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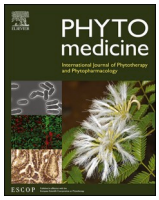

Original Article

Mulberry leaf benefits the intestinal epithelial barrier via direct anti-oxidation and indirect modulation of microbiota in pigs

Hui Yan [a,1,*], Shurui Yan [a,1], Zaiyao Li [a], Tingting Zhang [a], Jun He [a], Bing Yu [a], Jie Yu [a], Junqiu Luo [a], Aimin Wu [a], Junning Pu [a], Quyuan Wang [a], Huifen Wang [a], Xingyu Liu [b], Daiwen Chen [a,*]

[a] Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China

[b] Sichuan Shanghao Tea Co., Ltd., Nanchong, Sichuan 637000, China

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ABSTRACT

Background: Diarrhea and intestinal dysfunction commonly occur in young mammals, causing malnutrition and growth retardation in both human and livestock. As the traditional Chinese herb, mulberry leaf contains various bioactive compounds and showed several health benefits, such as regulating glucose and lipid metabolism, and modulating gut microbiota. Mulberry leaf exhibits the potential to modulate redox homeostasis and improve gut health, but the function and underlying mechanisms remains elucidative.

Purpose: To investigate the benefit of mulberry leaf on intestinal barrier in weanling pigs, illustrate the possible involvement of Keap1-Nrf2 mediated anti-oxidation and gut microbiota.

Methods: Chemical compositions of mulberry leaf powder (MLP) and mulberry leaf extract (MLE) were determined. The effects of MLP on growth performance, intestinal barrier integrity, anti-oxidative capacity, immune function and gut microbiota were evaluated in weaned pigs. The regulation of redox homeostasis by MLE and the involvement of Keap1-Nrf2 signaling were further determined in H_2O_2 induced oxidative stress (OS) model in IPEC-J2 cells via determining reactive oxygen species (ROS) production by flow cytometry and related protein abundance by western blot analysis.

Results: In weanling pigs, MLP reduced diarrhea incidence, and increased villus height, intestinal integrity and expression of tight junctions in intestinal mucosa. The improvement of intestinal barrier by MLP was associated with the enhancement in anti-oxidative capacity and the changes in gut microbiota and related short chain fatty acids production. Our study further revealed the direct regulation of MLE on tight junction expressions and ROS production to alleviate H_2O_2 induced OS in IPEC-J2 cells via the activating Keap1-Nrf2 signaling pathway.

Conclusions: Mulberry leaf in diet improved epithelial barrier via the direct anti-oxidation through the activation of Keap1-Nrf2 signaling pathway and the indirect modulation of gut microbiota in weaned pigs.

Abbreviation: AA, acetic acid; ADF, acid detergent fiber; ADFI, average daily feed intake; ADG, average daily gain; BA, butyric acid; CAT, catalase; CF, crude fiber; CP, crude protein; DI, diarrhea incidence; F/G, feed to gain ratio; GIT, gastrointestinal tract; GPX, glutathione peroxidase; HO-1, heme oxygenase 1; IDF, insoluble dietary fiber; Ig, immunoglobulin; IPEC-J2, porcine jejunal epithelial cells; Keap1, kelch like ECH associated protein 1; MDA, malondialdehyde; MLE, mulberry leaf extract; MLP, mulberry leaf powder; NDF, neutral detergent fiber; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, quinoneoxidoreductase; OS, oxidative stress; PA, propionic acid; p-Nrf2, phospho- nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SDF, soluble dietary fiber; SCFAs, short chain fatty acids; sIgA, secretory immunoglobulin A; T-AOC, total anti-oxidative capacity; TDF, total dietary fiber; TNF-α, tumor necrosis factor-α; SOD, superoxide dismutase; ZO-1, zonula occludens 1..

* Corresponding authors.
E-mail addresses: yan.hui@sicau.edu.cn (H. Yan), chendwz@sicau.edu.cn (D. Chen).

[1] Hui Yan and Shurui Yan contributed equally to this work.

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Introduction

The gastrointestinal tract (GIT) of young mammals, such as weanling pigs and infants, are vulnerable to weaning stress due to changes in diet composition, nutritional structure and eating habits (Indrio et al., 2022; Tang et al., 2021), contributing to intestinal barrier injury, diarrhea and growth retardation. Weaning stress also triggers mitochondrial dysfunction and over-production of reactive oxygen species (ROS), which disrupts redox homeostasis and results in oxidative stress (OS) in intestines (Sies, 2015). Excessive ROS induces epithelial apoptosis by the down-regulating Bcl-2 family protein and activating apoptotic caspase (Ashkenazi et al., 2017; Shi, 2002). The unclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, a classic redox signaling, has been reported to regulate expressions of intestinal tight junction proteins, such as claudins, zonula occludin-1 (ZO-1) and occludin, which are critical to maintain intestinal epithelial barrier integrity (Karam et al., 2017; Singh et al., 2019). Therefore, maintaining redox homeostasis through Nrf2 activation can enhance intestinal barrier function, reducing the risk of diarrhea and other GIT dysfunctions.

Moreover, previous study indicated that the intestinal function, mainly nutrient digestion and absorption, is positively correlated with anti-oxidative capacity of weanling pigs (Yan et al., 2022). Gut microbiota is also closely associated with OS and gut barrier integrity (Shandilya et al., 2022). Briefly, OS increases gut permeability, allowing detrimental gut bacteria and the noxious metabolites to invade, which exacerbates oxidative damage and leads to inflammation (Karam et al., 2017). Thus, targeting the regulation of redox homeostasis to alleviate OS and enhance barrier integrity is a feasible and efficient approach to improve intestinal health in young animals, particularly by reducing the incidence of diarrhea.

Mulberry (*Morus alba* L.) leaves have been used in traditional Chinese medicine as a natural supplement. Mulberry leaves are highly nutritious containing high levels of protein and calcium, and numerous bioactive flavonoids, polyphenols, alkaloids and polysaccharides (Jan et al., 2021; Zeng et al., 2019). Recent studies indicated that mulberry leaves and their extract offered health benefits by alleviating hyperlipidemia, hyperglycemia, inflammation and atherosclerosis, with minimum adverse effects, making them promising candidates for treating metabolic diseases, such as heart disease and diabetes (Cai et al., 2019; Maqsood et al., 2022; Parida et al., 2023; Radojkovic et al., 2012). Among these bioactive compounds, several are potent antioxidants, and mulberry leaf extract has been shown to reduce DNA damage caused by OS. The capacity of mulberry leaves to regulate redox homeostasis endows them the therapeutic potential to mitigate intestinal injury caused by OS. Given the favorable palatability and minimal side effects, mulberry leaf powder (MLP) a natural solution to protect weanling pigs from diarrhea, ultimately promoting overall gut health and growth.

The physiological structure and metabolic processes of the pig gastrointestinal tract (GIT) closely resemble those in humans, making pigs an ideal model for studying the intestinal barrier. In this study, we examined the dose-dependent effects of dietary mulberry leaf powder (MLP) on growth performance, diarrhea incidence, and intestinal barrier integrity in weanling pigs. We also explored the underlying mechanisms involving anti-oxidative capacity and gut microbiota regulation. The protective effect of mulberry leaf extract (MLE) on the intestinal epithelium was confirmed in an *in vitro* model of H_2O_2-induced oxidative stress in porcine jejunal epithelial cells (IPEC-J2). Our findings demonstrated that mulberry leaf promotes intestinal health by directly enhancing anti-oxidative capacity and indirectly modulating gut microbiota and short chain fatty acids (SCFAs) production. This study suggests mulberry leaf as a safe dietary supplement to improve intestinal health in young mammals.

Materials and methods

Nutrient composition of mulberry leaf powder and preparation of mulberry leaf extracts

The mulberry leaves used in this study were the "Guisangyou12" variety supplied by Shanghao Mulberry Tea Co., Ltd., in Sichuan, China. Mulberry leaves were processed through drying, crushing and sifting for the preparation of MLP. The nutritional compositions of MLP, including dry matter, crude protein, ether extract, crude fiber, amino acids, calcium and phosphorus, were determined as described by AOAC International (Lee, 1995). Total dietary fiber (TDF), soluble dietary fiber (SDF), insoluble dietary fiber (IDF), acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined according to the national standard analysis methods of GB 5009.88-2014, GB/T 20806 and NY/T 1459-2022 in China, respectively.

The MLE was prepared from aforementioned mulberry leaves by ethanol extraction using an ultrasonic machine (Scientz-II D, Scientz, China), followed by concentration using a rotary evaporator (Rotavapor R-200, BUCHI, Switzerland) and a frozen dryer (FDU-2110, Eyela, Japan). The content of polysaccharides, polyphenols, and flavonoids in MLP and MLE was determined using fluorometric enzyme labeling analyzer (SpectraMax 190, Molecular Devices, San Jose, USA).

GC-MS analysis of mulberry leaf extract

Chemical compositions of MLE were analyzed by using gas chromatography-mass spectrometry (GC-MS). For the analysis, 10 mg of MLE samples were dissolved in 2 mL methanol. The chromatographic analysis conditions consisted of an HP-5MS capillary column (30m × 0.25mm × 0.25μm), a flame ionization detector (FID), an injection port temperature of 200 °C, and a column temperature program from 40 °C to 220 °C at a rate of 10 °C/min. Mass spectrometry analysis utilized an electron impact ionization source at an interface temperature of 280 °C and an ion source temperature of 230 °C, with an electron energy of 70 eV and a scan range of 30-500 u.

Animals, experimental design, and management

All animal experiments were performed in the research facility of the Institute of Animal Nutrition under the approved protocols by the Animal Care and Use Committee at Sichuan Agricultural University, China (dated 5th April 2023, No. 20230409, Sichuan, China) in accordance with ethics requirements.

A total of 160 weanling pigs (Duroc × Landrace × Yorkshire, half barrows and half gilts) at 24-day of age with an initial body weight of 7.41 ± 0.24 kg were randomly allocated to 4 treatment diets: CON (basal diet), 1 % MLP (1 % MLP replaced 0.5 % soybean meal and 0.5 % corn in basal diet), 3 % MLP (3 % MLP replaced 1.5 % soybean meal and 1.5 % corn in basal diet), and 5 % MLP (5 % MLP replaced 2.5 % soybean meal and 2.5 % corn in basal diet). Each treatment was consistent of 8 pens with 5 pigs per pen. All diets were formulated with equal amounts of crude protein and energy, and met the nutrient requirements (NRC 2012) for weanling pigs. The diet compositions and nutrient levels of the experimental diets were listed in Table 1.

All piglets were housed in nursery crates within a controlled environment, and were fed *ad libitum* with free access to water. The animal trial lasted 42 days. The body weight (BW) and feed intake (FI) were recorded every 2 weeks. The average daily gain (ADG), average daily feed intake (ADFI), feed to gain ratio (F/G) were calculated. The numbers of diarrhea pigs were counted daily, and diarrhea incidence rate (DI) was calculated as DI (%) = Σ (the number of pigs with diarrhea × days of diarrhea)/ (total number of pigs × number of experimental days) × 100. On day 43, a representative pig from each replicate pen with the median body weight was anaesthetized with *i.v.* injection of sodium pentobarbital (40 mg/kg BW) and euthanized for sample

Table 1
Feed ingredients and nutrient composition of experimental diets (As-fed basis, %).

Ingredient	CON	1 %MLP	3 %MLP	5 %MLP
Corn	50.00	49.50	48.50	47.50
Soybean meal	14.50	14.00	13.00	12.00
Extruded soybean	5.00	5.00	5.00	5.00
Soybean protein concentrate	5.00	5.00	5.00	5.00
Mulberry leaf powder	0	1.00	3.00	5.00
Low protein whey powder	8.00	8.00	8.00	8.00
Fishmeal	5.00	5.00	5.00	5.00
Soybean oil	4.20	4.30	4.40	4.50
Sucrose	4.00	4.00	4.00	4.00
Limestone	0.32	0.22	0.12	0.02
Dicalcium phosphate	1.00	1.00	1.00	1.00
L-Lys·HCl	0.50	0.50	0.50	0.50
DL-Met	0.14	0.14	0.14	0.14
L-Thr	0.18	0.18	0.18	0.18
L-Trp	0.06	0.06	0.06	0.06
Val	0.10	0.10	0.10	0.10
NaCl	0.50	0.50	0.50	0.50
Chloride choline	0.10	0.10	0.10	0.10
Vitamin premix[1]	0.20	0.20	0.20	0.20
Mineral premix[2]	0.20	0.20	0.20	0.20
Feed-grade lactic acid	1.00	1.00	1.00	1.00
Nutrient levels[3]				
ME, kcal/kg	3394	3397	3394	3391
CP	19.74	19.66	19.50	19.33
Ca	0.81	0.83	0.86	0.90
Total P	0.67	0.67	0.67	0.67
SID Lys	1.39	1.39	1.38	1.37
SID Met	0.44	0.44	0.43	0.43
SID Thr	0.83	0.82	0.82	0.82
SID Trp	0.25	0.25	0.25	0.25
SID Val	0.88	0.88	0.88	0.88

[1] The vitamin premix provided per kg of the diet: VA 3,000 IU, VD_3 1,000 IU, VE 8 IU,VK 1 mg, VB_1 1 mg, VB_2 2.5 mg, niacin 10 mg, pantothenic acid 5 mg, folic acid 0.5 mg, VB_6 1.2 mg, biotin 0.5 mg, VB_{12} 120 μg, limestone 1.8 g.

[2] The mineral premix provided per kg of the diet: Cu ($CuSO_4·5H_2O$) 6 mg, I (KI) 0.14 mg, Fe ($FeSO_4·H_2O$) 100 mg, Mn ($MnSO_4·H_2O$) 4 mg, Se (Na_2SeO_3) 0.3 mg, Zn ($ZnSO_4·H_2O$) 100 mg.

[3] All the data were calculated values.

collection. Blood samples were collected from the vena cava for serum collection. The intact jejunum and ileum segments were collected and preserved in 4 % paraformaldehyde solution for intestinal morphology analysis. The ileal mucosa and cecum contents were collected and stored at -80 °C.

Intestinal morphology

The intestinal segments fixed in 4 % paraformaldehyde were dehydrated in gradient ethanol solution, embedded in paraffin, and cross-sectioned at 5 μM thickness for hematoxylin and eosin (H&E) staining. At least 10 structurally intact villi and crypts from each section were imaged at a 40 × magnification, and the villus length and crypt depth were measured using Image Pro Plus 6.0 software. The villus height-to-crypt depth ratio (V:C) were calculated.

Tight junction structure under electron microscope

The middle of ileum from pigs was collected, cut into 1 cm samples, and placed in glutaraldehyde fixative. The samples were then cut into 1 mm^3 pieces and fixed with 1 % osmium tetroxide in PBS for 2 h and rinsed again. Dehydration was carried out sequentially with ethanol and acetone, followed by embedding resin infiltration at 37 °C for 12 h. Polymerization occurred in a 60 °C oven for 48 h. The blocks were then sectioned with an ultramicrotome (RMC MTX). Sections were stained with 2 % uranyl acetate and lead citrate, washed with ultrapure water, and air-dried overnight. The sections were observed under a transmission electron microscope (HT7700, Hitachi, Tokyo, Japan) and images were analyzed using Image Pro Plus 6.0 software.

Anti-oxidation and immune associated markers in serum and intestinal mucosa

The total anti-oxidative capacity (T-AOC), the activities of catalase (CAT), total superoxide dismutase (T-SOD) and glutathione peroxidase (GPX), and the level of malondialdehyde (MDA) in serum and ileal mucosa were determined using the commercial assay kits (Jiancheng Company, Nanjing, China), respectively, according to the manufacturer's instruction.

The levels of D-lactic, IgG, IgA, IgM, sIgA and TNF-α in serum and/or ileal mucosa were measured using the commercial ELISA kits (MLBIO Biotechnology Ltd., Shanghai, China) with a spectrophotometer (Biomate 5, Thermo Electron Corp., Rochester, NY, USA).

ROS levels in ileal mucosa

The levels of ROS in the ileal mucosa were detected using a commercially available tissue ROS assay kit (MLBIO Biotechnology Ltd., Shanghai, China). The ROS levels were normalized to the protein concentration measured by a BCA protein assay kit (MLBIO Biotechnology Ltd., Shanghai, China). All procedures were performed strictly according to the manufacturer's instructions. The results were expressed as fluorescence intensity per mg of protein (RCF/mg prot).

Short-chain fatty acids concentration in cecum digesta

The concentrations of short-chain fatty acids mainly propionic, butyric, and acetic acids were determined using a CP-3800 gas chromatograph (Varian, Inc., CA, USA). The cecum digesta was thawed and diluted in sterilized water. The supernatants from diluted digesta were mixed with 210 mmol/L crotonic acid and 25 % metaphosphoric acid for SCFA extraction. The polar capillary columns with a polyethylene glycol were used for gas chromatograph analysis.

16s RNA Sequencing and analysis

Microbial DNA from cecal chyme samples was extracted using the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Norcross, GA, USA). PCR amplified the V4-V5 region of the bacterial 16S ribosomal RNA gene with primers 515F and 907R. PCR conditions were 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR reactions included 20 μL mixtures of FastPfu Buffer, dNTPs, primers, FastPfu Polymerase, and template DNA. Amplicons were purified from agarose gels and quantified using QuantiFluor™ -ST. Purified amplicons underwent paired-end sequencing (2 × 250) on an Illumina MiSeq platform. Raw fastq files were demultiplexed and quality-filtered with QIIME (version 1.17), truncating reads <300 bp with average quality <20, exact barcode matching, removal of reads with ambiguous characters, and assembly of sequences with overlaps >10 bp. Operational Units (OTUs) were clustered at a 97 % similarity cutoff using UPARSE (version 7.1). Data were analyzed by using the commercial bio-informatics platform (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China).

Cell culture and treatments

The intestinal porcine epithelial cell line (IPEC-J2) was used for *in vitro* experiment. Cells were grown in Dulbecco's modified Eagle's medium: F-12 (DMEM/F12; Sigma-Aldrich, St. Louis, MO) with 5 % fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA)) supplemented with 1 % insulin-transferrin-selenium premix (ITS: 1 mg/ml insulin, 0.6 mg/ml transferrin, and 0.6 μg/ml selenium; Corning Inc., Corning, NY), 5 ng/ml epidermal growth factor (EGF: Sigma-Aldrich, St. Louis, MO), and 1 % penicillin-streptomycin mixture (Mediatech. Inc.,

Manassas, VA). Cells were seeded into 96-well or 24-well plates (BD Falcon, Corning Inc., Corning, NY) at 0.5 × 10^5 cells/ml to form a confluent monolayer and within 4 days, and then switched to the FBS free medium to induce differentiation for 7 days as previous mentioned (Yan & Ajuwon, 2017). Cells were cultured in a humidified incubator at 37°C under 5 % CO_2.

On day 7 post-differentiation, cells were treated with mulberry leaf extract (MLE) for 24 h, and then were challenged with H_2O_2 for 2 h to induce oxidative stress. The optimal concentration of MLE (ranging from 10 to 1000 μg/ml) and H_2O_2 (ranging from 50 to 500 μM) were determined by assessing their impacts on cell viability using the CCK-8 determination kit (Beyotime, Shanghai, China), as shown in **Figure S1**.

Intracellular ROS levels

The intracellular ROS of IPEC-J2 cells were determined as the indication of cellular oxidative stress. After MLE treatment and H_2O_2 challenge, cells were incubated with PBS solution containing 5 μM DCFH-DA probes (Nanjing Jiancheng, Nanjing, China) at 37 °C for 40 min, and then digested in 0.05 % typsin-EDTA solution and suspended in fresh culture medium. The ROS in cells were immediately measured using a flow cytometry (FACS Verse, BD Biosciences, East Rutherford, NJ, USA) and analyzed using FlowJo 10.9.0 software.

Western blotting

Western blotting was carried out as previously described (Gu et al., 2023; Yan, Liu, et al., 2024), and protein concentration of ileal mucosa and IPEC-J2 cells was determined using the bicinchoninic acid assay (BCA) kit (A045-3-2, Nanjing, China). Western blotting was performed using primary antibodies against ZO-1 (Absin, abs122482), Occludin (Bioss, bs-10011R), Claudin-1 (Absin, abs146616), Nrf2 (Bioss, bs-1074R), p-Nrf2 (Bioss, bs-2013R), HO-1 (Bioss, bs-2075R), NQO1 (Bioss, bs-2184R), Keap1 (Bioss, bs-3648R) and β-actin (CST, 4970S). All primary antibodies were diluted 1:1000, and secondary antibody HRP conjugated anti-rabbit (Absin, Shanghai, China) was used in 1:3000 dilution. Visualization was performed using a ChemiDoc XRS Imager System (Bio-Rad), and protein quantification was conducted using Image Lab Version 6.2.

RNA isolation and analysis of quantitative of real-time PCR

Total RNA was extracted from IPEC-J2 cells and ileal mucosa using Trizol reagent (TaKaRa, Dalian, China), followed by reverse transcription using the PrimeScriptTM Reagent Kit (TaKaRa, Dalian, China). The quality and concentration of RNA were determined by agarose gel electrophoresis and a spectrophotometer (Coulter, DU800, Fullerton, CA, USA), respectively. The mRNA expression of tight junctions *ZO-1, Occludin*, and *Claudin-1*, mucus-coding genes *MUC1* and *MUC2*, and anti-oxidation related genes *Keap1, NQO1, CAT, GPX1, Nrf2* and *HO-1* were investigated. The sequences of PCR primers were listed in **Table S1**. The double-delta CT method was used to analyze the relative mRNA expression, and *β-actin* was used as the internal reference.

Statistical analysis

Data were analyzed though one-way analysis of variance (ANOVA) using the GLM procedure in SAS software (version 9.4, SAS Inst. Inc., Cary, NC, USA). Tukey multiple-comparisons test was performed to determine significance of differences among treatment means. Data were expressed as mean ± SE. Spearman correlation analysis was used to determine the correlations among cecum microbial genera, metabolites, and anti-oxidative indicators. Significant difference was set at P-value less than 0.05, whereas a P-value between 0.05 and 0.1 was considered a tendency.

Results

The chemical composition of mulberry leaf and mulberry leaf extracts

The chemical compositions of MLP and MLE were analyzed. The MLP contained 21.2 % crude protein (CP), 5.1 % ether extract (EE), 37.5 % total dietary fiber (TDF), 7.2 % soluble dietary fiber (SDF), 30.3 % % insoluble dietary fiber (IDF), 17.8 % acid detergent fiber (ADF), 21.9 % neutral detergent fiber (NDF), 50.3 % nitrogen free extract (NFE), and 10.4 % ash. The MLP also contains 3.31 % polysaccharides, 0.89 % polyphenols and 3.32 % flavonoids (Table 2).

The average extraction yield of MLE was 13 %. The MLE was dark brown with herbal aroma, and contained 251.85 mg/g polysaccharides, 67.80 mg/g polyphenols and 252.53 mg/g flavonoids (Fig. 1**A**). Furthermore, the GC-MS analysis revealed the 53 components of MLE, primarily composed of esters (33.84 %), ketones (18.54 %), acids (17.69 %), alcohols (13.59 %), amines (6.57 %), alkanes (4.18 %), salts (2.10 %), phenols (1.87 %), aldehydes (0.95 %), and oximes (0.67 %) (Fig. 1**B** and **Table S2**).

Dietary MLP diet improves intestinal barrier of weanling pigs

The effects of dietary MLP on the intestinal barrier of piglets were evaluated from various aspects, including incidence of diarrhea, intestinal morphology and barrier integrity. Weaning stress led to diarrhea with a ~30 % DI in the initial two weeks. Although there was the alleviation in the subsequent four weeks, a ~20 % DI persisted. Dietary 1 % to 5 % MLP supplementation significantly reduced DI to approximately 7 % during 15-42 days, and the overall diarrhea rate was reduced to ~15

Table 2
Nutritional composition of mulberry leaf powder (Air-dry basis, %).

Item	Content, %
Dry matter (DM)	93.4
Crude protein (CP)	15.2
Ether extract (EE)	3.13
Total dietary fiber (TDF)	46.9
Soluble dietary fiber (SDF)	8.09
Insoluble dietary fiber (IDF)	38.8
Acid detergent fiber (ADF)	17.5
Neutral detergent fiber (NDF)	21.8
Nitrogen free extract (NFE)	50.6
Crude fiber (CF)	12.9
Crude ash (Ash)	11.5
Gross energy (GE, kCal/kg)	4300
Calcium	1.98
Total Phosphorus	0.41
Essential amino acids	
Lys	1.18
Met	0.28
Met + Cys	0.42
Thr	0.87
Trp	0.27
Val	1.13
Leu	1.67
Ile	0.92
His	0.47
Arg	1.09
Phe	1.06
Non-essential amino acids	
Ala	1.12
Asp	2.21
Glu	2.53
Gly	1.05
Ser	0.92
Tyr	0.68
Pro	0.95
Bioactive compounds	
Polysaccharides	3.31
Polyphenols	0.89
Flavonoids	3.32

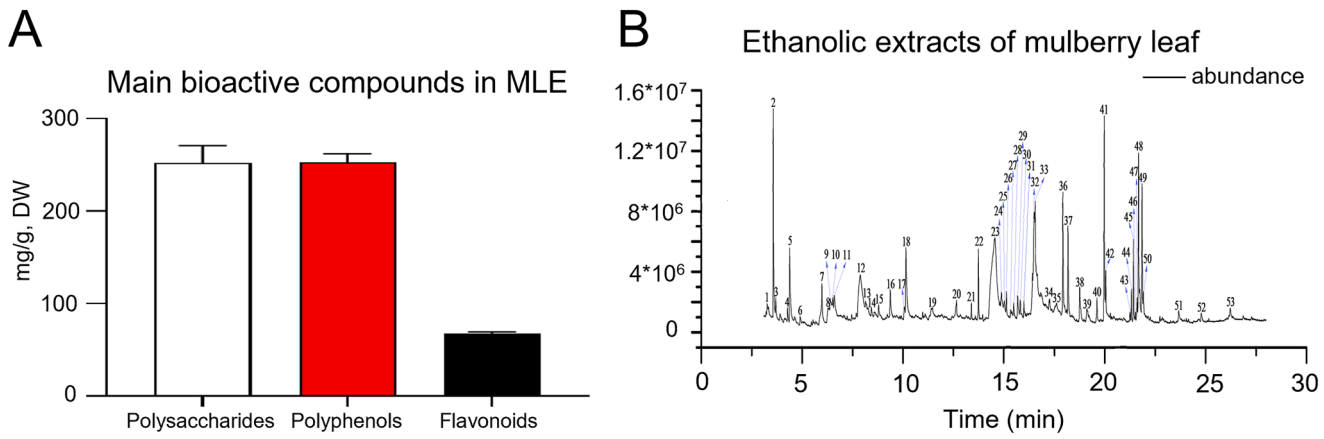

Fig. 1. Chemical analysis of MLE. (A) The main bioactive compounds in mulberry leaf extracts (MLE). (B) Gas chromatography-mass spectrometry (GC-MS) profiles of MLE. DW refers to dry weight of MLE. Results are shown as mean ± SEM. n = 3.

%, indicating the alleviation of weaning stress induced intestinal injury in piglets (Table 3). Furthermore, dietary MLP improved the morphology of jejunum and ileum as shown by the HE staining of intestinal sections (Fig. 2**A and Figure S1A**). Dietary 1 % and 3 % MLP significantly increased villus height and V/C ratio in both jejunum and ileum of piglets, suggesting a better development of intestinal mucosa, compared with control pigs (Fig. 2**C and D, Figure S1B and C**). The ultrastructure of intestinal epitheliums from the MLP groups exhibited relatively intact cell boundaries and tight junctions, with microvilli appearing longer, more uniform, and neatly arranged; in contrast, the epithelium from the control group had unclear cell boundaries, disrupted tight junctions, and fractured and disorganized microvilli (Fig. 2**B**). Dietary 1 % to 5 % MLP significantly reduced serum D-lactate, an indicator of intestinal permeability, suggesting the reduction in intestinal permeability and improvement in barrier integrity (Table 4). Tight junctions are main components of intestinal barrier. Compared with control group, dietary 1 % and 3 % MLP significantly up-regulated mRNA expressions of tight junctions *ZO-1, occludin, Claudin-1* and *MUC1* in ileal mucosa, confirming the improvement on barrier function (Fig. 2**E**). In addition, we notice that 1 % and 3 % MLP supplementation had no effect on growth performance of weanling pigs, while only 5 % MLP decreased ADG during day 15 to 42.

Taking into account the better improvement on villus length and expressions of tight junctions observed with 1 % and 3 % supplementation, coupled with the absence of adverse effects on growth performance, 1 % MLP in diet was recommended to alleviate weaning stress-induced intestinal injury in piglets, adhering to the principle of minimum effective dose.

Table 3
Effect of dietary MLP on growth performance of weanling pigs.

Item[1]	CON	1 %MLP	3 %MLP	5 %MLP	SEM	*P*-value
Day 0 BW, kg	7.38	7.40	7.41	7.46	0.24	0.21
Day 14 BW, kg	10.75	10.80	10.65	10.53	0.22	0.83
Day 42 BW, kg	27.07^{a}	27.01^{ab}	25.88^{ab}	25.68^{b}	0.55	0.10
Day 1-14						
ADG (g/d)	240.76	243.21	230.92	219.55	22.57	0.71
ADFI (g/d)	359.74	354.52	362.43	343.69	14.38	0.72
F/G	1.48	1.46	1.56	1.56	0.16	0.92
DI, %	30.94	31.88	29.69	30.94	2.94	0.95
Day 15-42						
ADG (g/d)	582.85^{a}	578.83^{ab}	544.06^{ab}	541.05^{b}	15.62	0.07
ADFI (g/d)	1084.47	1054.17	1060.09	1043.81	30.62	0.60
F/G	1.86	1.83	1.96	1.93	0.05	0.30
DI, %	20.70^{a}	7.42^{b}	8.20^{b}	7.42^{b}	2.77	<0.01
Day 0-42						
ADG(g/d)	468.82^{a}	466.95^{ab}	439.68^{ab}	433.88^{b}	14.65	0.09
ADFI(g/d)	843.09	820.86	827.54	810.43	22.67	0.54
F/G	1.62	1.59	1.65	1.73	0.08	0.44
DI, %	24.11^{a}	15.57^{b}	15.37^{b}	15.26^{b}	2.61	<0.01

[1] BW = body weight, ADG = average daily gain, ADFI= average daily feed intake, F/G = feed to gain ratio; DI: diarrhea incidence (%) = Σ (the number of pigs with diarrhea per pen × days of diarrhea)/ (total number of pigs × number of experimental days) × 100.

n = 8 replicates per treatment and 5 pigs per replicate. SEM = standard error of the mean, means with different superscript letters across rows are significantly different ($P < 0.05$).

MLP diet enhances anti-oxidation and regulates immunoglobulins levels in serum and ileum mucosa of weanling pigs

Weaning stress induces oxidative stress, which is negatively associated with intestinal function. Dietary 1 % to 5 % MLP diet significantly decreased the ROS levels in ileal tissue compared to the control group (Fig. 2**F**). The activities of anti-oxidative enzymes T-AOC, SOD, CAT and GPX, and the level of MDA, an indicator of OS, were investigated in serum and ileal mucosa of piglets (Fig. 2**G, H**). Dietary 1 % to 5 % MLP significantly reduced MDA level and increased activities of T-AOC, SOD, CAT and GPX in serum, indicating the enhancement of global anti-oxidative capacity (Fig. 2**G**). Dietary 1 % to 5 % MLP also enhanced mucosal anti-oxidative capacity as shown by the increased activities of T-AOC and SOD, and reduced MDA in ileal mucosa (Fig. 2**H**). Nrf2-HO-1 signaling pathway has a central role in the regulation of redox homeostasis. Dietary 1 % to 5 % MLP increased abundance of Nrf2, HO-1 and NQO1, confirming the enhanced anti-oxidative capacity in ileal mucosa (Fig. 2**I**). In addition, Dietary 1 % to 5 % MLP increased levels of IgM and IgA, and decreased IgG in the serum of piglets, while the pro-inflammatory cytokine TNF-α was unchanged (Table 4). Moreover, 5 % MLP increased levels of the serum IgM and IgA compared to 1 %MLP and 3 %MLP groups, suggesting that 5 % MLP diet may be more beneficial for enhancing the immune function of weaned pigs (Table 4).

MLE directly ameliorates H_2O_2 induced oxidative stress in IPEC-J2 cells

The antioxidant capacity of mulberry leaves served as a potential factor in enhancing intestinal barrier of weanling pigs. We prepared MLE from aforementioned MLP, and further investigated the direct protection of MLE against H_2O_2-induced oxidative stress in IPEC-J2 cell (Fig. 3**A**). Firstly, MLE exhibited no adverse effects on cell viability as the concentrations increasing from 10 to 1000 μg/ml, and MLE at 100, 400 and 600 μg/ml even increased cell viability (**Figure S2A**). H_2O_2 at 100 μM was used to induce oxidative stress, which decreased ~40 % of cell

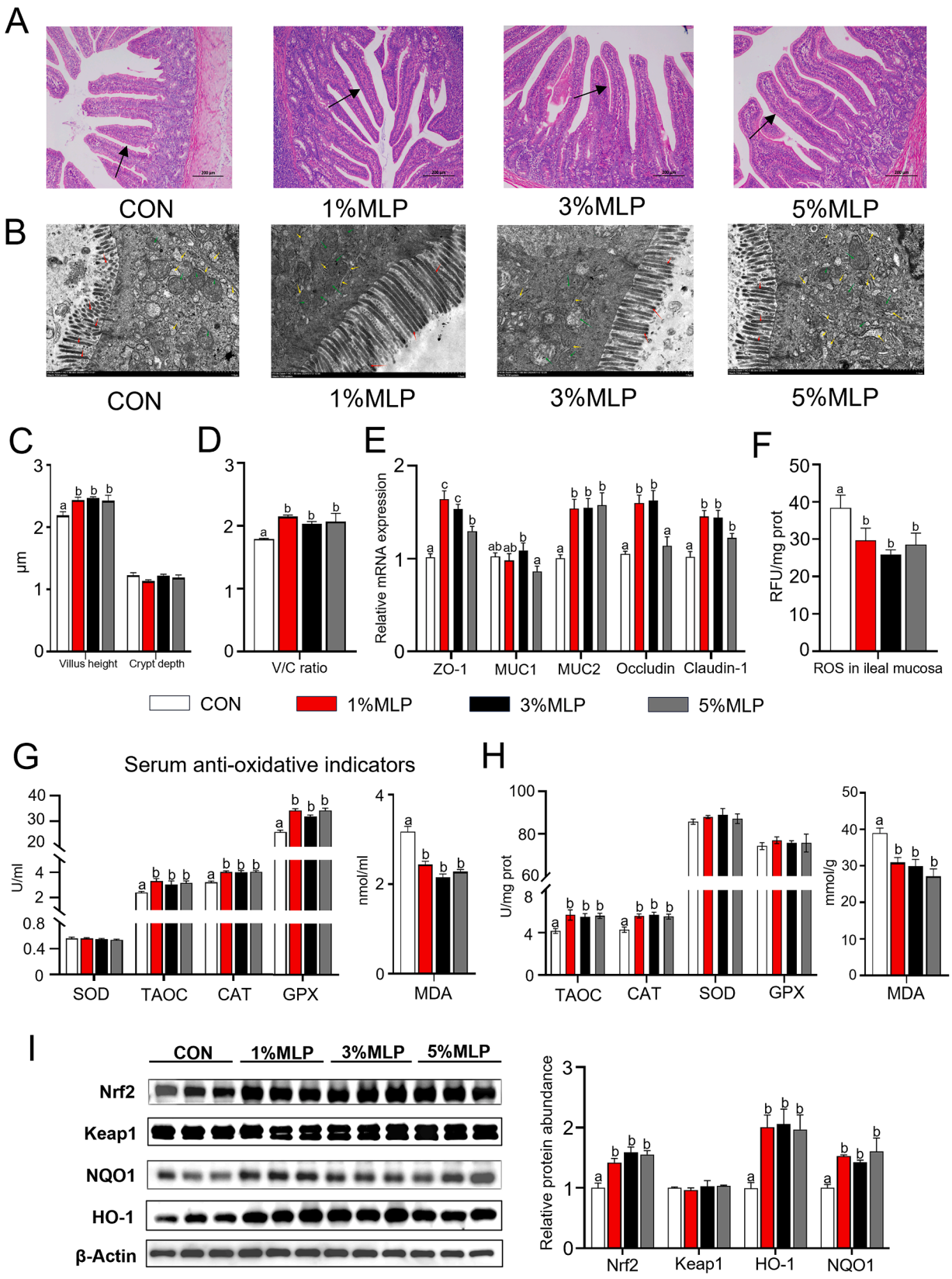

Fig. 2. Effects of dietary MLP on the intestinal health and the anti-oxidation of weanling pigs. (A) Representative images of ileal morphological images (H&E; × 100). (B) Representative images showing the microstructure of ileal epithelia by TEM. Red arrow: intestinal villi; green arrow: mitochondria; yellow arrow: endoplasmic reticulum. (C) Villus height and crypt depth, and (D) villus height to crypt depth (V/C) ratio in ileum. (E) mRNA expression of intestinal tight junctions and mucin coding genes. (F) The ROS levels in ileal tissue. (G-H) The anti-oxidation associated markers in serum and ileal mucosa, respectively. (I) Protein abundances of Keap1-Nrf2 signaling pathway related proteins. Values were presented as mean ± SEM. Bar marks with different superscripts represent a significance ($P < 0.05$). n = 8.

Table 4
Effect of dietary MLP on immune parameters in serum and ileal mucosa of weanling pigs.

Item[1]	CON[2]	1 %MLP	3 %MLP	5 %MLP	SEM	*P*-Value
Serum						
D-lactic, ng/L	1.39[b]	1.13[a]	1.10[a]	1.12[a]	0.04	<0.01
TNF-α, pg/mL	1.78	1.84	1.84	1.81	0.03	0.40
IgM, μg/mL	33.41[a]	38.26[b]	40.14[b]	43.92[c]	1.06	<0.01
IgA, μg/mL	34.15[a]	40.62[b]	40.96[b]	44.33[c]	0.85	<0.01
IgG, μg/mL	326.09[b]	279.19[a]	280.97[a]	285.35[a]	6.98	<0.01
Ileal mucosa						
TNF-α, pg/mL	485.66	475.35	476.66	489.26	9.39	0.83
sIgA, μg/mL	34.41	32.81	31.30	32.00	1.21	1.15

[1] TNF-α = tumor necrosis factor-α; IgM = immunoglobulin M; IgA = immunoglobulin A; IgG = immunoglobulin G; sIgA = secretory immunoglobulin A.

n=8 replicates per treatment and 5 pigs per replicate. SEM = standard error of the mean, means with different superscript letters across rows are significantly different ($P < 0.05$).

viability (**Figure S2B**). In H_2O_2 challenged cells, MLE at 10, 100 and 500 μg/ml concentrations significantly alleviated H_2O_2-incuded the decrease in cell viability (Fig. 3**B**). Excessive intracellular ROS was produced upon H_2O_2 challenge, while MLE at 100 μg/ml significant reversed the ROS over-production (Fig. 3**C**). MLE also reversed the effect of H_2O_2 on mRNA expressions of redox-associated genes, such as *CAT, GPX1, Keap1, HO-1* and *Nrf2* (**Figure S2C**). The effects of MLE on the tight junctions of IPEC-J2 cells were investigated. In unchallenged cells, MLE at 100 μg/ml significantly up-regulated mRNA expressions of *ZO-1, Occludin, Claudin-1* and *MUC2*, and increased protein abundance of ZO-1. Upon induction of oxidative stress, H_2O_2 impaired epithelium integrity by significantly decreased protein abundance and mRNA expressions of *ZO-1, Occludin* and *Claudin-1* as well as mRNA expressions of *MUC1* and *MUC2*, while pre-treatment of MLE prevented H_2O_2 induced decreases in tight junctions and protected IPEC-J2 cells from oxidative stress-induced epithelium injury (Fig. 3**D, E**).

Keap1-Nrf2 signaling pathway mediates the anti-oxidation of MLE in IPEC-J2 cells

As mentioned, MLP modulated protein abundance of Nrf2, HO-1 and NQO1 in ileal mucosa of weaning pigs. We further investigated the involvement of Keap1-Nrf2 signaling pathway in mediating anti-oxidative capacity of MLE in OS of IPEC-J2 cells. Prior to H_2O_2 challenge, MLE significantly increased pNrf2 and HO-1 protein abundance, and numerically decreased Keap1, a negative regulator for Nrf2, indicating enhancement of Keap1-Nrf2 signaling (Fig. 3**F**). H_2O_2 challenge significantly decreased the protein abundance of pNrf2, total Nrf2, HO-1 and NQO1, and increased Keap1 protein abundance compared with control group, causing oxidative stress (Fig. 3**F**). In H_2O_2 challenged cells, pretreatment with MLE significantly increased the protein abundance of Nrf2, HO-1 and NQO1, and decreased Keap1 protein abundance compared to H_2O_2 group, which totally reversed H_2O_2-induced the inhibition of Keap1-Nrf2 signaling (Fig. 3**F**). Moreover, the Nrf2 activator oltipraz (OPZ) and inhibitor ML385 were introduced to illustrate the importance of Nrf2 in the mediation of MLE function. We noticed that proper activation of Nrf2 by OPZ at 5 μM increased cell viability and protected against H_2O_2-induced oxidative stress in IPEC-J2 cells (Fig. 3**G and Figure S2D**). MLE also exhibited the protective effect, but not as strong as Nrf2 specific activator (Fig. 3**G**). Inhibition of Nrf2 by ML385 significantly decreased cell viability (**Figure S2E).** The combination of ML385 with MLE blocked the protective effect of MLE against oxidative stress (Fig. 3**G**). Furthermore, in H_2O_2 challenged cells, pretreatment with MLE or OPZ significantly increased the protein abundance of Nrf2, HO-1 and NQO1 while had no influence on Keap1, compared to H_2O_2 group, which further validated that MLE could activate the Nrf2 signaling pathway (Fig. 3**H**).

Collectively, MLE had no cytotoxicity even at high concentration on IPEC-J2 cells. The anti-oxidation of MLE, particularly under oxidative stress, was mediated though the activation of Keap1-Nrf2 signaling pathway.

The modulation of dietary MLP on the microbiota and SCFAs production in cecum of weanling pigs

The regulation of redox homeostasis has been associated with gut microbiota and the SCFAs production. Dietary 1 % to 5 % MLP significant increased acetic acid, butyric acid and total SCFAs concentrations in cecal content of weanling pigs (Fig. 4**A**). Dietary 5 % MLP significantly decreased the ratio of acetic acid, and increased the ratio of butyric acid, compared with control group (Fig. 4**B**).

For the gut microbiota analysis, a total of 2208 OTUs were detected, with 771 shared OTUs in all animals, and 390, 106, 148 and 224 OTUs appearing unique in the control, 1 % MLP, 3 % MLP, and 5 %MLP groups, respectively (Fig. 4**C**). Dietary 1 % to 5 % MLP also decreased total OTUs and unique OTUs (Fig. 4**D**). α-diversity analysis indicated dietary 1 % to 5 % MLP significantly increased Simpson index, and decreased Sobs, Ace and Chao indices, indicating MLP diets increased the dominance and richness decreased of cecal microbiota in weanling pigs. (Fig. 4**E**). The principal component analysis (PCA) analysis revealed the all MLP groups were clustered together at genus levels, and the separation of all treatment was not significant at phylum level (Fig. 4**F and G**).

The compositions of cecal bacterial communities at phylum and genus levels were analyzed. *Firmicutes* (70.62 %), *Bacteroidota* (22.92 %), *Proteobacteria* (3.14 %) and *Spirochaetota* (1.62 %) were the most dominant bacteria at phylum level **(**Fig. 5**A).** *Lactobacillus* (8.06 %), *Clostridium_sensu_stricto_1* (6.70 %), *Prevotella* (4.58 %), *Streptococcus* (6.90 %), *Alloprevotella* (2.61 %), UCG-005 (6.30 %) and *Faecalibacterium* (0.42 %) were the most dominant bacteria at genus level (Fig. 5**B**). Notably, the phylum *Spirochaetota* and *Desulfobacterota* were significantly decreased in in all MLP groups, compared with control group (Fig. 5**C**). At genus levels, MLP groups significantly increased *Clostridium*_sensu_stricto_1*, Prevotella, Faecalibacterium, Blautia, Dialister and et al.,* while decreased *Prevotellaceae*_UCG-005, UCG-003, UCG-001, *Lachnospiraceae* sub-genus, *Muribaculaceae, Osicillospira*, and *et al.* (Fig. 5**D**).

Dietary MLP modulates anti-oxidation of weanling pigs via indirectly regulation of SCFAs and gut microbiota

Aforementioned, dietary MLP significantly enhanced global and mucosal anti-oxidation in weanling pigs. We further investigated the association between the anti-oxidation and microbial compositions at genus level in weanling pigs using Spearman correlation analysis. The cecal abundances of *Clostridium*_sensu_stricto_1*, Faecalibacterium* and *Prevotell* were positively associated with activities of T-AOC, GPX and CAT, while negatively associated with MDA levels in both serum and ileal mucosa of weanling pigs (Fig. 5**E**). On the contrary, bacterial UCG-005 were negatively associated with activities of T-AOC and GPX, while positively associated with MDA level (Fig. 5**E**). It was indicated that these key bacteria were involved in the regulation of the anti-oxidation of weanling pigs. Of note, the abundances of these key bacteria were also significantly associated with the production of SCFAs in cecum (Fig. 5**E**). We next investigated whether SCFAs production modulated the anti-oxidation of MLP. Importantly, cecal acetic acid, propionic acid, butyric acid and total SCFAs production exhibited a strong correlation to the activities of T-AOC, CAT and GPX in both serum and ileal mucosa (Fig. 5**F**). These data suggested that the anti-oxidation of MLP was fulfilled partially via the indirectly regulation of gut microbiota and SCFAs production (Fig. 6).

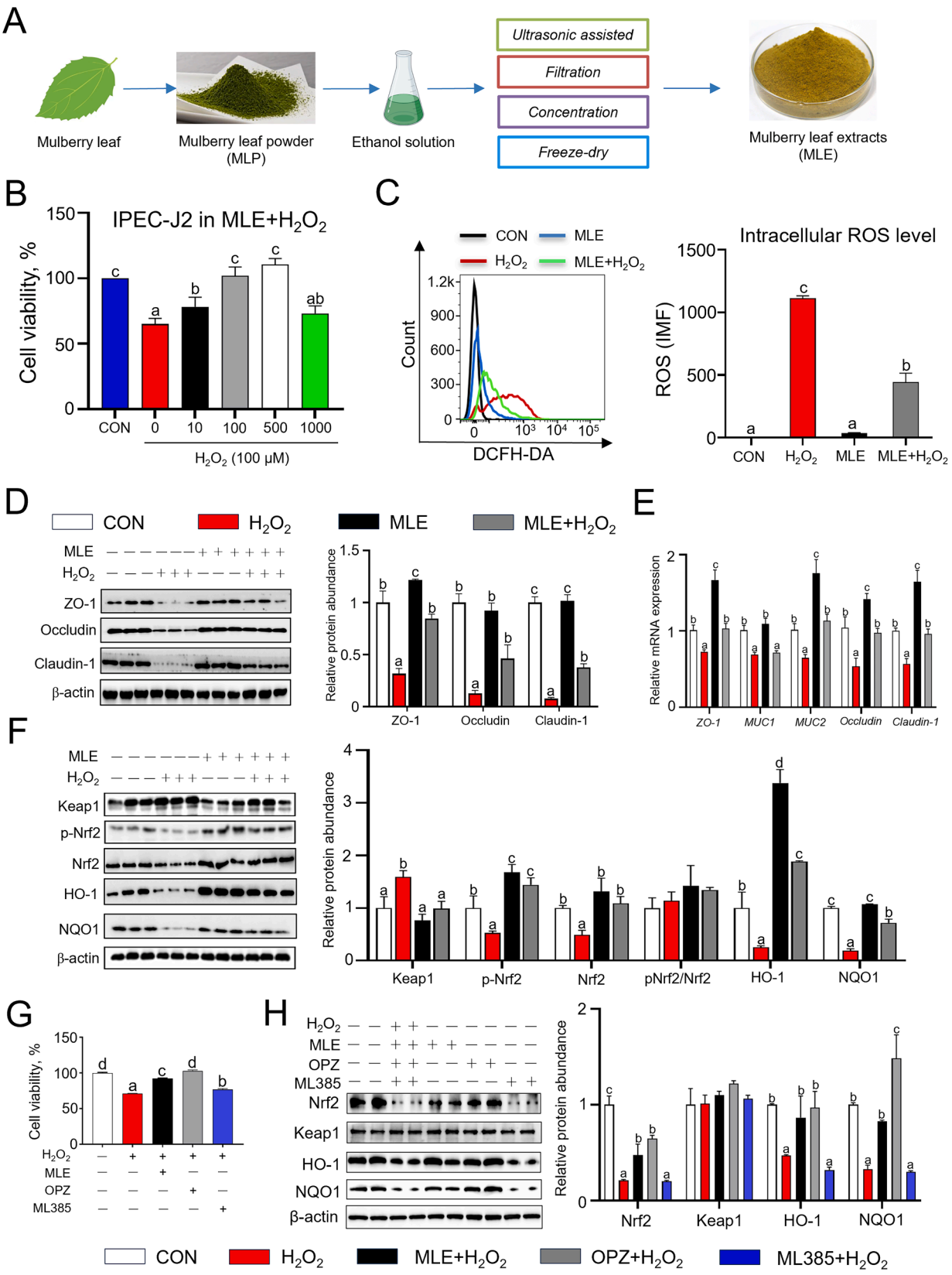

Fig. 3. Effects of MLE on H_2O_2-induced oxidative stress in IPEC-J2 cells. (A) Flow chart for the ethanolic extraction process of mulberry leaves. (B) The cell viability of IPEC-J2 cells treated with varying concentrations of MLE for 24 h, followed by 100 μM H_2O_2 for 2 h. (C) Intracellular ROS levels of IPCE-J2 cells treated with MLE and H_2O_2. (D) Intestinal tight junction related protein abundance of IPEC-J2 cells treated with MLE and H_2O_2. (E) Intestinal tight junction related relative mRNA expression of IPEC-J2 cells treated with MLE and H_2O_2. (F) Anti-oxidation related protein abundance of IPEC-J2 cells treated with MLE and H_2O_2. (G) The cell viability of IPEC-J2 treated with H_2O_2 and/or MLE, OPZ, ML385. (H) Anti-oxidation related protein abundance of H_2O_2 challenged IPEC-J2 cells pretreated with MLE, OPZ or ML385. Data were presented as mean ± SEM. Values in each column with different superscripts have significant differences ($P < 0.05$). n = 3-6.

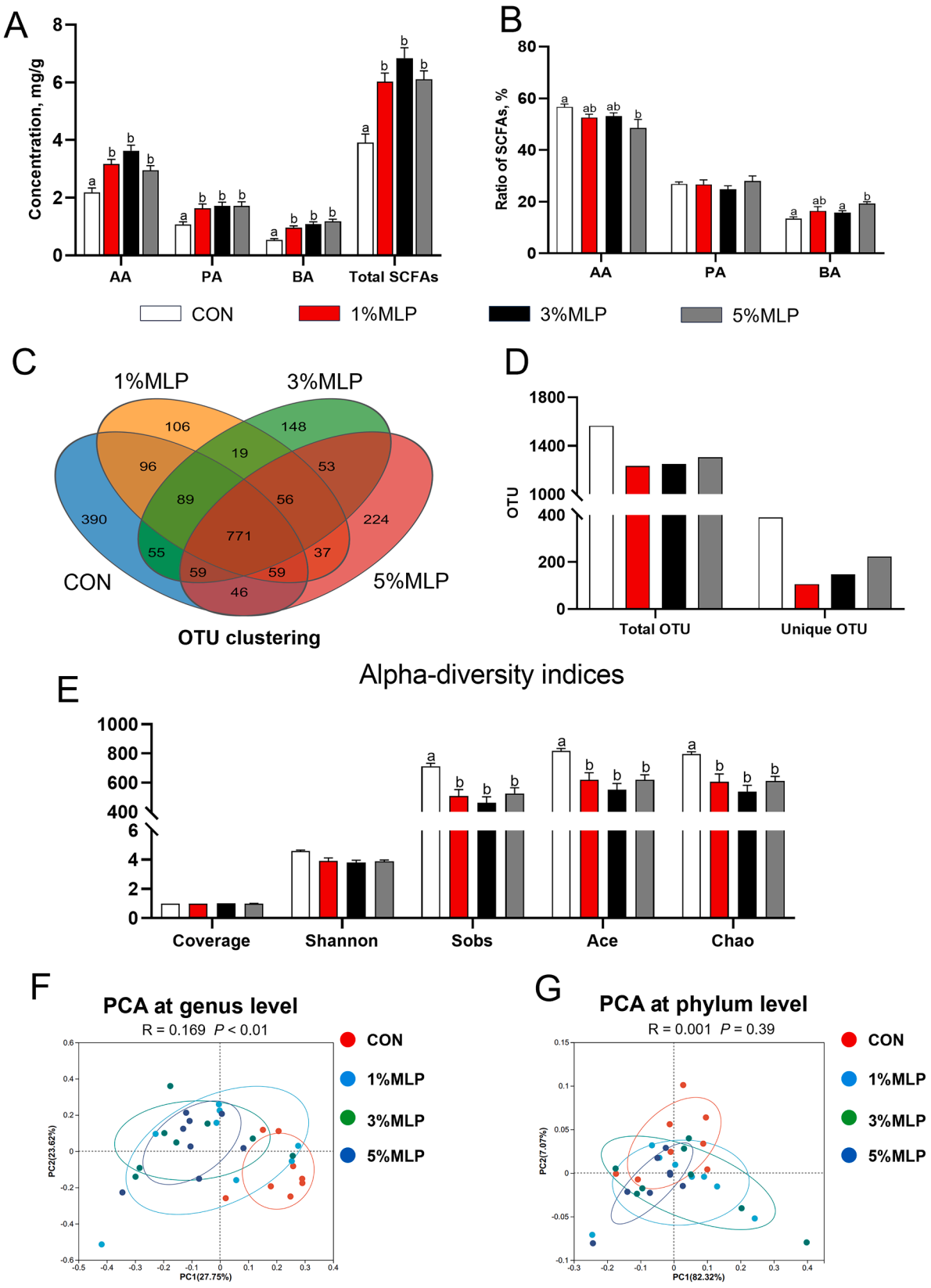

Fig. 4. Effects of dietary MLP on SCFAs concentrations and microbial diversity in cecum content of weanling pigs. (A) The concentration of cecal chyme acetic acid (AA), pentatonic acid (PA), butyric acid (BA) and total SCFAs. (B) The ratio of AA, PA and BA. (C) The Venn map of OTUs. (D) The numbers of total and unique OTUs. (E) The alpha-diversity indices. (F) Principal Component Analysis (PCA) at genus level. (G) Principal Component Analysis (PCA) at phylum level. Values were presented as mean ± SEM. Bar marks with different superscripts represent a significance ($P < 0.05$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n=8.

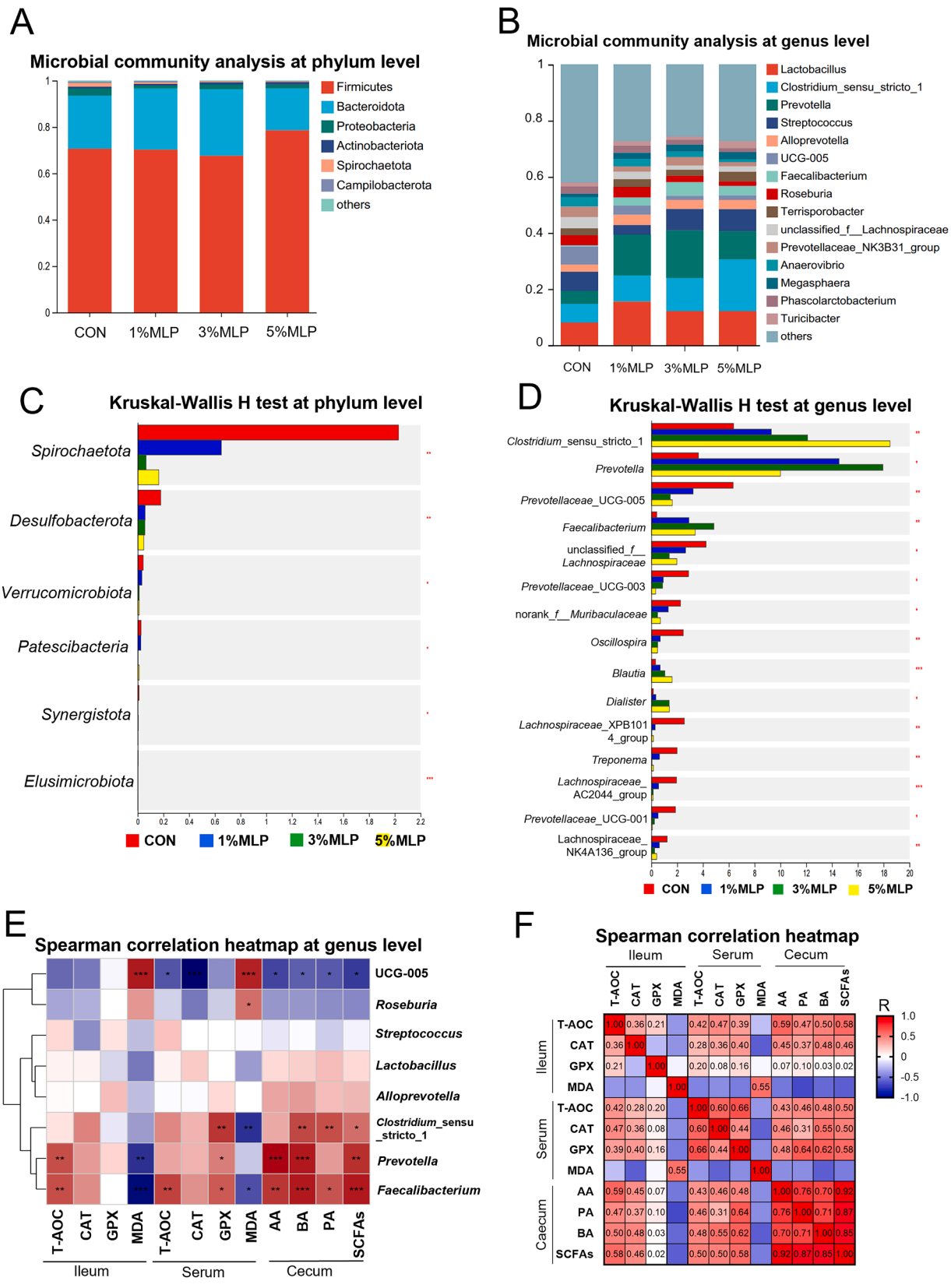

Fig. 5. Effects of dietary MLP on cecal bacteria and the correlation to anti-oxidation. (A) Cecal bacteria community barplot analysis at phylum level. (B) Cecal bacteria community barplot analysis at genus level. (C) Kruskal-Wallis H test heatmap of cecal bacteria at genus level. (D) Kruskal-Wallis H test heatmap of cecal bacteria at phylum level. (E) The heatmap of Spearman correlation analysis between cecal bacteria at genus level and metabolites. (F) The heatmap of Spearman correlation between cecal metabolites and antioxidative indicators. Bar marks with different superscripts represent a significance ($P < 0.05$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n=8.

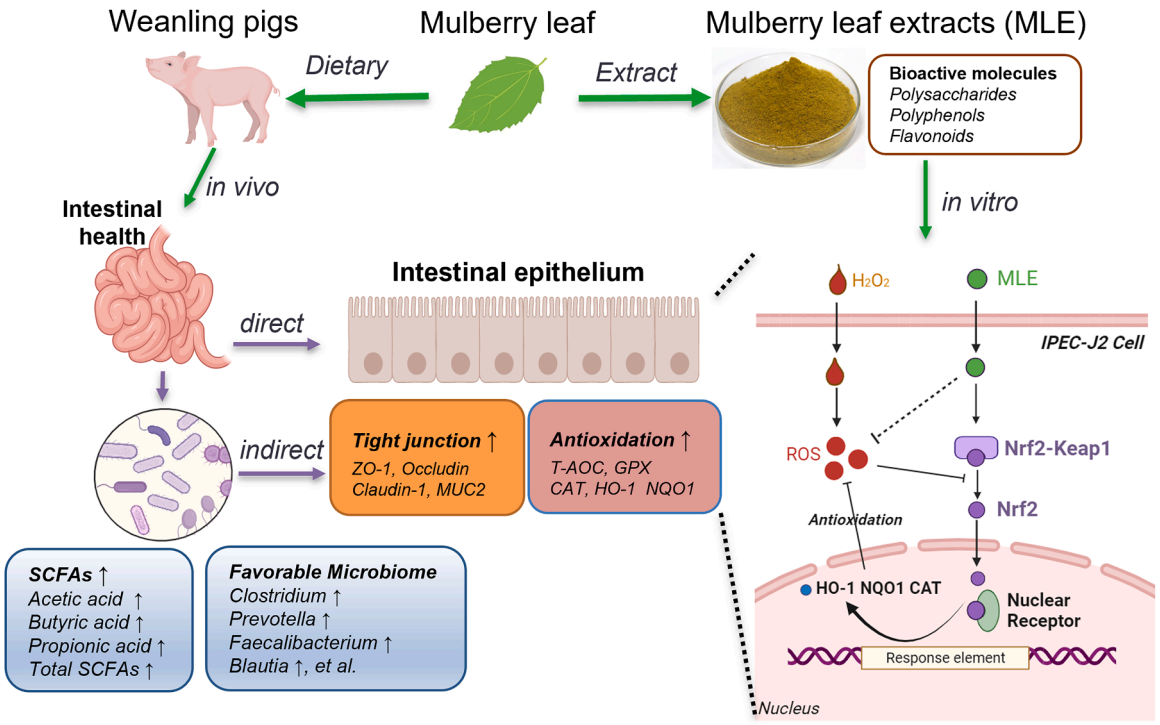

Fig. 6. Schematic diagram of mechanisms for mulberry leaf in the protection of intestinal health of weanling pigs.

Discussion

Diarrhea frequently occurs in young mammals, such as weanling pigs and infants, and is a serious health issue causing growth retardation and even death (Lallès et al., 2007). Due to the incomplete development of gastrointestinal system, weanling pigs are vulnerable to various stresses, such as weaning stress and pathogen infection, (Lallès et al., 2007). Weaning stress is caused by the multi-factors, such as changes in diet composition, nutritional structure and eating habits, leading to intestinal damage and subsequent diarrhea (Indrio et al., 2022; Tang et al., 2021). In our study, the weanling pigs exhibted a ~30 % of diarrhea incidence in the first two weeks of weaning, and still maitaied at ~20 % of DI in the subsequent four weeks, indicating the occurrence of weaning stress.

Mulberry (*Morus alba* L.) leaves have been used in traditional Chinese medicine as a natural supplement, and involves in the regulation of lipid and glucose metabolism, the alleviation of inflammation, and *et al.* (Cai et al., 2019; Maqsood et al., 2022). In this study, dietary MLP supplementation alleviated diarrhea incidence even at low level. Subsequently, we illustrated the protection of mulberry leaves on intestinal barrier through various experiments. Dietary MLP diet increased villus height and V/C ratio in the jejunum and ileum sections, indicating the better development intestinal villi and potentially enhanced nutrient absorption capacity (Nahidi et al., 2012). D-lactate is poorly absorbed in intestines, but can pass into circulation via the paracellular passage in "leaky gut" (Remund et al., 2023; Venn et al., 2020). Dietary MLP decreased serum D-lactate levels, indicating the decreased paracellular permeability of intestines in weanling pigs. Epithelial tight junctions maintain the paracellular permeability of the intestinal barrier (Horowitz et al., 2023). We found the MLP and MLE all increased expression of tight junctions *in vivo* and *in vitro*, respectively. In general, our study confirmed the protection of MLP on intestinal barrier against weanling stress induced intestinal injury and diarrhea. Notably, dietary 1 % and 3 % MLP had no adverse effect on growth of weanling, while 5 % MLP caused growth retardation, probably resulted from the regulation on lipid metabolism (Du et al., 2022; Zhao et al., 2022).

Oxidative stress commonly occurs in weanling pigs when experiencing diarrhea and intestinal injury, leading to mitochondrial dysfunction, inflammation and disruption of intestinal barrier function (Aruoma, 1998; Novais et al., 2021). Mulberry leaf contains a variety of bioactive compounds, such as polysaccharides, flavonoids, and polyphenols, which exhibit as antioxidants to neutralize free radicals, inhibit lipid peroxidation and mitigate OS-induced cellular damage (Aramwit et al., 2013; Guo et al., 2003; Kim et al., 2014). It is reported that mulberry leaf polysaccharides reduces the incidence of diarrhea in early-weaned piglets (Zhao et al., 2015), and the ethanol extract improved intestinal morphology (Song et al., 2023). In our study, dietary MLP decreased the ROS level in ileum and increased activities of antioxidant enzymes, such as CAT, GPX and T-AOC, and decreased MDA in both serum and ileum mucosa of weanling pigs. The MLE also reduced ROS production and prevented H_2O_2-induced oxidative stress in intestinal epithelial cells. Some other *in vivo* and *in vitro* studies also confirmed that mulberry leaf displayed potent anti-oxidative capacity (Lin et al., 2017; Wang et al., 2010). The association between anti-oxidative capacity and intestinal function, such as nutrient digestion and absorption and intestinal barrier function, has been previously established (Yan et al., 2022; Yan, Xing, et al., 2024). The herbal plant and the extract have been used to improve anti-oxidation of weanling pigs to enhance gut health (Yan, Xing, et al., 2024). As a result, the anti-oxidation of MLP and MLE direct mediates their benefits on intestinal barrier.

The Keap1-Nrf2 signaling pathway serves as a fundamental mechanism for safeguarding cells against oxidative damage (Matzinger et al., 2018). Recently study reported that Nrf2 regulates expressions of tight junction proteins such as claudins, zonula occludin-1 (ZO-1) and occludin, which are critical to maintained gut barrier integrity (Singh et al., 2019). Our study confirmed that the activation of Nrf2 by MLP and MLE increased expressions of tight junctions in weanling pigs and IPEC-J2 cell, respectively. Previous studies showed that some natural plants rich in polyphenols activate the Nrf2 signaling pathway (Erlank et al., 2011; Liu et al., 2022). Nrf2 is activated by phosphorylation. We notice that MLP increased total Nrf2 abundance in ileal mucosa, and MLE increased total and phosphorylated Nrf2 but had no effect on pNrf2/Nrf2 ratio in IPEC-J2 cells. These results indicated that the MLP

and MLE actually increased protein expression of Nrf2 to enhance Nrf2 signaling pathway. The mRNA expression of *Nrf2* was induced by MLE confirming that MLE enhanced Nrf2 signaling via genomic regulation. Keap1 acts as a negative regulator of Nrf2 preventing Nrf2 activation and trans-nucleus (Matzinger et al., 2018). In our findings, both *in vivo* and unchallenged *in vitro* experiments demonstrated that MLE and MLP had limited influence on Keap1 abundance. However, under strong H_2O_2 challenge, MLE exhibited decreased Keap1 abundance, which facilitated the activation of Nrf2. When nuclear translocation, Nrf2 promotes the synthesis of antioxidant enzymes (SOD, CAT and GPX1) and detoxifying enzymes (HO-1 and NQO1) (Diallo, 2014). *In vivo* study confirmed that MLP enhanced activity of antioxidant enzymes. Consistently, MLP and MLE increased abundance of HO-1 and NQO1 in both *in vivo* and *in vitro*, respectively. In our study, we provide evidence that mulberry leaf enhances antioxidant capacity and intestinal barrier function in weanling pigs by activating the Nrf2 signaling pathway, and increasing antioxidant enzyme activity. However, as our data shown, mulberry leaf contains a variety of antioxidant compounds, and further investigation is still needed to determine which specific substances play the key role in its antioxidant effect.

Gut microbes and the metabolites are critical for intestinal barrier (Hooper & Gordon, 2001). In our study, dietary 1 % to 5 % MLP all increased SCFAs concentrations in cecum of piglets, particularly the butyric acid. The SCFAs not only serve as 80 % energy source of intestinal epithelial cells, but also protect intestinal barrier via maintaining mucosa immune cell activity and the integrity of intestinal epithelium (Diao et al., 2019). Butyric acid enhances epithelial cell proliferation and synthesis of tight junction via activating Akt/mTOR signaling pathway (Nie et al., 2015; Yan & Ajuwon, 2017), confirmed by the increases in villus height and abundances of tight junctions in our study. Notably, the concentrations of SCFAs in cecum were positively associated with anti-oxidative capacity of weanling pigs. Recent study reported that SCFAs modulate Nrf2 redox signaling through specific free fatty acid receptors, and butyrate induced Nrf2 nuclear translocation (Gonzalez-Bosch et al., 2021), indicating the SCFAs production also mediated the anti-oxidation of MLP.

The dietary MLP influenced the bacterial diversity in cecum. The key bacteria response to MLP and associating with anti-oxidation were identified in this study. *Clostridium*_sensu_stricto_1, *Prevotella* and *Faecalibacterium* were positive correlated to of SCFAs production and anti-oxidation. *Clostridium*_sensu_stricto_1 is linked to decreased protein utilization but enhances energy metabolism (Fan et al., 2017; Gao et al., 2019), potentially enhances villus growth. *Prevotella* and *Faecalibacterium* are the primary fiber-degrading bacteria to promote propionic and butyric acids production, accounting for the increased SCFAs production in this study. *Prevotella* and *Faecalibacterium* are also positively correlated to the activity of the antioxidant enzyme such as GPX (Hu et al., 2022; Ren et al., 2021), and act as safeguard of GIT against pathogen infection (Long et al., 2021). Some bacteria in *Prevotellaceae* family, such as UCG-001, UCG-003, UCG-005, were decreased by MLP supplementation and were negatively correlated to anti-oxidative capacity in weanling pigs. UCG-005 was reported a vicious bacteria that abundance of UCG-005 was positively correlated to the diarrhea incidence of piglets (Liang et al., 2021). Certain members of the *Spirochaetota* phylum are associated with diarrhea in pigs and humans (Bohrer et al., 2022; Wu et al., 2022), and MLP diet decreased the abundance of *Spirochaetota*. MLP diet increased the abundance of *Blautia*, which was reported to alleviate inflammation in gastrointestinal diseases, such as inflammatory bowel disease (Liu et al., 2021). Dietary MLP decreased abundance of Unclassified_f_*Lachnospiraceae*, which is associated with the incidence of intestinal diseases (Vacca et al., 2020). The mechanism about how MLP influences gut microbiota is still unclear. The dietary fiber in MLP is one of factors influencing bacterial composition (Lindberg, 2014). Other studies also reported that some bioactive compounds, such as polyphenols (Liao et al., 2021; Qiu et al., 2021), polysaccharides (Zhao et al., 2015), flavonoids (Shi et al., 2022), influence gut microbial composition and alleviate OS and inflammation. Collectively, our findings demonstrated that MLP diets promote higher abundance of beneficial bacteria (*Blautia, Prevotella, Faecalibacterium* and *Clostridium*_sensu_stricto_1) along with more SCFAs and better anti-oxidative capacity, and decreased the abundance of detrimental bacteria (unclassified_f_*Lachnospiraceae* and UCG-005), consequently resulting in lower diarrhea incidence and a better intestine health. Although the study identified key gut microbiota in response to mulberry leaf, particularly those associated with its antioxidant effects, further studies are still required to determine the relative contribution of mulberry leaf's direct antioxidant activity versus its indirect modulation of gut microbiota in improving intestinal barrier function.

Conclusion

In sum, mulberry leaves prevented diarrhea induced by weaning stress and improved intestinal barrier, which was associated with enhanced anti-oxidation and changes in gut microbiota. The in vivo study indicated mulberry leaves increased activities of antioxidant enzymes, such as GPX and CAT, potentially via increasing Nrf2 protein abundance. The further *in vitro* study confirms that the bioactive compounds in mulberry leaves directly increases Nrf2 protein abundance to induce downstream target HO-1 and NQO1, subsequently decreased ROS production. Mulberry leaves also modulated intestinal barrier and anti-oxidation via indirectly changing gut microbial composition and SCFAs production. The beneficial bacteria (*Clostridium*_sensu_stricto_1, *Prevotella, Blautia* and *Faecalibacterium*) and the detrimental bacteria (*Lachnospiraceae and Prevotellaceae* family) were identified to mediate the anti-oxidation and protective effect of mulberry leaves on intestinal barrier.

CRediT authorship contribution statement

Hui Yan: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Shurui Yan:** Writing – original draft, Investigation, Formal analysis. **Zaiyao Li:** Investigation, Formal analysis. **Tingting Zhang:** Investigation. **Jun He:** Writing – review & editing, Validation. **Bing Yu:** Visualization. **Jie Yu:** Methodology. **Junqiu Luo:** Methodology. **Aimin Wu:** Visualization. **Junning Pu:** Formal analysis. **Quyuan Wang:** Project administration. **Huifen Wang:** Validation. **Xingyu Liu:** Resources. **Daiwen Chen:** Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.156217.

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