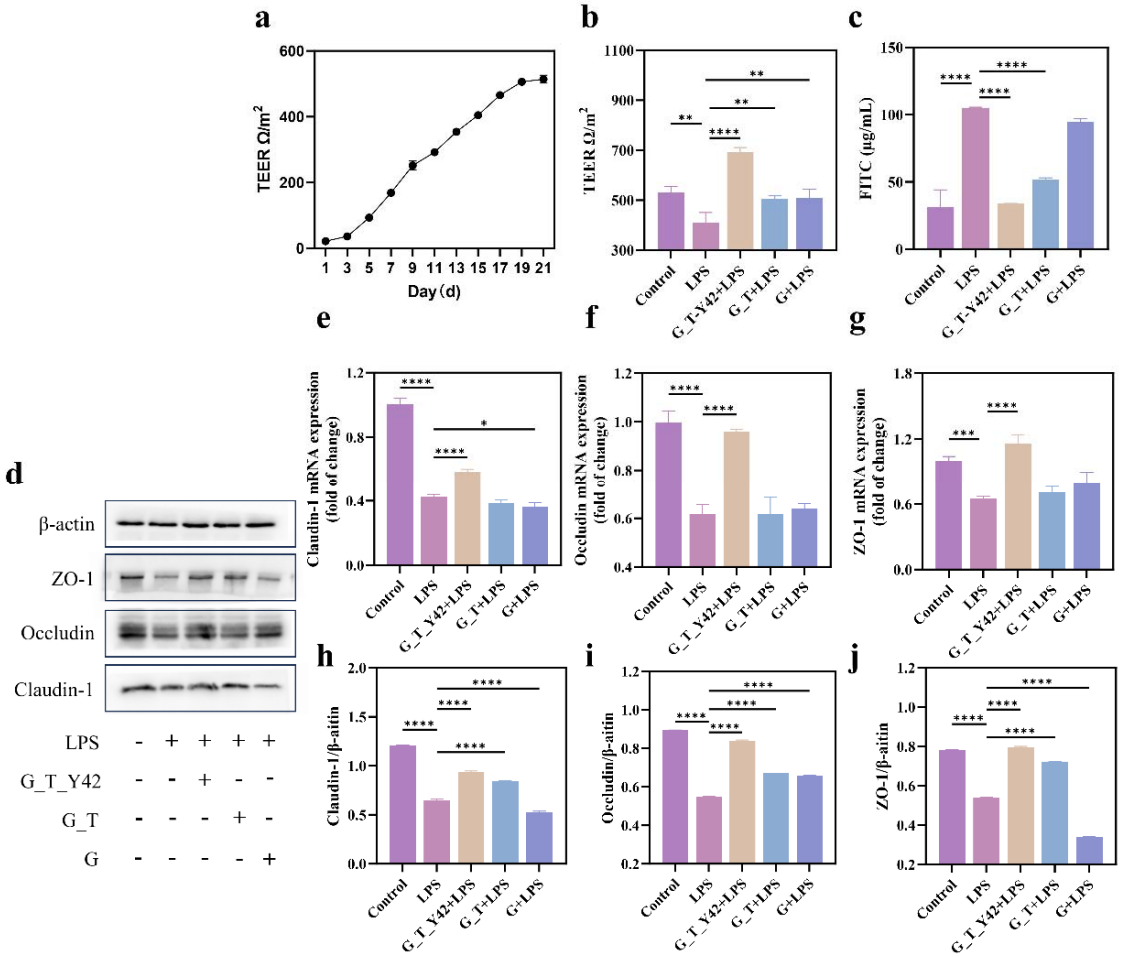

Fig. 1 Effect of G, G_T and G_T_Y42 on the permeability of Caco-2 cells. (**a**) TEER value of Caco-2 cell culture process. (**b**) TEER values in cells after treatment with G_T_Y42. (**c**) FITC-dextran levels. (**d**) Western blot analysis of the tight junction proteins. The mRNA expression of Claudin-1 (**e**), Occludin (**f**) and ZO-1 (**g**). The relative levels of Claudin-1 (**h**), Occludin (**i**) and ZO-1 (**j**) with β-actin.

At the same time, the addition of LPS significantly increased the content of IL-6 (Fig. 2b) and decreased the content of IL-10 in the Caco-2/RAW264.7 co-culture (Fig. 2c). The treatments by G_T_Y42 not only significantly increased IL-10, but also significantly reduced IL-6 in AP chamber. Therefore, the supernatant from the co-culture of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan reduced the permeability of Caco-2 cell monolayers by increasing the expression of tight-junction protein and regulating the expression of inflammatory factors, and thus ameliorated the intestinal epithelial injury induced by LPS.

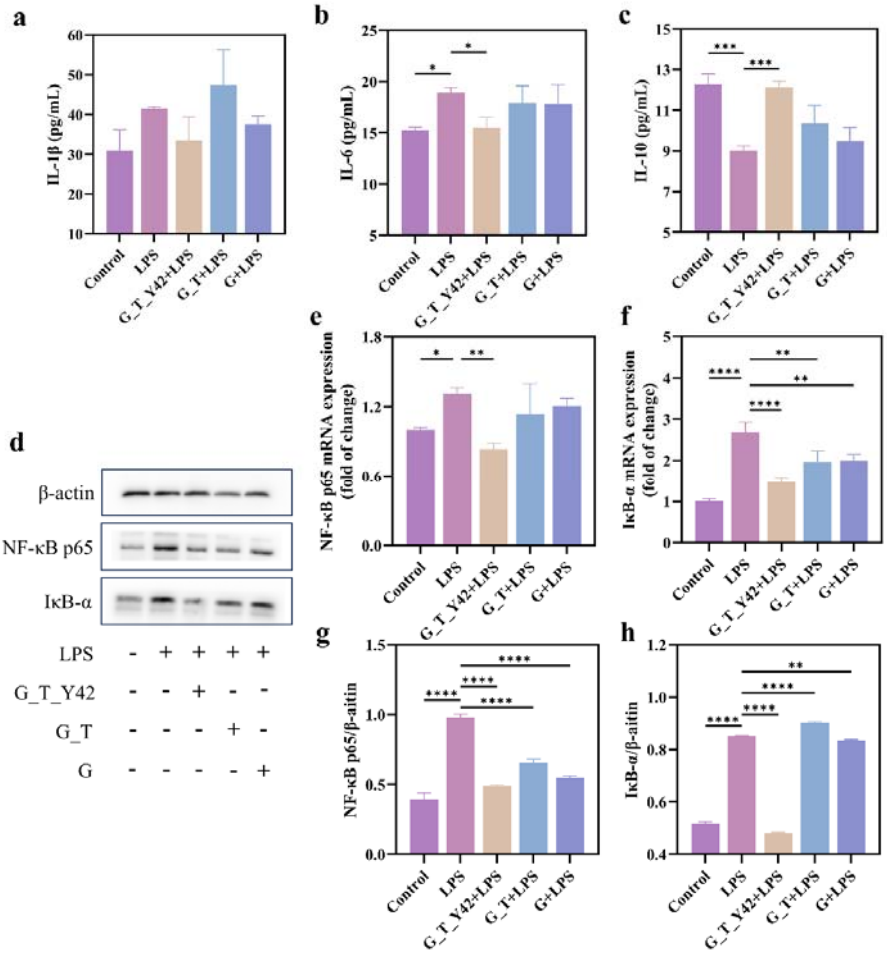

Fig. 2 Effect of G, G_T and G_T_Y42 on NF-κB signaling pathways in Caco-2 monolayer cells induced by LPS. The levels of (**a**) IL-1β, (**b**) IL-6 and (**c**) IL-10. (**d**) Western blot analysis of the NF-κB p65 and IκB -α proteins. The mRNA expression of *NF-κB p65* (**e**) and *IκB-α* (**f**). The relative levels of the NF-κB p65 (**g**) and IκB -α (**h**) proteins with β-actin.

3.2 Effect of the supernatant from the co-culture of intestinal flora from healthy human feces, L. plantarum Y42 and tryptophan on NF-κb, Nrf2 and AhR signaling pathways in Caco-2 cell monolayers exposed to LPS

NF-κB played an active role in stimulating the production of pro-inflammatory genes such as IL-1, IL-6, TNF-α and iNOS. Therefore, this study investigated the effects of G_T_Y42, G_T and G on the expression of key proteins in NF-κB signaling pathways induced by LPS in Caco-2 cells. LPS significantly increased the expression of NF-κB p65 and IκB-α, key proteins of NF-κB signaling pathway, in Caco-2 monolayer cells (Fig. 2e, f, g and h). Pretreatment with G_T_Y42, G_T and G decreased the expression of NF-κB p65 and IκB-α proteins to varying degrees. It was noted that the expression of NF-κB p65 and IκB-α proteins in G_T_Y42 group was lower than that in G_T and G group. Many studies have shown that Nrf2 signaling pathway also plays an important role in alleviating inflammation, and there is a certain relationship with NF-κB signaling pathway. Therefore, we also detected the expression of key proteins in the Nrf2 signaling pathway. As shown in Fig. 3, G_T_Y42, G_T and G significantly increased the expression of Nrf2 and HO-1 protein, and the expression of Nrf2 and HO-1 protein in G_T_Y42 group was higher than that in G_T and G group. This suggests that G_T_Y42 may reduce LPS-induced inflammatory damage in Caco-2 cells by inhibiting NF-κB signaling pathway and activating Nrf2 signaling pathway.

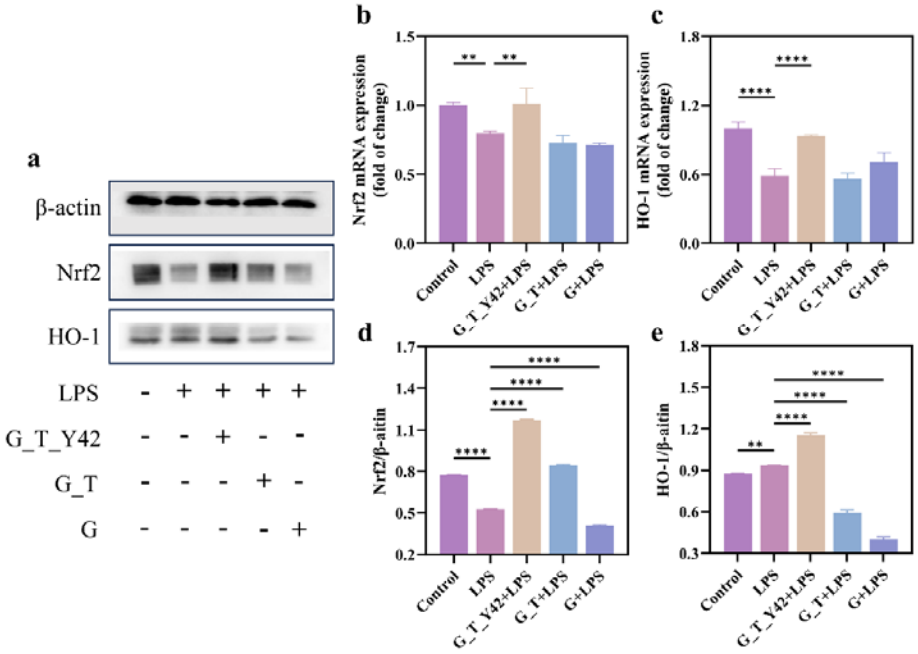

Fig. 3 Effect of G, G_T and G_T_Y42 on Nrf2 signaling pathways in Caco-2 monolayer cells induced by LPS. (**a**) Western blot analysis of the Nrf2 and HO-1 proteins. The mRNA expression of *Nrf2* (**b**) and *HO-1* (**c**). The relative levels of the Nrf2 (**d**) and HO-1 (**e**) proteins with β-actin.

AhR signaling pathway played an important role in maintaining the stability of intestinal epithelial barrier function. In this study, the expression of AhR and Cyp1a1 proteins was detected by RT-qPCR and Western Blot. Compared to the LPS group, the G_T_Y42 intervention in Caco-2 monolayers cells significantly promoted the expression of AhR and Cyp1a1 proteins and genes and was significantly more effective than the treatment with G_T and G of Caco-2 cells (Fig. 4). Therefore, G_T_Y42 might alleviate LPS-induced damage to Caco-2 monolayer cells by activating AhR signaling pathway.

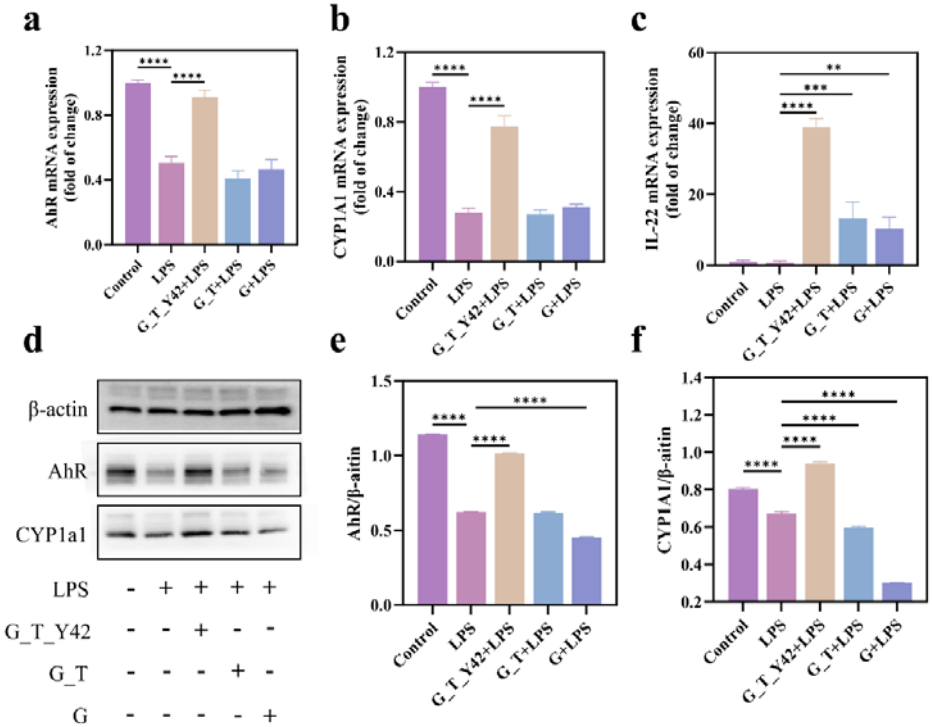

Fig. 4 Effect of G, G_T and G_T_Y42 on AhR signaling pathways in Caco-2 monolayer cells induced by LPS. The mRNA expression of *AhR* (**a**), *CYP1a1* (**b**) and *IL-22* (**c**). (**d**) Western blot analysis of the AhR and CYP1a1 proteins. The relative levels of the AhR (**e**) and CYP1a1 (**f**) proteins with β-actin.

3.3 The supernatant from the co-culture of intestinal flora from healthy human feces, L. plantarum Y42 and tryptophan ameliorated barrier damage of Caco-2 monolayer cells through AhR expression

In order to investigate whether G_T_Y42 alleviated the damage of Caco-2 monolayer cell barrier depending on AhR signaling pathway, the effect of AhR inhibitor (CH223191) on the damage of Caco-2 monolayer cell barrier was studied. LPS significantly reduces the expression of Claudin-1, Occludin and ZO-1 proteins in Caco-2 monolayer cells, and after prevention by G_T_Y42, the expression of Claudin-1, Occludin and ZO-1 proteins increases significantly. However, after CH223191 interferes with Caco-2 monolayer cells, G_T_Y42 pretreatment did not up-regulate the expression of Occludin and ZO-1 proteins. Meanwhile, compared with LPS group, G_T_Y42 significantly increased the expression of AhR and Cyp1a1 protein, and AhR and Cyp1a1 protein expression was indeed inhibited by AhR inhibitor (CH223191) addition. These results confirmed that G_T_Y42 mitigated damage to the Caco-2 monolayer cell barrier via the AhR signaling pathway.

3.4 Effect of activation of AhR signaling pathway on inhibition of NF-κB p65 expression in Caco-2 cells by the supernatant from the co-culture of intestinal flora from healthy human feces, L. plantarum Y42 and tryptophan

Literature studies showed that AhR and NF-κB p65 could form inactive complexes through binding, resulting in mutual inhibition. In this study, CH223191 (AhR inhibitor) was used to evaluate the effect of G_T_Y42 on the expression of Nrf2, NF-κB p65 and IκB-α in Caco-2 monolayer cells (Fig. 6). The G_T_Y42 pretreatment alleviated the increase in the expression of NF-κB p65 and IκB-α proteins and the decrease in the expression of Nrf2 protein in LPS-induced Caco-2 monolayer cells. When G_T_Y42 and CH223191 (AhR inhibitor) were co-pretreated, the expression of NF-κB p65 and IκB-α proteins was not reduced, but the expression of Nrf2 proteins was not affected. In summary, our results suggested that G_T_Y42 regulated inflammation and maintains Caco-2 monolayer cell barrier function mainly by activating AhR. G_T_Y42 inhibited NF-κB signaling pathways by directly inhibiting the activation of NF-κB p65 and indirectly reducing its expression by activating AhR signaling pathways.

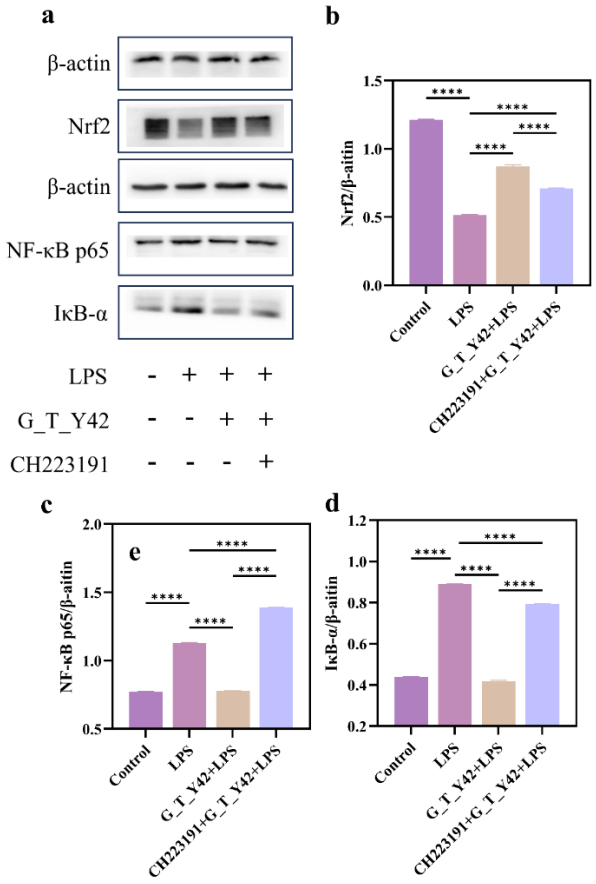

Fig. 6 The inhibitory effect of G_T_Y42 on NF-κB p65 was correlated with AhR. (**a**) Western blot analysis of the Nrf2, NF-κB p65 and IκB -α proteins. The relative levels of the Nrf2 (**b**), NF-κB p65 (**c**) and IκB -α (**d**) proteins with β-actin.

3.5 Tryptophan metabolism

In this study, tryptophan metabolites in G_T_Y42, G_T and G were detected by LC-MS. Based on the Volcano Plot analysis (Fig 7a), 22 kinds of tryptophan metabolites were up-regulated and 1 kinds of tryptophan metabolite was down-regulated in G_T group compared with G group. Compared with group G, 21 kinds of tryptophan metabolites were up-regulated and one kind of tryptophan metabolite was down-regulated in G_T_42 group. Compared with G_T group, 7 kinds of kinds of tryptophan metabolites were up-regulated and 6 tryptophan metabolites were down-regulated in G_T_42 group. PCA results (Fig. 7c) showed that there were significant differences among the groups of G_T_Y42, G_T and G, indicating that there were significant differences in tryptophan metabolism among the groups. The major metabolite changes are shown in Fig. 7b. Compared with G_T, the contents of ILA, Tryptamine, 3-indoleacrylicacid, 5-hydroxytryptophan, Rac-kynurenine, Nicotinic acid, Serotonin and Nicotinamide in G_T_Y42 were significantly increased, while the contents of IAld, IAA, Indole-3-acetamide, Indole-3-carboxylic acid and Picolinic acid were significantly decreased. The ILA content of G_T_Y42 was more than twice that of G_T and more than 1000 times that of G. Of all tryptophan metabolites except tryptophan, ILA content is much higher than other tryptophan metabolites. Therefore, ILA might play a key role in activating the AhR signaling pathway, promoting tight junction protein expression, and improving intestinal barrier function.

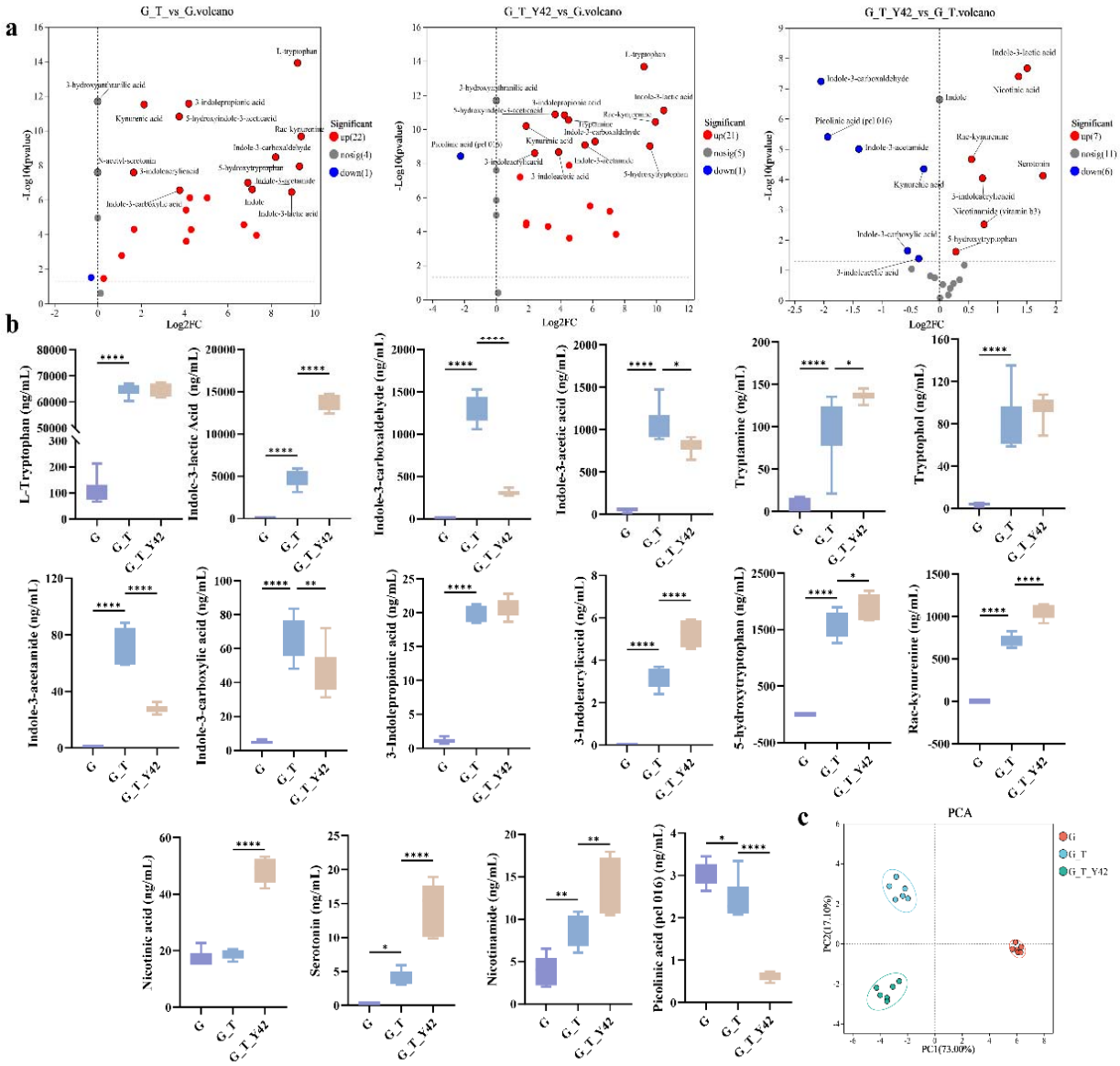

Fig. 7 Tryptophan metabolites in G, G_T and G_T_Y42. (**a**) volcano plot. (**b**) Levels and types of tryptophan metabolites. (**c**) PCA.

3.6 correlation analysis

Correlation analysis results showed that samples in group G were significantly negatively correlated with most of the metabolites of tryptophan, while samples in group G_T_Y42 and group G_T were significantly positively correlated with most of the metabolites of tryptophan (Fig. 8). Picolinic acid (pcl 016) was negatively correlated with other tryptophan metabolites. ILA showed significant negative correlation with IL-6, NF-κB p65, IκB-α, and significant positive correlation with IL-10, AhR, IL-22, Nrf2, Claudin-1, Occludin, ZO-1(Fig. 8). These results suggested that ILA was a major active substance that exerted anti-inflammatory function and maintained intestinal epithelial barrier function.

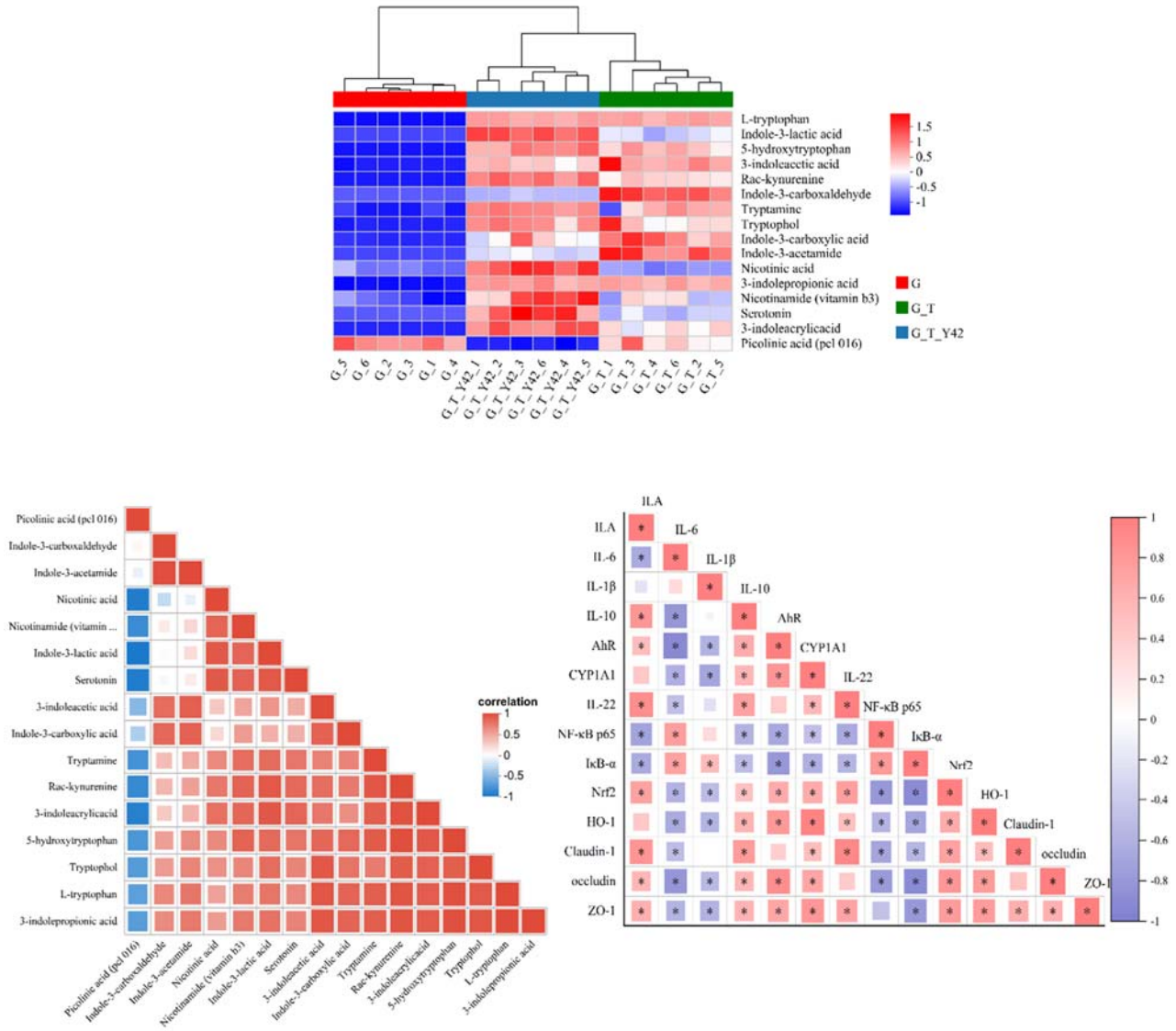

Fig. 8 Correlation analysis. Spearman correlation coefficient r ranges from -1 to 1, $r < 0$ is negative correlation, $r > 0$ is positive correlation.

4. Discussion

The intestinal barrier serves as the primary defense against pathophysiological stressors, playing a pivotal role in maintaining gastrointestinal homeostasis, and the destruction of barrier function has been established as the pathological basis of various systemic diseases[15, 16]. In this study, a co-culture model of Caco-2 cells and RAW264.7 cells was applied in order to simulate intestinal inflammation *in vitro*, and explore the action mechanism of co-culture supernatant of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan in alleviating intestinal inflammation and maintaining barrier function. Although there are some limitations in the *in vitro* models, they have the advantages of low cost, high accuracy and being free from ethical restrictions. The results showed that G_T_Y42 and G_T could significantly increase TEER value and significantly decrease FITC-glucan value in LPS-stimulated Caco-2/RAW264.7 co-culture model (Fig. 1b and c). LPS increased the content of IL-6 and decreased the content of IL-10 in AP chamber cell medium, and G_T_Y42 pretreatment reversed this phenomenon, while G and G_T were unable to do so. (Fig. 2b and c). Meanwhile, the content of ILA in the supernatant of G_T_Y42 was

significantly higher than that of G and G_T (Fig. 7b). Excessive secretion of pro-inflammatory cytokines is one of the important factors in the development of intestinal inflammation[17]. Inhibiting the excessive secretion of pro-inflammatory cytokines (such as IL-6) and promoting the production of anti-inflammatory cytokines (IL-10) can alleviate inflammation[17]. LPS stimulated RAW264.7 cells on BL chamber of co-culture model, resulting in increased secretion of pro-inflammatory cytokines, and then destroyed the Caco-2 monolayer cell structure on AP chamber. Intestinal inflammation is often accompanied by increased permeability of intestinal mucosal barrier, resulting in damage of TJ proteins (ZO-1, Occludin, Claudin-1)[18]. In this study, LPS significantly decreased the expression of Claudin-1, Occludin and ZO-1 proteins in Caco-2 monolayer cells model, while G_T_Y42 and G_T pretreatment significantly increased their expression (Fig. 1h, i and z). In the Caco-2/RAW264.7 co-culture model, pasteurized *Akkermansia muciniphila* mitigated 5-FU-induced cell barrier dysfunction and inflammatory response by increasing TEER values and tight junction protein expression[11]. TJs lied between epithelial and endothelial cells and controled the permeability of paracellular cells[19]. The lack of intestinal TJs leaded to increased antigen penetration, leading to more severe intestinal inflammation, and strengthening the intestinal TJ barrier helped to treat intestinal inflammation[20]. This suggests that G_T_Y42 could prevent LPS-induced inflammation of Caco-2 monolayer cells and alleviate barrier damage.

Many studies had confirmed that regulation of NF-κB p65 played an important role in protecting the intestinal epithelial barrier and immune homeostasis[21]. Normally, NF-κB and IκBα existed in the form of a complex, and when the cell was stimulated, NF-κB was released from the complex and translocated into the nucleus, which in turn produced a variety of pro-inflammatory cytokines[22, 23]. Activation of NF-κB leaded to upregulation of pro-inflammatory cytokines (IL-6, IL-8), which further increased barrier permeability and inflammation[24]. Angelica polysaccharide increased the expression of tight junction proteins ZO-1 and Occludin by inhibiting NF-κB signaling pathway, and protected intestinal mucosal injury[25]. Therefore, the inhibition of NF-κB activation by G_T_Y42 explains why pretreatment with G_T_Y42 results in the restoration of monolayer barrier function (reduced permeability, increased expression of tight junction protein), decreased production of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines in Caco-2 cells. Nrf2 is recognized as an important signaling pathway for controlling oxidative stress, but it is also critical in promoting barrier function and anti-inflammatory function[26, 27]. Inhibition of Nrf2 signaling pathway can enhance the expression of NF-κB, promote the increase of inflammatory cytokines , and NF-κB can control the expression of downstream genes by regulating Nrf2[28]. In this study, preconditioning of G_T_Y42 inhibited LPS-induced NF-κB signaling in Caco-2 cells (Fig. 2g and h) while activated the Nrf2 signaling pathway (Fig. 3). These results suggested that G_T_Y42 alleviated LPS-induced intestinal epithelial barrier damage and inflammation in Caco-2 cells by inhibiting NF-κB signaling and activating Nrf2 signaling.

AhR can regulate Nrf2 and NF-κB signaling pathways, reduce oxidative stress damage, regulate inflammatory response, and repair the intestinal epithelial barrier[18]. AhR had a positive effect on

maintaining intestinal homeostasis and intestinal health (including epithelial renewal and barrier integrity)[29]. Normally, AhR existed in the cytoplasm and bound to several chaperone proteins[30]. When AhR bound to ligand and separated from chaperone protein and entered the nucleus, it formed dimer with AHR nuclear translocator, and exerted functional activity by initiating cytochrome P450 1A1 (CYP1A1) and CYP1B1 gene transcription[30]. Studies have shown that many tryptophan metabolites such as ILA, IAld, indole and tryptamine could be used as ligands for AhR[31]. After the tryptophan metabolite bound to AhR, AhR entered the nucleus and promoted transcription of cytokines such as IL-22, which in turn promoted mucosal healing, thereby helping to regulate intestinal inflammation and maintain barrier function[32]. IL-22 could increase intestinal tight junctions to enhance intestinal barrier function[33], and increase antimicrobial peptides and mucins to enhance mucosal defense[34]. IL-22 is mainly produced by group 3 innate lymphoid cells (ILC3s), and ILC3s are regulated by AhR[35]. IAld reduced inflammatory responses and restored intestinal epithelial barrier function partially via AhR[36]. Bifidobacterium used tryptophan to produce indole derivatives such as ILA to activate aromatic hydrocarbon receptors, thereby enhancing intestinal barrier function[37]. LPS treatment significantly decreased the expression of AhR and CYP1a1 in Caco-2 cells, while G_T_Y42 pretreatment significantly increased the expression (Fig. 4). The expression of IL-22 was significantly increased after pretreatment with G_T_Y42. These results suggested that G_T_Y42 could alleviate LPS-induced intestinal epithelial barrier damage in Caco-2 cells by activating the AhR signaling pathway and promoting the expression of tight junction protein.

All these results suggest that G_T_Y42, G_T and G could activate AhR and Nrf2 signaling pathway, inhibit NF-κB signaling pathway, promote the expression of tight junction protein, and reduce the intestinal barrier damage of Caco-2 cells induced by LPS. The supernatant in G_T_Y42, G_T and G mainly consisted of tryptophan, ILA, IAld and IAA, of which ILA was the largest (Fig. 7). It is worth noting that G_T_Y42 has a more significant effect in regulating the intestinal epithelial barrier function (Claudin-1, Occludin and ZO-1) and inflammatory response (IL-6 and IL-10) compared to G_T and G. Moreover, the ILA content in G_T_Y42 is significantly higher than that in G_T and G. ILA showed significant negative correlation with IL-6, NF-κB p65, IκB-α, and significant positive correlation with IL-10, AhR, IL-22, Nrf2, Claudin-1, Occludin, ZO-1 (Fig. 8). Among the tryptophan metabolites of *L. plantarum* DPUL-S164 DPUL-S164[14], *Bifidobacterium*[38], and *Lactobacillus rhamnosus MN-431*[39], ILA acts as a ligand for AhR, binds to AhR, and activates AhR, playing a crucial role in enhancing the intestinal epithelial barrier function and regulating the immune response. A large number of studies have shown that ILA has the effects of alleviating inflammation, regulating immune function, and maintaining intestinal barrier function. Therefore, in this study, ILA was not directly verified. In this study, the co-cultured supernatant of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan alleviated inflammatory damage and intestinal barrier dysfunction by activating the AhR pathway (Fig. 5). In addition, previous studies have found that the key substance used by G_T_Y42 to relieve inflammatory damage is ILA[40]. And the content of ILA varied the most among the samples from different groups. It was inferred that ILA may have played a key role in

protecting the intestinal epithelial barrier damage of LPS-induced Caco-2 cells in the co-cultured supernatant of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan.

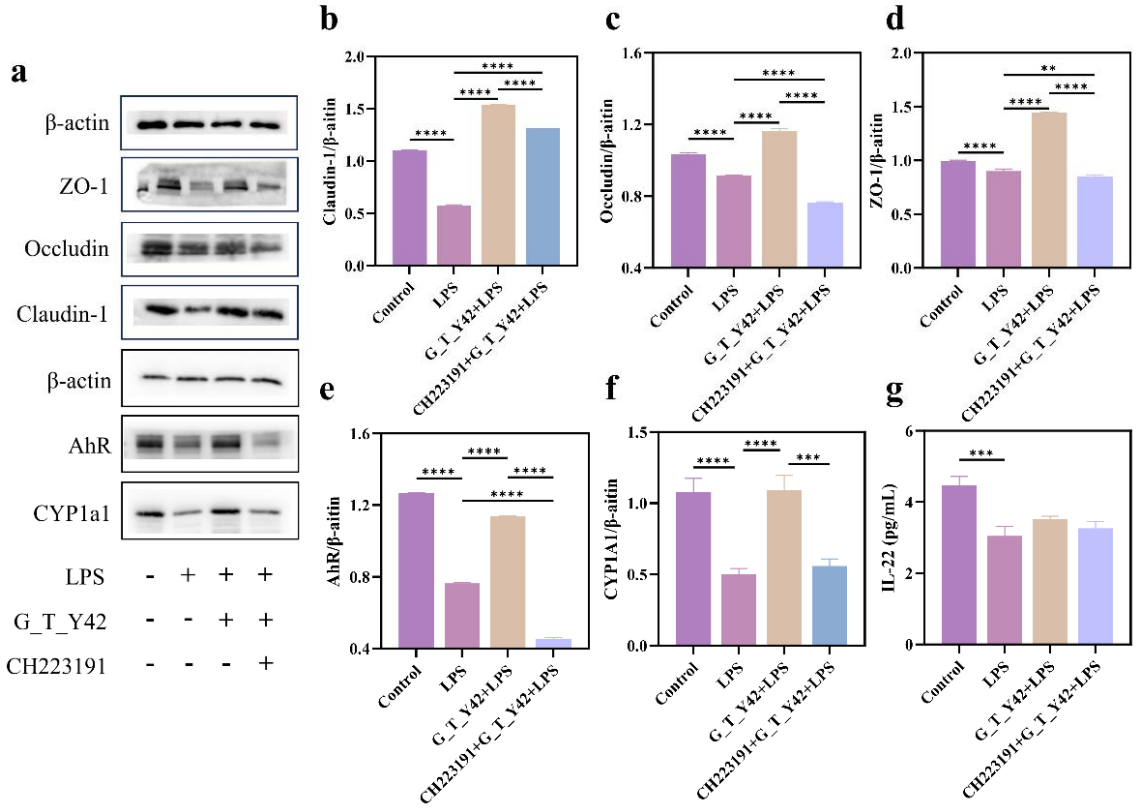

Fig. 5 G_T_Y42 improved barrier damage of Caco-2 monolayer cells through AhR expression. (**a**) Western blot analysis of the AhR, CYP1a1 and tight junction proteins. The relative levels of the Claudin-1 (**b**), occludin (**c**) ZO-1 (**d**), AhR (**e**) and CYP1a1 (**f**) proteins with β-actin.

To determine whether the reduction of intestinal epithelial barrier damage by G_T_Y42 was dependent on the activation of the AhR signaling pathway, LPS-induced Caco-2 cells were pretreated with an AhR inhibitor (CH223191) and G_T_Y42. CH223191 is a specific antagonist of AhR, which can compete with AhR ligands and thereby block the AhR signaling pathway[41]. Compared with LPS group, co-pretreatment with G_T_Y42 and CH223191 did not increase the expression of tight junction protein, AhR, and CYP1a1 proteins (Fig. 5). The promotion of tight junction protein expression by *L. plantarum* DPUL-S164 tryptophan metabolites depended on the expression of AhR in HT-29 cells[14]. This is similar to the results of this study. G_T_Y42 had a significant regulatory effect on NF-κB, Nfr2 and AhR, and literature studies have shown that there is a mutual inhibitory relationship between AhR signaling pathway and NF-κB signaling pathway, and the activation of AhR can inhibit the activation of NF-κB. Therefore, it was necessary to explore how G_T_Y42 affected the interaction of AhR with NF-κB and Nrf2. Zhilining Formula could inhibit NF-κB signaling through AHR, or directly inhibit NF-κB signaling, and the regulation of AHR signaling by Zhilining Formula was independent of NF-κB signaling[42]. The addition of CH223191 will inhibit the activation of the NF-κB/NLRP3 inflammatory pathway, thereby weakening the cardioprotective effect of IPA on septic rats.[43]. There is a physical interaction between AhR and NF-κB RelA, resulting in the mutual inhibition of the two signaling pathways[44]. The activation of AhR inhibits the

NF-κB pathway, thereby exerting an anti-inflammatory effect[43]. Dinatale et al. discovered that after TCDD treatment, the AhR/ARNT heterodimer needs to first activate IL-6 before AhR can exert its inhibitory effect on the NF-κB signaling pathway[45]. The results of this study indicate that G_T_Y42 depends on AhR to inhibit the NF-κB signaling pathway, and G_T_Y42 can also directly activate the Nrf2 signaling pathway to alleviate inflammation and damage to the intestinal barrier function (Fig. 6). *L. plantarum* DPUL-S164 tryptophan metabolites activated Nrf2 signaling pathway dependent on AhR, and inhibited NF-κB signaling pathway independent of AhR[14]. This point is different from this study.

In summary, the co-culture supernatant of intestinal flora from healthy human feces, *L. plantarum* Y42 and tryptophan promoted the expression of tight junction protein, alleviated the LPS-induced damage of Caco-2 monolayer. The main metabolites in the co-culture supernatant were ILA, IAld and IAA, which as the ligand of AhR activate AhR and Nrf2 signaling pathways, and then inhibit the NF-κB signaling pathway. ILA is the main functional active substance that maintains the barrier function of intestinal epithelial cells and plays a role in anti-inflammatory among the metabolites from the co-cultured supernatant.

Declaration of competing interest

None.

Data availability

The data that has been used is confidential.

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