Baicalin protects against APEC-induced colibacillosis by regulating gut microbiota and its metabolites in Chickens

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Abstract

BACKGROUND AND PURPOSE Chicken colibacillosis, caused by avian Escherichia coli (APEC), results in huge economic losses to the poultry industry. Baicalin exerts protective effects during the development of colibacillosis. In this study, we mainly explored the mechanism of this protective effects with regard to gut microbiota. EXPERIMENTAL APPROACH The chicken colibacillosis model was established by intratracheal instillation of APEC. The gut microbiota-depleted chicken model was established with broad-spectrum antibiotics. Viscera index measurement and Haematoxylin and eosin stain were applied to assess histological changes of tissues. ELISA were used to measure the cytokines and Quantitative-PCR were used to evaluate the gene expression. The gut microbiota and its metabolite were detected by 16srDNA and ultrahigh-performance liquid chromatography (LC-MS). KEY RESULTS Depletion of gut microbiota exacerbated the tissue damage and weakened the protective effects of baicalin during APEC-induced chicken colibacillosis while pretreatment of baicalin reduced these changes and inflammatory response induced by APEC. Moreover, APEC infection led to dysbiosis of gut microbiota and its metabolites. However, the pretreatment of baicalin remodeled the gut microbiota featured with increased abundance of Intestinimonas and its associated with beneficial metabolites. CONCLUSIONS Gut microbiota played a protective role in the prevention of chicken colibacillosis and the pharmacological action of baicalin. The altered specific gut bacterial and/or metabolites may be served as indicators to predict the occurrence and prognosis of chicken colibacillosis. Our findings may provide a paradigm for the mechanistic studies of compounds and aid the exploration of the mechanisms and pathways underlying the function of herbal medicines.

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EXPERIMENTAL APPROACH

The chicken colibacillosis model was established by intratracheal instillation of APEC. The gut microbiota-depleted chicken model was established with broad-spectrum antibiotics. Viscera index measurement and Haematoxylin and eosin stain were applied to assess histological changes of tissues. ELISA were used to

measure the cytokines and Quantitative-PCR were used to evaluate the gene expression. The gut microbiota and its metabolite were detected by 16srDNA and ultrahigh-performance liquid chromatography (LC-MS).

KEY RESULTS

Depletion of gut microbiota exacerbated the tissue damage and weakened the protective effects of baicalin during APEC-induced chicken colibacillosis while pretreatment of baicalin reduced these changes and inflammatory response induced by APEC. Moreover, APEC infection led to dysbiosis of gut microbiota and its metabolites. However, the pretreatment of baicalin remodeled the gut microbiota featured with increased abundance of Intestinimonas and its associated with beneficial metabolites.

CONCLUSIONS

Gut microbiota played a protective role in the prevention of chicken colibacillosis and the pharmacological action of baicalin. The altered specific gut bacterial and/or metabolites may be served as indicators to predict the occurrence and prognosis of chicken colibacillosis. Our findings may provide a paradigm for the mechanistic studies of compounds and aid the exploration of the mechanisms and pathways underlying the function of herbal medicines.

Key words : Chicken colibacillosis, baicalin, gut microbiota, metabolites

Introduction

Chicken colibacillosis, a localized and systemic disease caused by avian *Escherichia coli*(APEC), causedhuge economic losses to the poultry industry and threatened food security, which is responsible for the use of very significant volumes of antibiotics in commercial poultry farming(Peek, Halkes, Tomassen, Mes, & Landman, 2013). Despite being known for over a century and studied for a long time, the pathogenesis of colibacillosis remains poorly understood. Up until now, it is widely accepted that the systemic form of colibacillosis from a respiratory origin that induces colisepticemia. Colonization of the trachea and air sacs is considered the first step of a systemic infection by APEC, followed by the colonization of the lung, liver and the pericardium(Guabiraba & Schouler, 2015). Therefore, we considered that targeting the respiratory infection stage of APEC and stopping the APEC from entering the blood circulation may be an effective way to prevent and cure colibacillosis.

The gut microbiota, consisting of mostly bacteria, plays a positive player in the host defence system, regulating mucosal immunity and systemic immunity(Noverr & Huffnagel, 2004; Trompette et al., 2014). Recent studies have reported that gut microbiota modulated the pulmonary immune during respiratory disease through what is often called "gut-lung axis"(Budden et al., 2017; Dumas, Bernard, Poquet, Lugo-Villarino, & Neyrolles, 2018). Many respiratory diseases occurred and developed accompanying with intestinal flora alteration(Dickson, Martinez, & Huffnagle, 2014). Moreover, the gut microbiota contributed the protection against pneumococcal pneumonia and *Mycobacterium tuberculosis* in lung(Dumas, Corral, et al., 2018; Schuijt et al., 2016). What role does gut microbiota play in chicken colibacillosis, however, remains elusive.

Scutellaria baicalensis , a Chinese traditional medicine, has been widely used to treat various diseases, such as influenza, pneumonia, dysentery, and cancer(Z. L. Wang et al., 2018). Baicalin, a naturally occurring constituent, was extracted from the dry roots of *Scutellaria baicalensis* and possessed anti-inflammatory, anti-allergic, anti-oxidant, hepatoprotective, and anti-tumor activities. In the previous study, we have found that baicalin had a protective effect against colibacillosis. However, the precise mechanism is still unclear. Recent report has manifested that metabolic activities of intestinal microflora may be of a great importance for herbal medicines to express the pharmacological effects(An et al., 2019). Myung-Ah Jung et.al, has proved that orally administered baicalin was metabolized to baicalein and oroxylin A by intestinal microbiota, which enhances its anti-inflammatory effect by inhibiting NF-$\varkappa$B activation(Jung, Jang, Hong, Hana, & Kim, 2012a). Furthermore, modern studies discovered that the administrated herbal medicines also interacted with gut microbiota and regulated the composition of the flora to improve the disease, such as diabetes mellitus(Creely et al., 2007). Hence, we speculated that gut microbiota played an important role in the

process of baicalin exerting pharmacological effects. Similarly, baicalin may regulate the gut microbiota and its metabolite to protect chicken against colibacillosis.

Therefore, to understand the role of gut microbiota in the chicken colibacillosis and pharmacological effect of baicalin, as well as explore the protective mechanisms of baicalin on colibacillosis, we utilized APEC-O78 to establish the model of colibacillosis and applied the broad-spectrum antibiotic (Abx) to deplete the gut microbiota. Then, we measured the secretion of inflammatory cytokines, gene expression of inflammatory cytokines and tight junction, histopathological changes and viscera index. Furthermore, after orally administration of baicalin, we measure the alteration of gut microbiota and its metabolite by 16s rDNA sequencing and ultrahigh-performance liquid chromatography (LC-MS).

Materials and methods

Reagents and bacterial strain

Baicalin (purity>99%) was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). APEC-O78 strain (CVCC1418) was purchased from the Chinese Veterinary Culture Collection Center (CVCC).

Animals and experimental design

1-day-old male hyline-brown healthy chickens were provided by Jilin Academy of Agricultural Sciences (Changchun, Jilin, China). The whole experiment mainly included seven groups: control group (CON), Baicalin treatment alone group (BAI), broad-spectrum antibiotic group (Abx), APEC infection group (APEC), broad-spectrum antibiotic + APEC group (Abx+APEC), Baicalin + APEC group (BAI+APEC), Baicalin+ broad-spectrum antibiotic + APEC group (BAI + Abx + APEC), and each group included 10 chickens (n=10). The microbiota-deplete chickens were established with broad-spectrum antibiotic (ampicillin, neomycin, metronidazole and vancomycin) in their drinking water for two weeks. Baicalin was dissolved in a solvent mixture containing DMSO at a dose of 200 mg/kg was administrated by oral gavage 1 week before APEC infection. The model of chicken colibacillosis was induced by intratracheal inoculation with 2×10^9CFU APEC-O78 for 24 h. chickens had free access to feed and water during the entire rearing period. The chickens were reared in an environmentally controlled room: The temperature was maintained at 35 °C for the first 2 days, and then decreased gradually to 22 °C (45% humidity) under a 16/8 light/dark cycle.

Bronchoalveolar lavage fluid collection and measurement

The chickens were euthanatized and the cavity was opened by a midline incision. Then, the bronchoalveolar lavage fluid (BALF) were lavaged with sterile and ice of PBS (0.4 ml each time) for three times. After centrifuged at 2,000 g for 10 min at 4 °C, the supernatant was gathered to measure the concentration of total protein by BCA kits (Thermo, MA, USA).

RNA extraction and Quantitative RT-PCR

Total RNA of lung tissues was extracted using a TRIzol reagent (TaKaRa, Dalian, China) and was revered to cDNA by One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instruction. The gene expression of inflammatory cytokines and tight junction were performed using a 7500 real-time PCR system (Applied Biosystems, Foster, CA) with FastStart SYBR Green PCR Master Mix (Roche, Mannheim, Germany). Data were expressed as an n-fold difference relative to the expression of inner housekeeping gene β-actin. The primer sequences was shown in Table S11.

Hematoxylin and eosin staining

The partial heart, liver, spleen, lung and small intestine tissues of each group were removed and fixed with 10% formalin, were then dehydrated, and embedded in paraffin. To evaluate the pathological changes, the paraffin-embedded tissues were sliced into 5 μm thin sections to conduct hematoxylin and eosin staining, and then observed by a light microscope.

Viscera index measurement

After APEC infection for 24 h, the chickens of each group were euthanatized and obtain the weight. The complete heart, liver, spleen, lung, kidney and bursa of fabricius were harvested and weighed. The viscera index was calculated as each viscera weight divided by chicken weight.

16S rDNA

sequencing analysis

The microbial DNA from fecal and BALF samples was extracted using a E.Z.N.A.Stool DNA Kit (Omega, Inc., USA), dissolved in Elution buffer and stored at -80 °C until detection. Nuclear-free water was used for blank. The V3-V4 region of the bacterial 16s rDNA gene was amplified in a total volume of 25 μL reaction mixture containing 25 ng of template DNA, 12.5 μL PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. The amplification conditions were 98 °C for 30 seconds, 32 cycles of denaturation at 98 °C for 10 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 45 seconds; and then final extension at 72 °C for 10 minutes. The primers used in this study were as follows: 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3').

The samples were sequenced using an Illumina NovaSeq platform provided by LC-Bio. In 16sDNA analysis, the Alpha- and Beta- diversity were calculated by QIIME2 and graphed by R package. Blast was used for sequence alignment, and the feature sequences were annotated with SILVA database for each representative sequence. Other diagrams were implemented using the R package (v3.5.2).

Fecal metabolomics by LC/MS

In brief, the fecal samples were weighed for 100 mg and collected in 2 mL centrifuge tubes and added 600 μL 2-chlorophenylalanine (4 ppm) methanol (-20 °C), and then vortexed for 30 seconds. Subsequently, the mixture was sonicated at room temperature for 10 min and centrifuged at 14000 rpm at 4 °C for 10 min. The supernatant was filtered through 0.22 μm membrane to obtain the prepared samples for LC-MS.

The acquired MS data pretreatments including peak picking, peak grouping, retention time correction, second peak grouping, and annotation of isotopes and adducts was performed using XCMS software. LC-MS raw data fifiles were converted into mzXML format and then processed by the XCMS, CAMERA and metaX toolbox implemented with the R software. Each ion was identified by combining retention time (RT) and m/z data. Intensities of each peaks were recorded and a three dimensional matrix containing arbitrarily assigned peak indices (retention time-m/z pairs), sample names (observations) and ion intensity information (variables) was generated. The intensity of peak data was further preprocessed by metaX. Those features that were detected in less than 50 % of QC samples or 80 % of biological samples were removed, the remaining peaks with missing values were imputed with the k-nearest neighbor algorithm to further improve the data quality. PCA was performed for outlier detection and batch effects evaluation using the pre-processed dataset. Quality control-based robust LOESS signal correction was fitted to the QC data with respect to the order of injection to minimize signal intensity drift over time. In addition, the relative standard deviations of the metabolic features were calculated across all QC samples, and those $> 30\%$ were then removed. Student t-tests were conducted to detect differences in metabolite concentrations between 2 phenotypes. Supervised PLS-DA was conducted through metaX to discriminate the different variables between groups. The VIP value was calculated. A VIP cut-off value of 1.0 was used to select important features.

Statisticalanalysis

The analyses of chicken colibacillosis characteristics were performed using the Social Sciences (SPSS) version 19.0 (SPSS Inc., 2010 Chicago, IL, USA) and carried out in one-way analysis of variance (ANOVA) by Tukey-Kramer multiple comparison. The data were expressed as the means ± S.D. $P < 0.05$ was considered statistically significant.

Results

Protective effect of the gut microbiota during APEC-inducedchicken colibacillosis

To study the role the gut microbiota played in chicken colibacillosis, we first applied the broad-spectrum antibiotic (Abx) to deplete the gut microbiota. Administration of Abx resulted in a considerable reduction of richness (Fig. 1A) and diversity (Fig. 1B) of gut microbiota in chickens. Furthermore, principle coordinate analysis (PCoA) plot based on Unweighted Unifrac revealed that there was clear separation of clusters between control group compared with Abx-treated group chicken (Fig. 1C). In addition, Venn diagrams showing numbers of genera in Abx-treated group were significantly reduced (Fig. 1D). These results suggested that treatment of Abx significantly changes the structure of gut microbiota, and reduced the gut microbiota diversity and richness in chicken.

Furthermore, chicken from control and gut microbiota-depleted groups were intranasally challenged with APEC to induce the model of colibacillosis. Infection of APEC results in severe pathological injury of lung (Fig. 2A), and colon, heart, liver, and spleen (see supplementary fig. S1A), increased the production of proinflammatory cytokines TNF-α, IL-1β, and IL-6 both at gene and protein levels (Fig. 2B-D and supplementary Fig. S1B-D), as well as increased the bacterial outgrowth in the lung (Fig. 1E). In addition, infection of APEC also increased the viscera index of lung, heart, liver, spleen, and kidney (see supplementary Table S1). However, these inflammatory injuries induced by APEC were more pronounced in gut microbiota-depleted chicken when compared to the infected with control chicken (Fig. 2A-E and supplementary Fig. S1A-D and Table S1). Moreover, the concentration of total proteins in bronchoalveolar lavage fluid (BALF) and the expression of tight junction protein of lung tissues were measured to evaluate pulmonary air-blood barrier permeability. Infection of APEC increased the levels of total protein in BALF, and the levels of total protein were higher in Abx+APEC group than APEC group (Fig. 2F). In addition, the expression of junction protein of air-blood barrier, including occludin, claudin-3, and ZO-1 were significantly reduced after infected with APEC, and these changes are more obviously in gut Abx+APEC group (Fig. 2G-I). There results suggested that depletion of gut microbiota increase the severity of colibacillosis and increased the permeability of air-blood barrier induced by APEC.

The role of gut microbiota on the protective effects of baicalin in APEC-induced chicken colibacillosis

Gut microbiota plays a crucial role in herbal medicine therapy, whether gut microbiota was associated the protective role of baicalin on APEC-induced chicken colibacillosis was studied in present study. Treatment of baicalin after APEC infection significantly alleviated the pathological damages of lung (Fig. 3A), colon, heart, liver, and spleen pathological injuries see supplementary Fig. S2A), and viscera index (Table S2), reduced the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Fig. 3B-D and supplementary fig. S2B-D), and bacterial loads in the lung tissues (Fig. 3E). However, the protective effects of baicalin on APEC-induced colibacillosis were partly reversed when gut microbiota was depleted in chicken (Fig. 3A-E and supplementary Fig. S2A-C and Table S2). Furthermore, detection of the air-blood barrier permeability showed that depletion of gut microbiota partly counteracted the increase of the concentration of total protein in BALF (Fig. 3F) and the expression of tight junction protein including Occludin, claudin-3, and ZO-1 treated by baicalin (Fig. 3G-I). These results showed that gut microbiota played an important role on baicalin protects APEC-induced colibacillosis.

The changes of gut microbiota induced by treating with APEC and/or baicalin in chicken

The composition of gut microbiota in control (CON), baicalin-treated (BAI), APEC-treated (APEC), baicalin+APEC treatment (BAI+APEC) group chickens were differed in high-confidence OTUs, with lower diversity and richness in BAI group compared to CON group (Fig. 4A). In addition, infection of APEC significantly increased the microbiota diversity and richness compared to the CON group chickens (Fig. 4B-C). However, pre-treatment with BAI before APEC infection (BAI+APEC) reduced the higher levels of microbiota diversity and richness induced by APEC (Fig. 4B-C).

Furthermore, the changes of bacterial phyla in different treatment group chickens were showed that though gut microbiota in different treatment groups chicken were dominated by *Firmicutes*(means=87.32%-96.70%), *Proteobacteria* (means=0.62%-11.6%), *Tenericutes* (means=1.0%-2.16%), and *Actinobacteria*(means=0.20%-

0.79%) (Fig. 5A and Table S3). Infection of APEC reduced the relative abundance of *Proteobacteria* , and increased the relative abundance of *Actinobacteria* compared to the CON group chickens, while BAI treatment (BAI+APEC) group reversed those changes induced by APEC (Fig. 5B). In addition, the relative abundance of*Proteobacteria* in BAI group was higher than those in CON group chickens, while *Actinobacteria* was lower than those in CON group chickens (Fig. 5B).

Moreover, the characteristics of microbiota composition at the genus level were detected at the top 20 most abundant taxa indicated that the gut bacterial was dominated by *Lachnospiraceae_unclassified* ,*Ruminococcaceae_UCG-014* , *Ruminococcaceae_unclassified* ,*Intestinimonas* , *Blautia* , *Escherichia-Shigella* ,*Erysipelatoclostridium* , *Ruminococcus_torques_group* ,*Clostridiales_vadin BB60-group_unclassified* , and*Fournierella* (Fig. 5C and supplementary Table S4). Furthermore, t-test analysis revealed infection of APEC increased the abundance of*Ruminococcaceae_UCG-014* , *Ruminococcaceae_unclassified* ,*Clostridiales_vadinBB60_group_unclassified* , and*Ruminiclostridium_5* , while reduced the abundance of*Lachnospiraceae_unclssified* , *Blautia* ,*Escherichia-Shigella* , and *Pygmaiobacter* . However, those changes were reversed in BAI+APEC group (Fig. 5D).

In addition, the proportion of shared bacterial genera among CON, BAI, APEC, and BAI+APEC group chickens were 25.72%, 23,63%, 19.03%, and 22.01% (Fig. S3). Furthermore, to evaluated whether a unique bacterial was associated with the protective role of BAI during APEC-induced chicken colibacillosis, we conducted a biomarker analysis by linear discriminant analysis (LDA = 4.0) effect size (LEfSe) and a cladogram generated from LEfSe analysis on the microbiota on the different treatment group chickens. At both genus and species levels,*Clostridiales_vadinBB60_group_unclassified* ,*Ruminococcaceae_UCG_014* , and*Ruminococcaceae_unclassified* were enriched in the chicken of infected with APEC compared to the CON group chickens (Fig. 6A-B). While pretreatment of BAI partly inhibited these bacteria induced by APEC (Fig. 6C-D). In addition, compared to the CON group chickens,*Intestinimonas* genera was enriched in the chicken of treated with BAI (Fig. 6E-F). These results suggested that baicalin may protect against chicken colibacillosis partly by reversing the changes of gut microbiota induced by APEC.

The effect of baicalin on lung microbiota in chicken

To detect whether the protective role of baicalin in APEC-induced colibacillosis was associated with the changes of lung microbiota, we analyzed the lung bacterial community after treated with baicalin alone. The chao-1 and shannon index showed that there was no difference at both bacterial diversity and richness between the CON and BAI group chickens (Fig. 7A-B). In addition, PCoA with unweighted Unifrac distances indicated that CON and BAI groups were no significantly separated at the OUT levels (Fig. 7C). Further research showed that *Proteobacteria*(CON vs BAI=82.36% vs 78.43%), *Firmicutes* (CON vs BAI=7.78% vs 12.15%), *Bacteroidetes* (CON vs BAI=5.45% vs 4.91%), and*Actinobacteria* (CON vs BAI=1.24% vs 1.37%) were the dominant phyla (Fig. 7D and supplementary Table S5), and *Stenotrophomonas*(CON vs BAI=62.38% vs 56.84%), *Delftia* (CON vs BAI=3.7% vs 3.9%), *Moraxella* (CON vs BAI=2.0% vs 4.1%), and*Sphingomonas* (CON vs BAI=4.6% vs 2.9%) genus were the dominant genus in the lung microbiota of chicken, and there are no different were showed in CON and BAI group chickens (Fig, 7E and supplementary Table S6). These results suggested that the protective effect of baicalin on APEC-induced colibacillosis was associated with the changes of gut microbiota rather than the changes of lung microbiota.

The changes of gut metabolome induced by treating with APEC and/or baicalin in chicken

Principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) were conducted to the characterize the variations in the metabolic profiles of different treatment group chickens. PCA analysis showed that the axes 1 and 2 were up to 25.9% to 15.1% of the total variation, respectively, and the gut metabolites in different treated group chickens were clearly distinct showed in Fig. 8A. In addition, a PLS-DA analysis also revealed distinct clustering based on the different treatment groups chickens (Fig. 8B). These results revealed that infection of APEC changed the gut metabolic profiles, and BAI resulted in the creation of a new gut metabolite community that different with the CON and APEC group chickens. Furthermore, a total of 371 metabolites, including amines, amino acids, ortanic acids, fatty acids,

lipids, sugars, and nucleosides were identified in the gut of chicken. Among these, 65 metabolites were identified and 45 metabolites as differential between CON and APEC groups, characteristic by 9 metabolites were significantly up-regulated, while 36 metabolites were significantly up-regulated in APEC-treated group compared to the CON group chickens (Table S7). Moreover, when compared to the APEC and BAI+APEC group chickens. There are 61 metabolites were identified and 43 metabolites as differential between the two groups, and there are 9 metabolites were increased and 34 metabolites were reduced (Table S8). In addition, there are 82 metabolites were identified and 69 metabolites as differential between CON and BAI group chickens. There 14 metabolites were up-related and 55 metabolites were down-regulated in BAI groups compared to the CON group chickens (Table S9).

Metabolic pathway of differential metabolites

Function of the pathways associated with differential metabolites analysis by Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to understand how multiple pathways altered in response to treat with APEC and BAI in the present study. As shown in Fig. 9A, Phenylalanine metabolism; Phenylalanine, tyrosine and tryptophan biosynthesis; Cysteine and methionine metabolism; Riboflavin metabolism; Tryptophan metabolism; Alanine, aspartate and glutamate metabolism were enriched in the gut of the APEC group chicken compared to the CON group, while in addition to Alanine, aspartate and glutamate metabolism, the D-Glutamine and D-glutamate metabolism; Caffeine metabolism, Lysine biosynthesis; Histidine metabolism; Arginine and proline metabolism; TCA cycle; Retinol metabolism; and alpha-Linolenic acid metabolism were enriched in BAI+APEC group compared to the APEC group chickens (Fig. 9B). In addition, Ubiquinone and other terpenoid-quinone biosynthsis; Linoleic acid metabolism; Caffeine metabolism; Riboflavin metabolism; Alanine, aspartate and glutamate metabolism; Histidine metabolism; Glycine, serine and threonine metabolism; Cysteine and methionine metabolism; and alpha-Linolenic acid metabolism were enriched in the gut of the BAI group compared to the CON group chickens (Fig. 9C).

Correlation analysis between the gut microbiota and metabolome

To obtain insight into microbiota-metabolic axis, we conducted a correlation analysis on the significant microbial taxa and metabolites. Gut microbiota analysis showed that infection of APEC increased the abundance of *Clostridiales_vadinBB60_group* , and*Ruminococcaceae_UCG_014* (Fig. 6A). Correlation analysis showed that they were both positively associated with Xanthine, Deoxyinosine, 21-Hydroxypregnenolone, Serotonin, 3,4-Dihydroxyhydrocinnamic acid, 8,9-DiHETrE, Dihydrofolic acid, Corticosterone, and Dihydrocortisol, while were negatively associated with (S)-2-Amino-6-Oxohexanoate and Guanine (Fig. 10A). In contrast, pretreatment with BAI reduced the concentration of 21-Hydroxypregnenolone, Serotonin, 8,9-DiHETrE, Dihydrofolic acid, and Dihydrocortisol induced by APEC (Table S11). Except for reduced the abundance of*Clostridiales_vadinBB60_group* and*Ruminococcaceae_UCG_014* induced by APEC in the gut, pretreated with BAI also increased the abundance of *Blautia* , and*Fournierella* (Fig. 6B). Correlation analysis showed that they were both positively associated with 2-oxoarginine, while negatively associated with L-Asparagine, 3-Dehydroquinate, Prostaglandin G2, 5-Phosphoribosylamine, 5-Methoxyindoleacetate, and 4-Pyridoxic acid (Fig. 10B). In addition, gut microbiota analysis showed that*Intestinimonas* was enriched in the gut of chicken that treated with BAI compared to the CON group (Fig. 6C). As shown in Fig. 10C,*Intestinimonas* was positively associated with the abundance of Gentamicin C1a, 3,4-Dihydroxyhydrocinnamic acid, Selenodiglutathione, L-Arabinose, Serotonin, Norfloxacin, 2-Oxoarginine, Epinephrine, and Xanthine, while both negatively associated with Gulonic acid, 5-Methoxyindoleacetate, Prostaglandin G2, 3,4-Dihydroxyhydrocinnamic acid, Palmitic acid, Dehydroepiandrosterone, Isonicotinic acid, FMN, 2-Dehydro-3-Deoxy-L-Rhamnonate, 4-Pyridoxic acid, 12(S)-HpETE, Ubiquinone-1, D-Fructose, Picolinic acid, (S)-2-Amino-6-Oxohexanoate, 5-Phosphoribosylamine, Hydroquinone. These results suggested the protective effect of baicalin on colibacillosis induced by APEC was not only associated with regulating gut metabolites induced by APEC, but may also associated the production of new gut metabolites after baicalin metabolized by APEC.

Discussion

Chicken colibacillosis, characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis, initially develops in the respiratory tract and air sacs and then takes the form of sepsis, causing considerable mortality in poultry(Wernicki, Nowaczek, & Urban-Chmiel, 2017). Previous studies have reported that the air-exchange regions of the lung and the airsacs are important sites of entry of *E. coli* into the bloodstream of birds during the initial stages of infection and that resistance to phagocytosis may be an important mechanism in the development of the disease (Dho-Moulin & Fairbrother, 1999). Therefore, controlling the bacterial infection in lung and airsacs may be a pivotal method to protect against colibacillosis. Therefore, we established the model of colibacillosis by intratracheal inoculation with 2×10^9 CFU APEC-O78 to mimic the natural infection process.

The gut microbiome, which consists of trillions of bacterial, viral, and fungal microorganisms, is a complex ecosystem that can mediate the interaction of the host and their environment(Blum, 2017). Growing evidences have shown that gut microbiota is closely related to health and various diseases including neuropsychiatric disorders (Cenit, Sanz, & Codoner-Franch, 2017), inflammatory bowel disease (Frank et al., 2007), Type 1 Diabetes(Dedrick et al., 2020), atherosclerosis (D. Y. Li & Tang, 2017) and acute respiratory distress syndrome(Mukherjee & Hanidziar, 2018). Especially, the beneficial role of the gut microbiota through "gut-lung axis" during pulmonary bacterial infections has been explored in some researches (Dumas, Bernard, et al., 2018). Therefore, we conjectured that gut microbiota also played an important role in chicken colibacillosis, an avian respiratory disease. To explore the effects of gut microbiota during colibacillosis, we pretreated chicken with Abx for two weeks to deplete the microbiota(Dumas, Corral, et al., 2018). Two days later, the colibacillosis was induced and relevant indicators were examined. The experimental results indicated that infected with APEC induced significantly pathological damages of heart, spleen, liver, kidney, and lung especially, increased the inflammatory cytokines secretion and gene expression in lung tissues. Importantly, these changes were more severity in gut microbiota-depleted chickens when compared to the control chickens. The air-blood barrier is essential to maintain the homeostasis of the lung microenvironment (L. Y. Zhang et al., 2016). In the present study, the air-blood barrier integrity was markedly impaired showing by increased protein in BALF and decreased expression of tight junction induced by APEC when gut microbiota was depleted. Collectively, these results demonstrated that gut microbiota may contribute to protect against the chicken colibacillosis.

Baicalin, a main component of *scutellaria baicalensis*Georgi, , possesses extensive pharmacological activities, such as antioxidant, anti-apoptotic, anti-excitotoxicity and anti-inflammatory(Liang, Huang, & Chen, 2017). In previous study, we have proved that protective effects of baicalin against APEC-induced lung inflammation in chicken colibacillosis. However, the underlying mechanisms remains to be explored. Recently, there is accumulating evidence have reported the potential interactions between the gut microbiota and herbal medicines. These interactions mainly includes that gut microbiota can "digest" herbal medicines into absorbale active small molecules by enzymatically transforming, vice versa, herbal medicines can influence the composition and metabolite of gut microbiota(An et al., 2019; Weersma, Zhernakova, & Fu, 2020). As previously reported, codonopsis pilosula polysaccharide can be digested by gut microbiota to exert its pharmacological effect. Meanwhile, codonopsis pilosula polysaccharide can promote abundance of the beneficial gut microbiota and inhibit the colonization by pathogens(Jing et al., 2018). Whether baicalin has a same mechanism in the protection of chicken colibacillosis was still elusive.

Based on this, we firstly compared the pharmacological effect of baicalin in healthy chickens and microbiota-depleted chicken after infected by APEC to decipher the influence of gut microbiota on baicalin. The results showed that the effect of baicalin on ameliorating the air-blood permeability, inflammatory responses, lung bacterial loads and tissues damages were significantly diminished when gut microbiota was depleted. As we can see, the bacterial loads of lung tissue, BALF protein, production and gene expression of pro-inflammatory cytokines and histological lesion were distinctly elevated, and the expression of tight junction were markedly reduced incomparision with BAI+APEC group and BAI+Abx+APEC group. This phenomenon may imply that gut microbiota plays a pivotal role during the process of baicalin exerting pharmacological effects in colibacillosis. Refer to the previous literature(Jung, Jang, Hong, Hana, & Kim, 2012b), we supposed that baicalin may need to be metabolized by gut microbiota to play its therapeutic effect during chicken colibacillosis.

However, the specific mechanism still needs to be deeply explored. Furthermore, we explored the influence of baicalin on gut microbiota in APEC-induced colibacillosis. The results showed that infection with APEC significantly increased the gut bacterial diversity and richness, and increased the relative abundance of *Ruminococcaceae_UCG-014* ,*Ruminococcaceae_unclassified* , and*Clostridiales_vadinBB60_group_unclassified* , while these changes were reversed by pretreatment with baicalin.*Ruminococcaceae* family contains a large number of gut-associated butyrate producing bacteria, and they were positive association with total milk intake in piglets(Morissette, Talbot, Beaulieu, & Lessard, 2018). However, other studies suggested*Ruminococcaceae* was associated with the development of children with chronic pancreatitis(W. Wang et al., 2020). In addition, studies revelated that *Clostridiales* was associates with the development of inflammatory bowel diseases (IBD), and was negatively associated with the levels of SCFAs(Saitoh et al., 2015). Furthermore, we also found that treatment with baicalin increased the relative abundance of *intestinimonas* , which was associated with the production of SCFAs (Shomorony et al., 2020). These results suggested that gut microbiota and its metabolites may be play an important role in the pharmacological effects of baicalin on chicken colibacillosis.

In order to investigate the whether the changes of gut metabolites could provide further insight into specific gut microbiota-related changes and aimed to explore a new perspective targeting gut metabolism to find biomarkers of chickens colibacillosis and reveal the mechanism of the protective role of baicalin on chicken colibacillosis. We analyzed the gut metabolites in chickens from different treatment groups. The results showed that there are significant differences in metabolic composition among CON, APEC, BAI, and BAI+APEC group chickens. Combined analysis of gut microbiota and metabolites indicated that increased of concentration of Xanthine, Deoxyinosine, 21-Hydroxypregnenolone, Serotonin, 3,4-Dihydroxyhydrocinnamic acid, 8,9-DiHETrE, Dihydrofolic acid, and Corticosterone may be associated with the development of colibacillosis induced by APEC in chicken. Xanthine is a plant alkaloid which acts as an intermediate product on the pathway of purine degradation. It also serves as scaffold for various natural and synthetically derived bioactive molecules (Singh, Patra, & Patra, 2018). Deoxyinosine is an abnormal nucleoside and has hypoxanthine as its base moiety (Yoneshima et al., 2016). Studies indicated that exogenous deoxyinosine can be utilized to meet the carbon and energy requirements of growing cells (Carta et al., 2001). 21-Hydroxypregnenolone play an important role in steroid hormone biosynthesis, which does well in helping with memorization, anti-fatigue, and refreshing the neural system (C. Shao et al., 2018). However, other studies showed that 21-Hydroxypregnenolonemay be key biomarkers of hypothalamic-pituitary-adrenal (HPA) axis abnormalities in patients with chronic fatigue syndrome (CFS)(C. Z. Shao et al., 2017). Serotonin, synthesized mainly by enterochromaffin cells (ECs), is an important gut neuroendocrine factor which have ability to regulate gut motility (Ge et al., 2017). Previous studies demonstrated that fecal pellets facilitate serotonin release from enterochromaffin cells by regulating the activation of local mucosal reflexes and stretch reflexes of colonic migrating motor complex to facilitate propulsion (Ge et al., 2017; Heredia, Dickson, Bayguinov, Hennig, & Smith, 2009). Others studies also showed that treatment with prebiotics and probiotics have antidepressive effects by regulating the Serotonin metabolism(H. Li, Wang, Huang, Li, & Zhang, 2019). Oxylipins, namely 8,9-DiHETrE, has been showed that it was associated with development of Alzhemer's disease risk, type 2 diabetes cardiovascular disease, cerebrovascular events, and acute coronary syndrome (ACS) (Morris et al., 2019; Solati & Ravandi, 2019). Dihydrofolic acid can be converted into tetrahydrofolic acid, and tetrahydrofolic acid is activated to 5,10-methylenetetrahydrofolic acid by the action of serine hydroxymethylase (B. X. Zhang, Qi, & Cai, 2020). Corticosterone, a stress hormone, the main glucocorticoids released from hypothalamus-pitutary-adrenal (HPA) axis. Recently studies indicated that Corticosterone was elevated in mice that received mild stress at a neurobiological level(Fuentes-Verdugo et al., 2020), and others showed that Corticosterone impairs the recall of strong contextual fear memory after reactivation in the ovariectomized rat model of menopause (Kashefi et al., 2020). Importantly, BAI not only significantly reduced the levels of 21-Hydroxypregnenolone, Serotonin, 8,9-DiHETrE, Dihydrofolic acid, and Dihydrocortisol in the gut, but also increased the abundance of*Blautia* and *Fournierella* and the associated metabolites 2-oxoarginine in the chickens suffered colibacillosis. Studies showed that arginine/proline metabolic pathway compounds 2-Oxoarginine, a guanidino metabolite of arginine, were negatively associated with the glioma risk (Huang et al., 2017). Others also suggested that 2-Oxoarginine may play an important role to chelate Mo and reduce its toxicity(Xu et al., 2018). In addition, *Intestinimonas* and its positively associates metabolites Gentamicin

C1a, Selenodiglutathione, L-Arabinose, Serotonin, Norfloxacin, 2-Oxoarginine, Epinephrine, and Xanthine were increased in the gut of chicken that treated with BAI compared to the CON chicken. Gentamicin C1a is considered as a precursor to the synthesis of etimicin (Wei et al., 2019). Selenodiglutathione is a primary Se metabolite conjugated to two glutathione moieties, and it increase intracellular Se accumulation and is more toxic than selenous acid (Fisher et al., 2016; Tobe et al., 2017). L-arabinose, a kind of plant-specific five-carbon aldose, is a naturally occurring constituent of plant polysaccharides, and often extracted from vegetable gum, corn straw or beet(Feher, Gal, Feher, Barta, & Reczey, 2015; Kotake, Yamanashi, Imaizumi, & Tsumuraya, 2016; Moremi, Jansen Van Rensburg, & La Grange, 2020). Recently, L-Arabinose has been used as a food additive, nutritional supplement, and have many pharmacological activities, including inhibited intestinal sucrose activity and triglyceride in blood serum and fat accumulation, increased insulin resistance, promoted the proliferation of probiotics, increased the synthesis and production of organic acids and SCFAs, as well as ameliorates colitis and metabolic syndrome by regulating gut microbiota in mice(Kaats et al., 2011; Y. Li et al., 2019; Osaki, Kimura, Sugimoto, Hizukuri, & Iritani, 2001; Seri et al., 1996; Zhao et al., 2019). Norfloxacin, a fluoroquinolone antibiotic, has an activity and enhances a regulatory T cell-mediated inflammatory response properties(Juanola et al., 2016). These results suggested the protective effect of baicalin on chickens colibacillosis induced by APEC was not only associated with regulating gut metabolites, such as 21-Hydroxypregnenolone, Serotonin, 8,9-DiHETrE, Dihydrofolic acid, and Dihydrocortisol, induced by APEC, but may also associated the production of new gut metabolites by the gut microbiota metabolizing baicalin.

In conclusion, the present study showed that gut microbiota plays an important role in the development of colibacillosis and the protective effect of baicalin on chickens colibacillosis. Moreover, increases the abundance of *Clostridiales_vadinBB60_group* and*Ruminococcaceae_UCG_014* , as well as the levels of 21-Hydroxypregnenolone, Serotonin, 8,9-DiHETrE, Dihydrofolic acid, and Dihydrocortisol may be serve as the biomarkers of chicken colibacillosis. In addition, the pharmacological effects of baicalin on chicken colibacillosis may be not only by inhibiting the increase the specific gut microbiota and metabolites induced by APEC, but also by regulating the new metabolites produced by fermentation of baicalin by gut microbiota. However, these results need to be further verified by more experiments.

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ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Jilin University for animal experiments approvals published by Jilin University.

Author contributions

L.Y.P. and H.T.SH. performed the experiments and analyzed the data. L.Y.P., Z.X.G., J.H.L and J.N.H wrote the manuscript. P.F.Y., H.Q.SH. and B.D.F. conceived of project and designed experiments. All authors read and approved the paper.

Conflict of interest

The authors declare no conflicts of interest.

References

An, X. D., Bao, Q., Di, S., Zhao, Y. R., Zhao, S. H., Zhang, H. Y., ... Tong, X. L. (2019). The interaction between the gut Microbiota and herbal medicines.*Biomedicine & Pharmacotherapy, 118* doi:Unsp 109252

10.1016/J.Biopha.2019.109252

Blum, H. E. (2017). The human microbiome. *Adv Med Sci, 62* (2), 414-420. doi:10.1016/j.advms.2017.04.005

Budden, K. F., Gellatly, S. L., Wood, D. L. A., Cooper, M. A., Morrison, M., Hugenholtz, P., & Hansbro, P. M. (2017). Emerging pathogenic links between microbiota and the gut-lung axis. *Nature Reviews Microbiology, 15* (1), 55-63. doi:10.1038/nrmicro.2016.142

Carta, M. C., Mattana, A., Camici, M., Allegrini, S., Tozzi, M. G., & Sgarrella, F. (2001). Catabolism of exogenous deoxyinosine in cultured epithelial amniotic cells.*Biochim Biophys Acta, 1528* (2-3), 74-80. doi:10.1016/s0304-4165(01)00175-1

Cenit, M. C., Sanz, Y., & Codoner-Franch, P. (2017). Influence of gut microbiota on neuropsychiatric disorders. *World J Gastroenterol, 23* (30), 5486-5498. doi:10.3748/wjg.v23.i30.5486

Creely, S. J., McTernan, P. G., Kusminski, C. M., Fisher f, M., Da Silva, N. F., Khanolkar, M., ... Kumar, S. (2007). Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes.*Am J Physiol Endocrinol Metab, 292* (3), E740-747. doi:10.1152/ajpendo.00302.2006

Dedrick, S., Sundaresh, B., Huang, Q., Brady, C., Yoo, T., Cronin, C., ... Altindis, E. (2020). The Role of Gut Microbiota and Environmental Factors in Type 1 Diabetes Pathogenesis. *Front Endocrinol (Lausanne), 11* , 78. doi:10.3389/fendo.2020.00078

Dho-Moulin, M., & Fairbrother, J. M. (1999). Avian pathogenic Escherichia coli (APEC). *Vet Res, 30* (2-3), 299-316. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10367360

Dickson, R. P., Martinez, F. J., & Huffnagle, G. B. (2014). The role of the microbiome in exacerbations of chronic lung diseases. *Lancet, 384* (9944), 691-702. doi:10.1016/S0140-6736(14)61136-3

Dumas, A., Bernard, L., Poquet, Y., Lugo-Villarino, G., & Neyrolles, O. (2018). The role of the lung microbiota and the gut-lung axis in respiratory infectious diseases.*Cell Microbiol, 20* (12), e12966. doi:10.1111/cmi.12966

Dumas, A., Corral, D., Colom, A., Levillain, F., Peixoto, A., Hudrisier, D., ... Neyrolles, O. (2018). The Host Microbiota Contributes to Early Protection Against Lung Colonization by Mycobacterium tuberculosis. *Front Immunol, 9* , 2656. doi:10.3389/fimmu.2018.02656

Feher, C., Gal, B., Feher, A., Barta, Z., & Reczey, K. (2015). Investigation of commercial enzyme preparations for selective release of arabinose from corn fibre.*Journal of Chemical Technology and Biotechnology, 90* (7), 1329-1337. doi:10.1002/jctb.4440

Fisher, B., Yarmolinsky, D., Abdel-Ghany, S., Pilon, M., Pilon-Smits, E. A., Sagi, M., & Van Hoewyk, D. (2016). Superoxide generated from the glutathione-mediated reduction of selenite damages the iron-sulfur cluster of chloroplastic ferredoxin.*Plant Physiol Biochem, 106* , 228-235. doi:10.1016/j.plaphy.2016.05.004

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A, 104* (34), 13780-13785. doi:10.1073/pnas.0706625104

Fuentes-Verdugo, E., Pellon, R., Papini, M. R., Torres, C., Fernandez-Teruel, A., & Anselme, P. (2020). Effects of partial reinforcement on autoshaping in inbred Roman high- and low-avoidance rats. *Physiol Behav, 225* , 113111. doi:10.1016/j.physbeh.2020.113111

Ge, X. L., Ding, C., Zhao, W., Xu, L. Z., Tian, H. L., Gong, J. F., ... Li, N. (2017). Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. *Journal of Translational Medicine, 15* doi:ARTN 13

10.1186/s12967-016-1105-4

Guabiraba, R., & Schouler, C. (2015). Avian colibacillosis: still many black holes. *FEMS Microbiol Lett, 362* (15), fnv118. doi:10.1093/femsle/fnv118

Heredia, D. J., Dickson, E. J., Bayguinov, P. O., Hennig, G. W., & Smith, T. K. (2009). Localized release of serotonin (5-hydroxytryptamine) by a fecal pellet regulates migrating motor complexes in murine colon. *Gastroenterology, 136* (4), 1328-1338. doi:10.1053/j.gastro.2008.12.010

Huang, J. Q., Weinstein, S. J., Kitahara, C. M., Karoly, E., Sampson, J. N., & Albanes, D. (2017). A prospective study of serum metabolites and glioma risk. *Cancer research, 77* doi:10.1158/1538-7445.AM2017-2266

Jing, Y., Li, A., Liu, Z., Yang, P., Wei, J., Chen, X., ... Zhang, C. (2018). Absorption of Codonopsis pilosula Saponins by Coexisting Polysaccharides Alleviates Gut Microbial Dysbiosis with Dextran Sulfate Sodium-Induced Colitis in Model Mice.*Biomed Research International, 2018* , 1781036. doi:10.1155/2018/1781036

Juanola, O., Gomez-Hurtado, I., Zapater, P., Moratalla, A., Caparros, E., Pinero, P., ... Frances, R. (2016). Selective intestinal decontamination with norfloxacin enhances a regulatory T cell-mediated inflammatory control mechanism in cirrhosis. *Liver Int, 36* (12), 1811-1820. doi:10.1111/liv.13172

Jung, M. A., Jang, S. E., Hong, S. W., Hana, M. J., & Kim, D. H. (2012a). The Role of Intestinal Microflora in Anti-Inflammatory Effect of Baicalin in Mice.*Biomolecules & Therapeutics, 20* (1), 36-42. doi:10.4062/biomolther.2012.20.1.036

Jung, M. A., Jang, S. E., Hong, S. W., Hana, M. J., & Kim, D. H. (2012b). The role of intestinal microflora in anti-inflammatory effect of baicalin in mice. *Biomol Ther (Seoul), 20* (1), 36-42. doi:10.4062/biomolther.2012.20.1.036

Kaats, G. R., Keith, S. C., Keith, P. L., Leckie, R. B., Perricone, N. V., & Preuss, H. G. (2011). A combination of l-arabinose and chromium lowers circulating glucose and insulin levels after an acute oral sucrose challenge. *Nutr J, 10* , 42. doi:10.1186/1475-2891-10-42

Kashefi, A., Rashidy-Pour, A., Vafaei, A. A., Haghparast, A., Tomaz, C., & Ahmadalipour, A. (2020). Corticosterone impairs contextual fear recall after reactivation in the ovariectomized rat model of menopause. *Behav Brain Res, 394* , 112817. doi:10.1016/j.bbr.2020.112817

Kotake, T., Yamanashi, Y., Imaizumi, C., & Tsumuraya, Y. (2016). Metabolism of L-arabinose in plants.*J Plant Res, 129* (5), 781-792. doi:10.1007/s10265-016-0834-z

Li, D. Y., & Tang, W. H. W. (2017). Gut Microbiota and Atherosclerosis. *Curr Atheroscler Rep, 19* (10), 39. doi:10.1007/s11883-017-0675-9

Li, H., Wang, P., Huang, L., Li, P., & Zhang, D. (2019). Effects of regulating gut microbiota on the serotonin metabolism in the chronic unpredictable mild stress rat model.*Neurogastroenterol Motil, 31* (10), e13677. doi:10.1111/nmo.13677

Li, Y., Pan, H., Liu, J. X., Li, T., Liu, S., Shi, W., ... Wang, L. (2019). l-Arabinose Inhibits Colitis by Modulating Gut Microbiota in Mice. *J Agric Food Chem, 67* (48), 13299-13306. doi:10.1021/acs.jafc.9b05829

Liang, W., Huang, X., & Chen, W. (2017). The Effects of Baicalin and Baicalein on Cerebral Ischemia: A Review. *Aging Dis, 8* (6), 850-867. doi:10.14336/AD.2017.0829

Moremi, M. E., Jansen Van Rensburg, E. L., & La Grange, D. C. (2020). The Improvement of Bioethanol Production by Pentose-Fermenting Yeasts Isolated from Herbal Preparations, the Gut of Dung Beetles, and Marula Wine. *Int J Microbiol, 2020* , 5670936. doi:10.1155/2020/5670936

Morissette, B., Talbot, G., Beaulieu, C., & Lessard, M. (2018). Growth performance of piglets during the first two weeks of lactation affects the development of the intestinal microbiota. *J Anim Physiol Anim Nutr (Berl), 102* (2), 525-532. doi:10.1111/jpn.12784

Morris, J. K., Piccolo, B. D., John, C. S., Green, Z. D., Thyfault, J. P., & Adams, S. H. (2019). Oxylipin Profiling of Alzheimer's Disease in Nondiabetic and Type 2 Diabetic Elderly. *Metabolites, 9* (9)doi:Artn 177

10.3390/Metabo9090177

Mukherjee, S., & Hanidziar, D. (2018). More of the Gut in the Lung: How Two Microbiomes Meet in ARDS. *Yale J Biol Med, 91* (2), 143-149. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/29955219

Noverr, M. C., & Huffnagel, G. B. (2004). Does the microbiota regulate immune responses outside the gut? *Trends in microbiology, 12* (12), 562-568. doi:DOI 10.1016/j.tim.2004.10.008

Osaki, S., Kimura, T., Sugimoto, T., Hizukuri, S., & Iritani, N. (2001). L-arabinose feeding prevents increases due to dietary sucrose in lipogenic enzymes and triacylglycerol levels in rats. *Journal of Nutrition, 131* (3), 796-799. Retrieved from <Go to ISI>://WOS:000167405400016

Peek, H. W., Halkes, S. B., Tomassen, M. M., Mes, J. J., & Landman, W. J. (2013). In vivo screening of five phytochemicals/extracts and a fungal immunomodulatory protein against colibacillosis in broilers. *Avian Pathol, 42* (3), 235-247. doi:10.1080/03079457.2013.780121

Saitoh, Y., Suzuki, H., Tani, K., Nishikawa, K., Irie, K., Ogura, Y., ... Fujiyoshi, Y. (2015). Structural insight into tight junction disassembly by Clostridium perfringens enterotoxin. *Science, 347* (6223), 775-778. doi:10.1126/science.1261833

Schuijt, T. J., Lankelma, J. M., Scicluna, B. P., de Sousa e Melo, F., Roelofs, J. J., de Boer, J. D., ... Wiersinga, W. J. (2016). The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut, 65* (4), 575-583. doi:10.1136/gutjnl-2015-309728

Seri, K., Sanai, K., Matsuo, N., Kawakubo, K., Xue, C., & Inoue, S. (1996). L-arabinose selectively inhibits intestinal sucrase in an uncompetitive manner and suppresses glycemic response after sucrose ingestion in animals. *Metabolism, 45* (11), 1368-1374. doi:10.1016/s0026-0495(96)90117-1

Shao, C., Song, J., Zhao, S., Jiang, H., Wang, B., & Chi, A. (2018). Therapeutic Effect and Metabolic Mechanism of A Selenium-Polysaccharide from Ziyang Green Tea on Chronic Fatigue Syndrome. *Polymers (Basel), 10* (11)doi:10.3390/polym10111269

Shao, C. Z., Ren, Y. M., Wang, Z. N., Kang, C. Z., Jiang, H. K., & Chi, A. P. (2017). Detection of Urine Metabolites in a Rat Model of Chronic Fatigue Syndrome before and after Exercise. *Biomed Research International, 2017* doi:Artn 8182020

10.1155/2017/8182020

Shomorony, I., Cirulli, E. T., Huang, L., Napier, L. A., Heister, R. R., Hicks, M., ... Shah, N. (2020). An unsupervised learning approach to identify novel signatures of health and disease from multimodal data. *Genome Med, 12* (1), 7. doi:10.1186/s13073-019-0705-z

Singh, N., Patra, S., & Patra, S. (2018). Identification of Xanthine Derivatives as Inhibitors of Phosphodiesterase 9A Through in silico and Biological Studies. *Combinatorial Chemistry & High Throughput Screening, 21* (7), 476-486. doi:10.2174/1386207321666180821100713

Solati, Z., & Ravandi, A. (2019). Lipidomics of Bioactive Lipids in Acute Coronary Syndromes. *Int J Mol Sci, 20* (5)doi:10.3390/ijms20051051

Tobe, T., Ueda, K., Aoki, A., Okamoto, Y., Kojima, N., & Jinno, H. (2017). Selenium uptake through cystine transporter mediated by glutathione conjugation. *Journal of Toxicological Sciences, 42* (1), 85-91. Retrieved from <Go to ISI>://WOS:000393735300009

Trompette, A., Gollwitzer, E. S., Yadava, K., Sichelstiel, A. K., Sprenger, N., Ngom-Bru, C., ... Marsland, B. J. (2014). Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nature Medicine, 20* (2), 159-166. doi:10.1038/nm.3444

Wang, W., Xiao, Y., Wang, X., Zhou, Y., Wang, T., Xv, C., & Shen, B. Y. (2020). Disordered Gut Microbiota in Children Who Have Chronic Pancreatitis and Different Functional Gene Mutations. *Clin Transl*

Gastroenterol, 11 (3), e00150. doi:10.14309/ctg.0000000000000150

Wang, Z. L., Wang, S., Kuang, Y., Hu, Z. M., Qiao, X., & Ye, M. (2018). A comprehensive review on phytochemistry, pharmacology, and flavonoid biosynthesis of Scutellaria baicalensis. *Pharm Biol, 56* (1), 465-484. doi:10.1080/13880209.2018.1492620

Weersma, R. K., Zhernakova, A., & Fu, J. (2020). Interaction between drugs and the gut microbiome. *Gut* doi:10.1136/gutjnl-2019-320204

Wei, Z., Shi, X. A., Lian, R., Wang, W. B., Hong, W. R., & Guo, S. B. (2019). Exclusive Production of Gentamicin C1a from Micromonospora purpurea by Metabolic Engineering. *Antibiotics-Basel, 8* (4)doi:ARTN 267

10.3390/antibiotics8040267

Wernicki, A., Nowaczek, A., & Urban-Chmiel, R. (2017). Bacteriophage therapy to combat bacterial infections in poultry. *Virol J, 14* (1), 179. doi:10.1186/s12985-017-0849-7

Xu, S., Hu, C., Hussain, S., Tan, Q., Wu, S., & Sun, X. (2018). Metabolomics analysis reveals potential mechanisms of tolerance to excess molybdenum in soybean seedlings. *Ecotoxicol Environ Saf, 164* , 589-596. doi:10.1016/j.ecoenv.2018.08.062

Yoneshima, Y., Abolhassani, N., Iyama, T., Sakumi, K., Shiomi, N., Mori, M., ... Nakabeppu, Y. (2016). Deoxyinosine triphosphate induces MLH1/PMS2- and p53-dependent cell growth arrest and DNA instability in mammalian cells. *Sci Rep, 6* , 32849. doi:10.1038/srep32849

Zhang, B. X., Qi, X. J., & Cai, Q. (2020). Metabolomic study of raw and bran-fried Atractylodis Rhizoma on rats with spleen deficiency. *Journal of Pharmaceutical and Biomedical Analysis, 182* doi:Artn 112927

10.1016/J.Jpba.2019.112927

Zhang, L. Y., Yi, P. F., Guo, X., Wu, S. C., Fu, Y. X., Zhang, C., ... Wei, X. B. (2016). Astragaloside IV Inhibits the Inflammatory Injury of Chicken Type II Pneumocytes Induced by Avian Pathogenic Escherichia coli. *Inflammation, 39* (5), 1660-1669. doi:10.1007/s10753-016-0400-9

Zhao, L., Wang, Y., Zhang, G., Zhang, T., Lou, J., & Liu, J. (2019). L-Arabinose Elicits Gut-Derived Hydrogen Production and Ameliorates Metabolic Syndrome in C57BL/6J Mice on High-Fat-Diet. *Nutrients, 11* (12)doi:10.3390/nu11123054