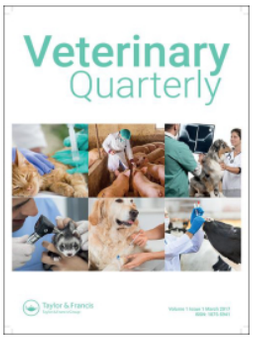

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Isatidis root polysaccharides ameliorates post-weaning diarrhea by promoting intestinal health and modulating the gut microbiota in piglets

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ABSTRACT

This study aimed to investigate the effects of dietary isatidis root polysaccharide (IRP) on diarrhea, immunity, and intestinal health in weanling piglets. Forty healthy piglets were randomly assigned to five groups receiving varying dosages of IRP. The findings indicated that different concentrations of IRP significantly reduced diarrhea scores ($p < 0.01$). Notably, the serum levels of immunoglobulin A and immunoglobulin G increased linearly and quadratically ($p < 0.01$), while immunoglobulin M also showed a linear increase ($p < 0.05$) in IRP-fed piglets. The secretory immunoglobulin A levels in ileal contents were significantly higher compared to control piglets ($p < 0.01$). Key intestinal health parameters, including villus height, villus height-to-crypt depth ratio, and goblet cell numbers, showed linear and quadratic increases in both the jejunum and ileum ($p < 0.05$), while crypt depth decreased significantly ($p < 0.01$). Additionally, the expression of *IL-10, ZO-1*, occludin, and mucin2 was upregulated linearly and quadratically in IRP-fed piglets ($p < 0.05$). In cultured IPEC-J2 cells, *ZO-1* and occludin expression levels significantly increased upon exposure to 400 μg/mL IRP ($p < 0.01$). Furthermore, the relative abundances of *Escherichia coli*, *Ralstonia pickettii*, and *Desulfovibrio fairfieldensis* decreased linearly with increasing dietary IRP concentration. In conclusion, IRP shows promise as an effective dietary supplement for mitigating diarrhea and enhancing intestinal health in early weaned piglets.

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Isatidis root polysaccharides; early weaning stress; diarrhea; intestinal health; gut microbiota

Introduction

Weaning stress is a crucial aspect of the pig farming industry and directly impacts profitability and production efficiency. In intensive production, early weaning of piglets (2–3 weeks old) results in a shorter slaughter cycle and improved reproductive efficiency, but also induces stress in weaning piglets, leading to reduced growth performance and an elevated incidence of diarrhea (Cao et al. 2019, 2022; Yu et al. 2021).

Early weaning-related diarrhea poses a significant threat to animal welfare and is the leading cause of mortality among weaned piglets. The occurrence of diarrhea in early weaned piglets generally reaches to 20%, imposing substantial economic losses that are of concern to the pig industry (Hu et al. 2018; René et al. 2023). Research has demonstrated that weaned piglets experience diarrhea primarily as a result of dietary and environmental changes following weaning, along with deficiencies in their digestive and immune systems (Colson et al. 2012; Wu et al. 2015). These factors can provoke intestinal injury and immune system dysfunction, which subsequently cause increased intestinal permeability, inflammatory reactions, and disruptions in the gut microbiota structure, and ultimately culminate in the development of diarrhea (Shao et al. 2022; Wang et al. 2022). Thus, improving intestinal health and optimizing the gut microbiota structure have emerged as fundamental strategies for mitigating diarrhea in early weaned piglets.

Nutritional supplementation is widely recognized as an effective approach for mitigating weaning stress. In recent years, the utilization of natural products (such as probiotics and phytogenic substances) has attracted increased amounts of attention owing to their potential role in safeguarding intestinal mucosal barrier function and alleviating symptoms associated with gastrointestinal disorders

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(Kim et al. 2022; Song et al. 2021; Yu et al. 2020). Additionally, the incorporation of natural nutritional supplements can contribute to reducing the adverse impacts of antibiotics, as well as high levels of zinc and copper, on public health and the environment (Long et al. 2019; Ma et al. 2021). Plant polysaccharides are widely distributed in nature and possess diverse biological activities, including immunomodulatory, antibacterial, anti-inflammatory, antiviral, antioxidant, and lipid-lowering functions (Mirzadeh et al. 2021; Yang et al. 2022; Yin et al. 2019). Prior investigations have indicated that these polysaccharides can stimulate the growth of weaned piglets, boost immunity, support intestinal development, and improve the composition of the gut microbiota, suggesting that they are a sustainable and natural alternative to antibiotics (Mao et al. 2019; Xi et al. 2017; Yang et al. 2019). Isatis root, derived from the cruciferous plant *Isatis indigotica*, holds a prominent position as a traditional Chinese medicinal material and is renowned for its heat-clearing ability, detoxification ability, ability to cool blood, and ability to soothes the sore throat (Chen et al. 2022; Feng et al. 2021; Wong et al. 2022). Clinical practitioners frequently employ rhizomes and crude extracts of isatis root in the treatment of viral diseases and bacterial infections (Wu et al. 2020; Yang et al. 2013). Isatidis root polysaccharide (IRP) is a key bioactive component found in Isatidis. It primarily consists of arabinose, galactose, glucose, rhamnose, mannose, and xylose (Gao et al. 2021; Liu et al. 2023). Currently, the merits of IRP, including its antibacterial, anti-inflammatory, antiviral, and antioxidant properties, have been widely identified (Li et al. 2019; Wang et al. 2018; Yuan et al. 2022). However, the impact of IRP on weaning stress in piglets has not been fully elucidated. Thus, the aim of this study was to investigate the effects of IRP on diarrhea, growth performance, liver and kidney function, immunity, intestinal barrier function, and the gut microbiota in post-weaning piglets. Furthermore, by understanding how IRP improves diarrhea and overall health in these piglets, this research seeks to establish a foundation for its application in enhancing their wellbeing.

Materials and methods

Animals, diets and management

Forty 21-day-old healthy Duroc×Landrace×Yorkshire castrated male weanling piglets (mean body weight =7.24±0.12 kg) with similar body, weights were randomly assigned to five groups, with eight replicates per group. Each group consisted of one pig per replicate, and the pigs were raised in individual pens. The experiment started the following day and lasted for 21 days. The control group received a basal diet, while the treatment groups were supplemented with 0.1%, 0.2%, 0.5%, or 1.0% IRP in their respective basal diets. The IRP (purity of 90%) was obtained from Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co., Ltd. The basal diet was formulated following the National Research Council (NRC) 2012 guidelines (Appendix Table A1) and was free of antibiotics. The piglets had ad libitum access to feed and water.

Cell culture and treatments

Porcine small intestinal epithelial cells (IPEC-J2) were cultured in DMEM/F12 medium (Gibco, 11320-033) following the methodology described by Hu et al. (2018). The culture medium contained 10% FBS (OPCEL, Inner Mongolia Opcel Biotechnology Co., Ltd., BS1101) and 1% penicillin-streptomycin (Gibco, 15140-122). The cells were incubated at 37 °C in a 5% CO_2 environment. IPEC-J2 cells exhibited 80% to 90% adherent growth in a 25 cm^2 cell culture flask. The cells were subsequently seeded at a density of 1×10^4 cells/mL in 96-well plates and cultured at 37 °C in a 5% CO_2 incubator until they reached 80% to 90% confluence. For storage, 10 mg of IRP was dissolved in 10 mL of autoclaved distilled water, resulting in a concentration of 1 mg/mL. Then, the 1 mg/mL IRP solution was further diluted with complete medium. The final concentrations of IRP in the treatment medium (DMEM/F12 medium, 10% FBS, 1% penicillin-streptomycin) were 0, 200, 400, 600, 800 and 1000 μg/mL, with 6 replicates per group. IPEC-J2 cells were treated with different concentrations of IRP in 96-well plates for 24 h. The cell proliferation rate of each group was determined *via* the CCK-8 method. The minimum safe dose of IRP that did not negatively affect the growth of IPEC-J2 cells was subsequently selected for subsequent tests.

IPEC-J2 cells with 80%-90% adherent growth in a 25 cm^2 cell culture flask were seeded at a density of 2×10^5 cells/mL into 12-well plates and cultured in a 37 °C incubator with 5% CO_2 until 80%-90% confluence was reached. The cells were subsequently divided into five groups, each consisting of 6 replicates. For processing IPEC-J2 cells with IRP, a safe concentration was used, and after 24 h, the cells were collected for RNA extraction. RNAiso Plus was used to isolate the cells, and the relative mRNA expression levels of the *IL-1β*, *IL-10*, *ZO-1*, occludin, and claudin-1 genes were analyzed *via* quantitative real-time PCR.

Sample collection

At the conclusion of the experiment, blood samples were collected from the anterior vena cava of all the piglets *via* a 10 mL blood collection tube. The samples were then left at room temperature for 20 min and centrifuged at a speed of 845 rcf (g) for 10 min. The upper serum was subsequently collected in sterile frozen tubes, preserved in liquid nitrogen, and stored at a temperature of −80 °C. The piglets were euthanized through exsanguination following anesthesia with sodium pentobarbital. The abdominal cavity was subsequently opened to separate the viscera and intestine. Two 2 cm sections of intestinal

tissue were obtained, one from the anterior segment of the jejunum and the other from the posterior segment of the ileum. The former was placed in a 4% paraformaldehyde solution for subsequent histological analysis. Another portion was promptly rinsed with normal saline, transferred to a 2 mL sterile cryostat, and stored in liquid nitrogen. Approximately 10 cm of the intestinal tract, from the anterior segment of the colon, was selected. A small incision was made *via* a scalpel, after which the ends were bonded together with a string. The gut contents were transferred to a sterile 2 mL cryostat and stored in liquid nitrogen. Additionally, the ileal digesta were collected, transferred into a sterile 2 mL cryostat, and stored in liquid nitrogen.

Growth performance and diarrhea score

Piglets were weighed on day 1 and day 21, and the daily food supply was recorded; the remaining data were used to calculate the average daily feed intake (ADFI), average daily gain (ADG), and feed to gain ratio (F/G). The average daily gain (ADG, kg/pig) was calculated by subtracting the initial body weight from the final body weight and dividing the result by the number of experimental days. The average daily feed intake (ADFI, kg/d) was determined by dividing the total feed weight by the number of experimental days. The feed-to-gain ratio (F/G) is computed by dividing the average daily feed intake by the average daily gain.

Diarrhea in the piglets was assessed daily at approximately 15:00 *via* the following scoring criteria: 1 - solid hard stool; 2 - slightly loose stool; 3 - soft stool, partially formed; 4 - semiliquid stool; and 5 - liquid stool separated from solid matter, according to Song et al. (2012).

Relative organ weight

After the piglets were slaughtered, the heart, liver, spleen, lung and kidney were removed, and the surface tissue fluid was dried with absorbent paper and weighed. The calculation formula was as follows: organ weight (%) = organ weight/body weight × 100%.

Serum biochemical parameter analysis

Serum levels of total protein (TP), total bile acid (TBA), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were measured *via* an automatic biochemical analyzer and corresponding reagents (KHB 450, Shanghai Kehua Bioengineering Co., Ltd., Shanghai, China).

Immunoglobulin analysis

Serum levels of IgA, IgG, and IgM were measured *via* ELISA kits obtained from Jiangsu Meimian Industrial Co., Ltd. (Jiangsu, China). Additionally, the sIgA content of the ileal digesta was determined *via* ELISA kits obtained from Quanzhou Ruixin Biological Technology Co., Ltd. (Quanzhou, China). All detection steps were performed according to the manufacturer's instructions for each kit.

Haematoxylin-eosin (HE) staining

In accordance with our previous study (Ma et al. 2022), intestinal tissue was fixed in a 4% paraformaldehyde solution. The tissues were subsequently cut, dehydrated, embedded in paraffin, sectioned, dewaxed, stained with hematoxylin and eosin, dehydrated again, and finally sealed with neutral glue. Intestinal villus height (VH) and crypt depth (CD) were measured through observation and image capture *via* a microscope imaging system (Carl Zeiss, Germany). For each slice, the ratio of villus height to crypt depth (V/C) was calculated on the basis of the analysis of five randomly selected fields.

Alcin blue-periodate scheff (AB-PAS) staining

In accordance with our previous study, tissue paraffin blocks were sectioned, deparaffinized, rehydrated, and stained with AB-PAS staining fluid. The samples were then sealed with neutral resin and subsequently observed and photographed using a Carl Zeiss Microimaging System (Carl Zeiss, Germany). The number of goblet cells was determined by selecting ten intact villi and crypts from each piglet sample, and the results are expressed as the number of goblet cells per villus.

Quantitative real-time PCR analysis

The intestinal tissue was frozen in liquid nitrogen and then ground. Total RNA extraction was performed *via* RNAiso Plus (TaKaRa, Dalian, China), followed by reverse transcription using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Fluorescence quantification was carried out *via* TB Green® Fast qPCR Mix on a LightCycler 480 System II. The porcine-specific primers used in this study were designed (Appendix Table A2). The PCR cycles and relative expression assays followed the methodology described in our previous study by Yin et al. (2018).

Microbial analysis

The total genomic DNA of the colonic contents was extracted *via* the Power Fecal DNA Extraction Kit (MOBIO, USA). The V3-V4 region of the 16S rRNA gene was amplified *via* the universal primers 341 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806 R (5′-GGAC TACHVGGGTWTCTAAT-3′). After purification, the PCR products were used to construct the library with the NEB NextB UltraTM DNA Library Prep Kit and New

England Biolabs, Inc.'s Lumina Library Construction Kit. Index codes were added on the basis of our previous study (Yin et al. 2020). The constructed library was quantified and verified *via* a Qubit instrument. Following the eligibility screening, on-machine sequencing was conducted *via* the Illumina MiSeq PE300 platform. The raw sequences were analyzed *via* QIIME version 1.7.0 and subjected to initial mass filtering, denoising, assembly, and removal of chimeric sequences. Only ASVs with a minimum of 2 reads and presence in more than 2 samples were retained.

In vitro antibacterial activity

The preserved *Escherichia coli* (*E. coli*) K88 (CVCC224) was activated under sterile conditions, inoculated into LB broth medium (tryptone, 10.0 g/L; yeast extract powder, 5.0 g/L; sodium chloride 10.0 g/L), and incubated at 37 °C for 18–24 h to adjust the bacterial concentration to 1.5×10^6 cfu/mL. The sterilized nutrient agar medium (tryptone, 10.0 g/L; yeast extract powder, 5.0 g/L; sodium chloride, 10.0 g/L) was poured into a plate *via* the Oxford cup method, following the protocol established by Li et al. (2019). Once set, 0.1 mL of the bacterial suspension was aspirated *via* a micropipettor, evenly spread with an applicator, and then placed into 4 Oxford cups at equal distances. Using a micropipettor, 200 µL of sterile water, gentamicin sulfate solution (40 mg/mL), 0.1 g/mL IRP, and 0.2 g/mL IRP solution were added to the respective Oxford cups. The cells were incubated at 37 °C for 18–24 h, after which the diameter of the inhibition zone was measured.

Statistical analysis

A single-factor randomized design was used in the animal experiment, in which each pig served as the statistical unit. Before analysis, the Kolmogorov-Smirnov and Levene tests in SPSS 26.0 (the significance level was set to 5%) were used to test the normality and uniformity of the variance of all the data and eliminate outliers. The chi-square analysis was conducted on the piglet diarrhea scores, while other indicators were analyzed through one-way ANOVA. In cases of significant group differences, Duncan's multiple comparison method was utilized. The impact of RIP supplementation concentration was evaluated *via* linear and quadratic orthogonal polynomial models. Cell qPCR results were analyzed *via* the Student's t-test. Spearman rank correlation coefficient analysis was performed in R software (version 3.2.4, peatmap package) to assess correlations. Graphs were generated *via* GraphPad Prism 9.0.0 software. Statistical significance, high significance and tendency were considered at $p < 0.05$, $p < 0.01$ and $0.05 \le p < 0.10$, respectively. The data are reported as the mean and standard error of the mean (SEM).

Results

Growth performance, diarrhea score, and relative organ weight

Table 1 shows that the average daily feed intake and average daily gain of the piglets were not affected by the dietary treatments ($p > 0.10$). However, the F/G decreased linearly with increasing IRP level in the diet ($p < 0.05$), and reduced the diarrhea score of weanling piglets ($p = 0.000$). Table 2 shows that the relative liver weight of the piglets changed in a quadratic relationship with the dietary IRP concentration ($p = 0.023$), but IRP had no significant effect on the relative weights of the kidneys and spleens of the piglets ($p > 0.10$).

Table 1. Effects of isatis root polysaccharides on the growth performance of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Initial body weight, kg/pig	7.13	7.32	7.17	7.35	7.23	0.12	0.981	0.951	0.869
Final body weight, kg/pig	11.45	11.22	11.85	11.88	12.23	0.28	0.822	0.285	0.817
ADG, kg/d	0.21	0.19	0.22	0.22	0.24	0.01	0.342	0.107	0.561
ADFI, kg/d	0.49	0.39	0.43	0.44	0.38	0.02	0.410	0.285	0.273
F/G	2.35	2.26	2.09	2.00	1.87	0.078	0.293	0.020	0.073
Diarrhea score	4.41[a]	2.92[b]	2.93[b]	2.93[b]	2.85[b]	0.13	0.000	0.000	0.000

ADG = average daily gain; ADFI = average daily feed intake; F/G = the ratio of average daily feed intake to average daily gain. SEM = standard error of the mean.
[a,b]Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 2. Effects of isatis root polysaccharides on relative organ weights of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Liver weight, %	2.80	2.80	2.86	2.96	2.74	0.03	0.063	0.638	0.023
Renal weight, %	0.63	0.60	0.62	0.61	0.61	0.01	0.515	0.843	0.870
Spleen weight, %	0.22	0.24	0.20	0.24	0.25	0.01	0.510	0.387	0.766

SEM = standard error of the mean.

Table 3. Effects of isatis root polysaccharides on the serum biochemical parameters of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
ALT, U/L	78.96^{a}	68.20^{a}	66.76^{a}	41.68^{b}	43.68^{b}	3.81	0.001	0.000	0.851
AST, U/L	157.09^{a}	130.36^{ab}	108.66^{abc}	71.37^{c}	89.85^{bc}	8.89	0.011	0.001	0.241
ALP, U/L	421.25^{a}	303.44^{b}	242.00^{c}	152.98^{d}	216.11^{c}	19.43	0.000	0.000	0.000
LDH, U/L	915.17^{a}	764.33^{ab}	744.20^{ab}	498.33^{c}	577.00^{bc}	40.13	0.002	0.000	0.325
TBA, umol/L	65.61	53.71	45.92	51.70	53.74	3.26	0.447	0.377	0.435
TP, g/L	47.18^{b}	63.50^{a}	69.51^{a}	41.81^{b}	40.84^{b}	2.58	0.000	0.000	0.000
GLU, mmol/L	6.51^{a}	5.88^{ab}	4.59^{bc}	4.34^{c}	5.42^{bc}	0.24	0.013	0.049	0.365

ALT = alanine aminotransferase; AST = aspertate aminotransferase; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; TBA = total bile acid; TP = total protein; GLU = glucose. SEM = standard error of the mean.
^{a,b,c,d}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 4. Effects of isatis root polysaccharides on the immunoglobulin contents of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Serum									
IgA, ug/mL	42.06^{c}	55.18^{b}	57.00^{b}	63.95^{a}	64.60^{a}	1.76	0.000	0.000	0.000
IgG, mg/mL	13.01^{b}	20.00^{a}	21.27^{a}	22.25^{a}	22.32^{a}	0.72	0.000	0.000	0.000
IgM, ug/mL	14.24	15.42	16.00	16.09	16.60	0.27	0.054	0.013	0.183
Ileal contents									
sIgA, ug/g	2.64^{b}	6.82^{ab}	12.62^{a}	11.92^{a}	6.55^{ab}	1.14	0.008	0.004	0.213

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; sIgA = secretory immunoglobulin A. SEM = standard error of the mean.
^{a,b,c}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Serum biochemical parameters and immunoglobulin contents

Linear decreases in the serum levels of ALT, AST, LDH, and GLU ($p < 0.05$), a linear and quadratic decreases in the ALP level ($p = 0.000$), and a linear and quadratic increases in the TP content were observed as the dietary IRP concentration increased ($p = 0.000$) (Table 3). Compared with the control group, 0.5% and 1.0% IRP significantly reduced serum levels of ALT, AST, LDH, and GLU ($p < 0.05$). 0.1% and 0.2% IRP significantly increased TP content ($p < 0.05$). Furthermore, 0.1%, 0.2%, 0.5%, and 1.0% IRP significantly decreased LDH levels ($p < 0.05$). Dietary IRP linearly increased serum IgA, IgG, and IgM levels ($p < 0.05$), and the effects of IgA and IgG also exhibited a quadratic change ($p < 0.05$) (Table 4). Compared with those in the control group, 0.1%, 0.2%, 0.5%, and 1.0% IRP significantly increased the serum contents of IgG and IgA ($p < 0.05$). Furthermore, Furthermore, the sIgA content in the ileal contents highly linearly increased with increasing dietary IRP concentration ($p = 0.004$), and 0.2% and 0.5% IRP significantly increased the sIgA content in the ileal contents ($p < 0.05$).

Intestinal morphology

As shown in Table 5 and Supplementary Figure S2, jejunal villus height and the ratio of villus height to crypt depth were linearly and quadratically increased in IRP-fed piglets ($p = 0.000$). Jejunal crypt depth linearly and quadratically decreased ($p = 0.000$). In the ileum, the ileal villus height and the ratio of the villus height to the crypt depth increased linearly and quadratically ($p < 0.05$), and the crypt depth decreased linearly and quadratically ($p = 0.000$) in IRP-fed piglets. Intestinal goblet cells were stained with AB-PAS (Supplementary Figure S3) and the results revealed a linear and quadratic increase in the number of goblet cells in the jejunal and ileal villi of piglets fed an IRP diet ($p < 0.01$). The 0.2%, 0.5%, and 1.0% IRP groups were significantly better than the control group in all parameters ($p < 0.05$).

Expression of inflammation-related genes

The effects of IRP on the expression of inflammation-related genes in the jejunum and ileum tissues of weaning piglets are shown in Table 6. In the jejunal tissue, *IL-1β* expression was quadratically down-regulated ($p = 0.011$) and *IL-10* expression was linearly and quadratically up-regulated ($p < 0.05$) in the IRP-fed piglets, and the expression of *IL-10* in 0.1%, 0.2%, 0.5% and 1.0% IRP groups was significantly higher than that in the control group. ($p < 0.05$). In the ileum, there was a linear and quadratic down-regulation of *IL-1β* expression ($p = 0.000$) and a linear and quadratic up-regulation of *IL-10* expression ($p < 0.05$) with the concentration of IRP supplementation, and the expression levels of *IL-1β* and *IL-10* in the 0.2%, 0.5% and 1.0% IRP groups were significantly different from those in control group.

Expression of intestinal barrier-related genes

The effects of IRP on the expression of genes related to intestinal barrier function in weanling piglets are shown in Table 7. The expression levels of *ZO-1*, claudin-1, occludin and mucin2 in the jejunum were linearly and quadratically up-regulated with the increasing concentrations of IRP ($p < 0.05$). The expressions of *ZO-1* and mucin2 in the ileum were quadratically up-regulated ($p < 0.05$), and the expression of occludin in the ileum was linearly and quadratically up-regulated with the increasing concentrations of IRP ($p < 0.05$). Compared with the control group, supplementation with 0.2% IRP up-regulated the

Table 5. Effects of isatis root polysaccharides on the histomorphology of the jejunum and ileum of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Jejunum									
Villus height, μm	237.21^{c}	292.02^{b}	311.16^{ab}	336.96^{a}	315.08^{ab}	7.79	0.000	0.001	0.000
Crypt depth, μm	205.01^{a}	154.23^{b}	153.59^{b}	152.89^{b}	153.33^{b}	4.29	0.000	0.000	0.000
Villus height/crypt depth	1.18^{c}	1.90^{b}	2.03^{ab}	2.22^{a}	2.07^{ab}	0.07	0.000	0.000	0.000
Goblet cells/villus	8.65^{b}	17.61^{a}	21.50^{a}	22.48^{a}	21.14^{a}	1.10	0.000	0.000	0.000
Ileum									
Villus height, μm	159.49^{c}	274.47^{b}	291.43^{ab}	315.68^{a}	295.41^{ab}	11.13	0.000	0.000	0.000
Crypt depth, μm	177.81^{ab}	188.49^{a}	156.62^{bc}	139.34^{c}	144.50^{c}	4.72	0.000	0.000	0.013
Villus height/crypt depth	0.92^{d}	1.46^{c}	1.87^{b}	2.26^{a}	2.06^{ab}	0.10	0.000	0.000	0.000
Goblet cells/villus	11.69^{d}	17.89^{c}	22.79^{b}	25.74^{a}	22.28^{b}	0.95	0.000	0.000	0.002

SEM = standard error of the mean.
^{a,b,c,d}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 6. Effects of isatis root polysaccharides on the relative expression of inflammatory-related genes in the intestinal tissues of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Jejunum									
IL-1β	1.64	1.66	0.70	0.60	1.40	0.16	0.070	0.557	0.011
IL-10	0.80^{b}	1.86^{a}	1.90^{a}	1.71^{a}	1.89^{a}	0.14	0.025	0.031	0.035
Ileum									
IL-1β	1.54^{a}	0.51^{b}	0.60^{b}	0.63^{b}	0.38^{b}	0.09	0.000	0.000	0.000
IL-10	0.91^{c}	1.70^{bc}	2.49^{b}	5.08^{a}	1.95^{bc}	0.31	0.000	0.019	0.000

IL-1β = interleukin-1β; *IL-10* = interleukin-10. SEM = standard error of the mean.
^{a,b,c}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 7. Effect of isatis root polysaccharides on the relative expression of intestinal barrier-related genes in weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Jejunum									
ZO-1	0.88^{b}	1.75^{b}	2.67^{a}	1.45^{b}	1.26^{b}	0.17	0.005	0.002	0.003
Claudin-1	0.83^{c}	2.00^{a}	1.52^{b}	1.12^{bc}	1.12^{bc}	0.10	0.000	0.000	0.000
Occludin	0.86^{c}	2.47^{b}	3.70^{a}	2.06^{b}	2.22^{b}	0.22	0.000	0.000	0.000
Mucin2	0.79^{c}	3.06^{a}	1.84^{b}	1.75^{b}	1.60^{b}	0.19	0.001	0.000	0.000
Ileum									
ZO-1	0.95^{b}	1.31^{b}	1.34^{b}	1.98^{a}	1.01^{b}	0.10	0.004	0.169	0.006
Claudin-1	0.90	0.82	0.89	0.68	0.65	0.05	0.449	0.812	0.684
Occludin	0.67^{b}	0.92^{b}	1.77^{a}	1.07^{b}	0.86^{b}	0.10	0.003	0.001	0.031
Mucin2	0.88	0.82	1.29	0.70	1.05	0.08	0.123	0.667	0.035

ZO-1 = tight junction protein 1. SEM = standard error of the mean.
^{a,b,c}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 8. Effects of isatis root polysaccharides on the relative expression of IPEC-J2 cell barrier and inflammation related genes.

	Isatis root polysaccharides addition levels, μg/mL			
Item	0	400	SEM	*P*-value
IL-1β	1.17	0.98	0.18	0.329
IL-10	0.77	0.93	0.19	0.432
ZO-1	0.98^{b}	1.49^{a}	0.07	0.000
Claudin-1	0.908	1.30	0.27	0.190
Occludin	0.98^{b}	1.27^{a}	0.03	0.000

IL-1β = interleukin-1β; *IL-10* = interleukin-10; *ZO-1* = tight junction protein 1. SEM = standard error of the mean.
^{a,b}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

expression of *ZO-1* in the jejunum ($p < 0.05$). Furthermore, 0.1% and 0.2% IRP up-regulated the relative expression of *Claudin-1* in the jejunum, whereas 0.1%, 0.2%, 0.5%, and 1.0% IRP up-regulated the relative expression of *Occludin* and *Mucin2* in the jejunum ($p < 0.05$). In the ileum, 0.5% IRP up-regulated the expression of *ZO-1*, and 0.2% IRP significantly increased the expression of *Occludin* ($p < 0.05$).

To further investigate the effect of IRP on intestinal epithelial cells, qPCR was used to evaluate the expression of genes associated with inflammation and barrier function in IPEC-J2 cells. As shown in Table 8, compared with the control treatment, 400 μg/mL IRP upregulated the relative expression of *ZO-1* and occludin ($p = 0.000$).

Intestinal bacterial composition

The colonic microbiota of the piglets was examined *via* 16S rDNA amplicon high-throughput sequencing

to identify changes. Initially, the impact of IRP on colonic microbial diversity was assessed (Figure 1A–D). The Shannon and Simpson indices indicated quadratic increases ($p = 0.084$ and $p = 0.030$, respectively), while a linear increase was observed in the Ace index ($p = 0.027$) and the Chao1 index ($p = 0.032$) in proportion to the IRP concentration. Furthermore, the principal coordinates analysis (PCoA) demonstrated a distinction in the microbiota compositions of the piglets supplemented with 1.0% IRP compared to those in the control group (Figure 1E).

Moreover, we analyzed the colonic microbiota composition at the phylum and species levels in weaning piglets. The dominant colonic microbial groups in weanling piglets were Firmicutes, Bacteroidetes, and Proteobacteria. The analysis of top-15 phylum-level microorganisms revealed a linear increase in the relative abundance of Firmicutes in piglets fed with IRP ($p = 0.000$). Conversely, Bacteroidetes, Campylobacterota, Deferribacteres, and Cyanobacteria displayed a linear decrease in relative abundance ($p < 0.05$), and was significantly lower in the treatment group than in the control group ($p < 0.05$). Additionally, Deferribacteres and Cyanobacteria showed a quadratic decrease ($p = 0.024$ and $p = 0.045$, respectively) (Table 9). At the species level (Table 10), The relative abundance of *Roseburia inulinivorans* increased linearly and quadratically with the dietary IRP concentration ($p = 0.021$ and $p = 0.014$, respectively), while that of *Escherichia coli*, *Ralstonia pickettii*, *Porphyromonadaceae bacterium_DJF_B175* and *Lactobacillus murinus* decreased linearly and quadratically with the dietary IRP concentration ($p < 0.05$). The relative abundance of *Ruminococcus_sp_N15.MGS-57* and *Desulfovibrio fairfieldensis* decreased linearly ($p = 0.030$ and $p = 0.002$, respectively). Compared with the control group, *Escherichia coli*, *Ralstonia pickettii*, *Porphyromonadaceae bacterium_DJF_B175*, *Lactobacillus murinus*, *Ruminococcus_sp_N15.MGS-57* and *Desulfovibrio fairfieldensis* were significantly decreased in the treatment group ($p < 0.05$).

The inhibitory effect of IRP on *E. coli* K88 was further studied *in vitro* (Appendix Table A3 and Supplementary Figure S4) and the results indicated

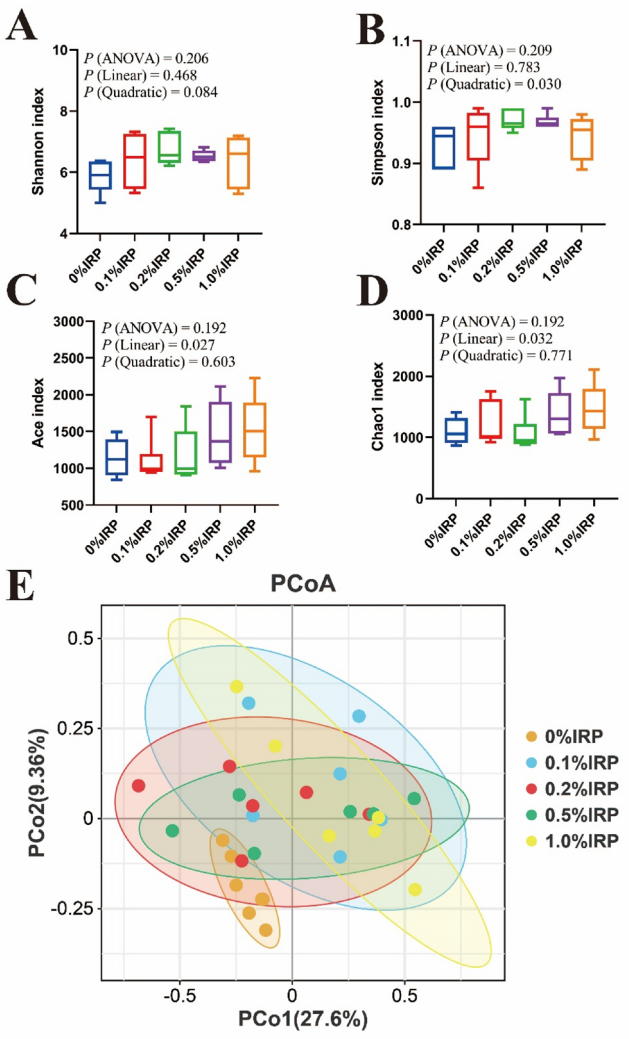

Figure 1. Effects of isatidis root polysaccharides on microbial diversity in Colon of piglets. (A) Shannon index. (B) Simpson index, (C) Ace index. (D) Chao1 index. (E) Principal coordinate analysis (PCoA) plot of bacterial communities based on bray_curti. 0% IRP = basal diet, 0.1% IRP = basal diet supplemented with 0.1% isatidis root polysaccharides, 0.2% IRP = basal diet supplemented with 0.2% isatidis root polysaccharides, 0.5% IRP = basal diet supplemented with 0.5% isatidis root polysaccharides, 1.0% IRP = basal diet supplemented with 1.0% isatidis root polysaccharides.

Table 9. Effects of isatidis root polysaccharide on the relative abundance of top 15 microorganisms at the phylum level in the colons of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Firmicutes, %	59.98^{b}	81.75^{a}	66.35^{b}	77.35^{a}	86.45^{a}	2.55	0.000	0.000	0.550
Bacteroidota, %	29.84^{a}	8.39^{b}	15.16^{b}	9.48^{b}	4.44^{b}	2.43	0.001	0.001	0.067
Campylobacterota, %	0.90^{a}	0.24^{b}	0.45^{b}	0.28^{b}	0.26^{b}	0.08	0.021	0.042	0.082
Proteobacteria, %	2.98	2.15	6.63	6.70	2.23	1.04	0.437	0.864	0.107
Spirochaetota, %	0.43	0.47	1.04	1.18	0.72	0.13	0.278	0.437	0.059
unidentified_Bacteria, %	2.28	2.38	1.73	2.84	1.82	0.32	0.850	0.804	0.588
Actinobacteriota, %	0.54	1.29	0.47	0.26	0.85	0.27	0.822	0.959	0.551
Euryarchaeota, %	0.32	0.02	0.01	0.14	0.30	0.08	0.653	0.570	0.349
Desulfobacterota, %	0.07	0.67	0.79	0.23	0.06	0.12	0.173	0.232	0.316
Fusobacteriota, %	0.00	0.00	0.00	0.00	0.19	0.04	0.450	0.099	0.402
Actinobacteria, %	0.04	0.06	0.09	0.05	0.05	0.01	0.786	0.753	0.687
Verrucomicrobiota, %	0.08	0.03	0.00	0.00	0.04	0.01	0.399	0.635	0.094
Chloroflexi, %	0.00	0.00	0.00	0.00	0.04	0.01	0.438	0.097	0.382
Deferribacteres, %	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00	0.010	0.028	0.024
Cyanobacteria, %	0.03^{a}	0.01^{b}	0.02^{ab}	0.01^{b}	0.01^{b}	0.00	0.044	0.047	0.045

SEM = standard error of the mean.
^{a,b}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 10. Effects of isatidis root polysaccharide on the relative abundance of the top 20 microorganisms at the species level in the colons of weanling piglets.

	Isatis root polysaccharides addition levels, %						*P*-value		
Item	0	0.1	0.2	0.5	1.0	SEM	Treatment	Linear	Quadratic
Lactobacillus johnsonii, %	1.78	1.74	1.06	2.93	1.73	0.26	0.271	0.588	0.261
Lactobacillus amylovorus, %	3.55^{a}	2.00^{ab}	0.81^{b}	1.01^{b}	0.81^{b}	0.38	0.084	0.051	0.095
Faecalibacterium prausnitzii, %	1.17	2.08	2.23	2.01	0.64	0.26	0.230	0.176	0.081
Megasphaera elsdenii, %	0.01	1.45	3.32	1.83	0.16	0.55	0.316	0.575	0.112
Lactobacillus reuteri, %	1.97	0.45	0.35	1.46	1.06	0.21	0.059	0.997	0.442
Roseburia inulinivorans, %	0.13^{b}	0.91^{a}	1.21^{a}	0.52^{ab}	0.73^{ab}	0.12	0.040	0.021	0.014
Eubacterium coprostanoligenes, %	0.47	0.90	0.76	0.36	0.46	0.14	0.765	0.518	0.929
Escherichia coli, %	1.70^{a}	0.05^{b}	0.16^{b}	0.01^{b}	0.00^{b}	0.21	0.016	0.039	0.036
Actinobacillus minor, %	1.01^{a}	0.02^{b}	0.07^{b}	0.02^{b}	0.03^{b}	0.13	0.024	0.062	0.046
Lactobacillus salivarius, %	0.08	0.11	0.34	0.26	0.12	0.05	0.312	0.996	0.089
Ralstonia pickettii, %	0.91^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.09	0.000	0.000	0.000
Olsenella_sp_GAM18, %	0.07	0.05	0.11	0.07	0.33	0.06	0.611	0.166	0.521
Porphyromonadaceae bacterium_DJF_B175, %	0.56^{a}	0.01^{b}	0.03^{b}	0.01^{b}	0.01^{b}	0.06	0.000	0.001	0.000
Weissella jogaejeotgali, %	0.00	0.37	0.11	0.06	0.01	0.07	0.467	0.445	0.787
Lactobacillus murinus, %	0.47^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.01^{b}	0.05	0.000	0.000	0.000
Ruminococcus_sp_N15.MGS-57, %	0.08	0.07	0.10	0.06	0.03	0.01	0.140	0.030	0.661
Actinobacillus porcinus, %	0.12	0.01	0.05	0.02	0.02	0.02	0.171	0.147	0.257
Treponema bryantii, %	0.03	0.00	0.00	0.04	0.03	0.01	0.113	0.248	0.928
Actinobacillus rossii, %	0.03	0.01	0.02	0.01	0.00	0.01	0.434	0.123	0.534
Desulfovibrio fairfieldensis, %	0.03^{a}	0.01^{bc}	0.02^{ab}	0.00^{c}	0.00^{c}	0.00	0.003	0.002	0.112

SEM = standard error of the mean.
^{a,b}Different superscript letters in the same row indicate significant differences ($p < 0.05$).

that IRP (0.1 g/mL and 0.2 g/mL) had a marked inhibitory effect on *E. coli* K88.

Correlation analysis of the gut microbiome, piglet performance and serum parameters

Spearman correlation analysis was also conducted to investigate the relationships among growth performance, immunoglobulin levels, and the gut microbiota of the piglets (Figure 2). Intestinal bacteria, including *Escherichia coli*, *Actinobacillus minor*, *Ralstonia pickettii*, *Porphyromonadaceae bacterium DJF B175*, and *Lactobacillus murinus*, were negatively correlated with the serum immunoglobulin concentration ($p < 0.05$). Moreover, *Lactobacillus amylovorus*, *Escherichia coli*, *Actinobacillus minor*, *Ralstonia pickettii*, *Porphyromonadaceae bacterium DJF B175*, and *Lactobacillus murinus* s were significantly positively correlated with the severity of diarrhea in piglets. Additionally, *Megasphaera elsdenii* and *Lactobacillus salivarius* were significantly positively correlated with the sIgA concentration in the ileal contents.

Discussion

Early weaning stress often results in disorders pertaining to intestinal development, digestion, and immune function. These disorders manifest as small intestinal villus atrophy, nutrient and electrolyte absorption issues, and compromised intestinal barrier function, ultimately leading to diarrhea, growth inhibition, and a host of other problems (Campbell et al. 2013; Cao et al. 2022). Previous studies have demonstrated that plant polysaccharides possess antibacterial, antioxidant, antiviral, immunomodulatory, and growth-promoting properties, suggesting that they

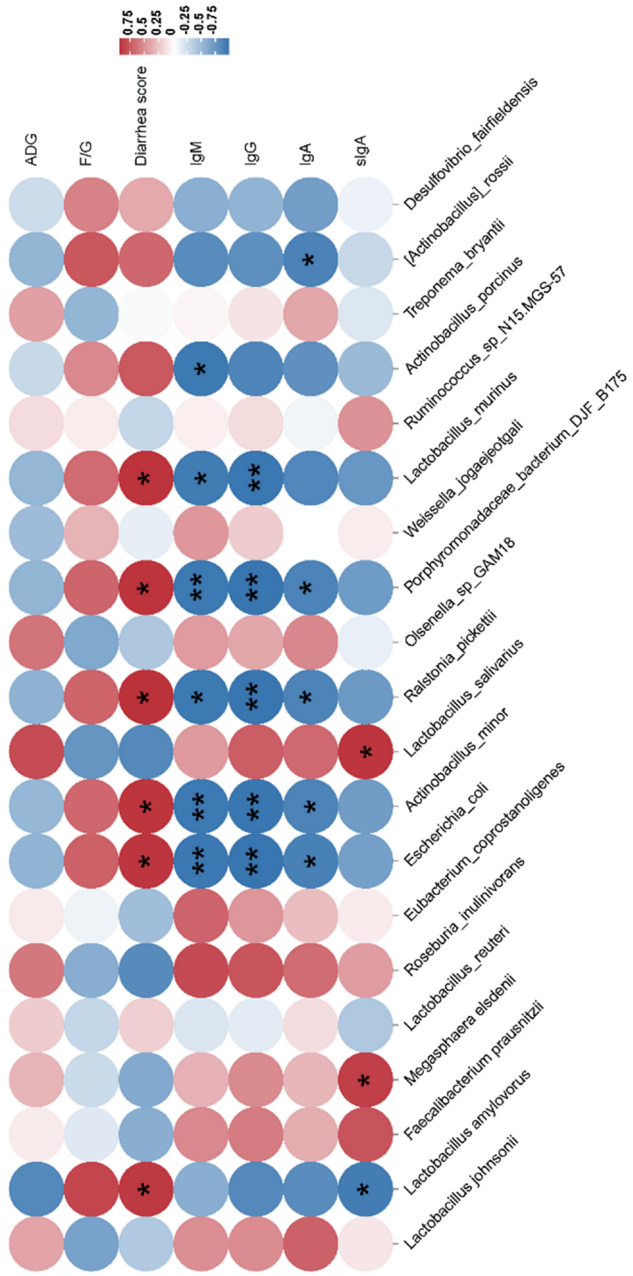

Figure 2. Correlation coefficients between microbial species and growth performance and immune parameters.
ADG = average daily gain; F/G = the ratio of average feed intake to average daily gain. IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; sIgA = secretory immunoglobulin A.
Differences were set to $^{*}P < 0.05$ and $^{**}P < 0.01$.

are potential nutritional supplements for alleviating early weaning stress in piglets (Gao et al. 2021; Meng et al. 2017; Yang et al. 2019; Yin et al. 2021). In this study, the inclusion of varying levels of IRP in the diet did not significantly impact the average daily gain, average daily feed intake, or gain-to-feed ratio of early-weaned piglets. These results contradict those of several previous studies, on the one hand they utilized polysaccharides from different plant sources, as did weanling piglets, which are relatively older and have a longer prefeeding (Mao et al. 2019; Yang et al. 2019). Another possible reason may be attributed to the relatively short duration of our experiment (only 21 days), which may not have allowed sufficient time for the growth performance effects of IRP to fully manifest. Hence, future long-term feeding trials are needed to investigate the influence of IRP on the growth performance of weaned piglets. Furthermore, IPR had no significant effect on the organ weight of piglets. In conclusion, the inclusion of IRP did not significantly adverse affect piglet growth performance.

Diarrhea remains a significant challenge in early weaning piglets. In large-scale pig production, the standard practice for managing diarrhea involves the administration of antibiotics and zinc compounds. However, this approach raises concerns regarding its environmental impact and implications for public health (Xie et al. 2016; Yang et al. 2022). Our findings indicate that dietary supplementation with IRP significantly alleviates diarrhea induced by early weaning stress. These findings suggest that IRP may serve

as a viable alternative intervention to antibiotics and zinc compounds in addressing early weaning diarrhea. Given the increasing concerns over antibiotic resistance and the detrimental effects of zinc on the environment, it is imperative to investigate alternative strategies such as IRP that can effectively mitigate this issue. IRP not only has immunomodulatory properties that enhance gut health and integrity but also promotes a balanced gut microbiota, which is crucial for optimal digestive function in weaned piglets (Chen et al. 2023; Feng et al. 2021; Gao et al. 2021). By incorporating IRP into dietary regimens, producers can reduce their reliance on traditional antimicrobials while concurrently enhancing the overall health and productivity of piglets. This innovative approach not only aligns with sustainable livestock production principles but also facilitates the development of comprehensive strategies that protect against diarrhea, thereby improving the welfare of young pigs during this critical growth and transition phase.

Blood biochemical indicators reflect the metabolic and health status of animals. The liver serves as the primary organ for substance metabolism in the body and functions as the largest detoxification site. ALT, AST, and LDH are crucial enzymes found in the liver cytoplasm and mitochondria, and serve as sensitive indicators of liver injury (Tamber et al. 2023). When liver cells are damaged, the resulting free radicals can harm the cell membrane and organelles, leading to cell swelling or even necrosis. Simultaneously, ALT, AST, and LDH are released from cells into the bloodstream, increasing in the serum ALT, AST, and LDH levels (Woreta and Alqahtani 2014). However, early weaning stress can compromise liver function (Wang et al. 2022; Zaitsev et al. 2022). In the present study, the serum levels of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase in piglets significantly linearly decreased with increasing dietary IRP concentration, and the serum level of alkaline phosphatase highly significantly linearly and quadratically decreased with increasing dietary IRP concentration. These findings are consistent with previous studies conducted by Meng et al. (2019) and Wang et al. (2020), demonstrating the effectiveness of polysaccharides in inhibiting the elevation of serum ALT, AST, and LDH activities resulting from liver injury. Moreover, they contribute to the stabilization of liver cell membranes and the enhancement of liver tissue healing. Furthermore, we found highly significant linear and quadratic increases in total protein levels in the serum of piglets with increasing levels of IRP in the diet, reflecting the ability of the liver to synthesize proteins.(Zaitsev et al. 2021) These findings suggest that IRP may have beneficial effects on liver metabolism, contributing to the health of weanling piglets.

The levels of immunoglobulins, such as IgA, IgG and IgM, are crucial indicators of the body's immune function.(Chen et al. 2023) They are vital antibodies with antiviral, neutralizing, antibacterial, and immunomodulatory properties (Bruzeau et al. 2022; Poonsuk and Zimmerman 2018). Plant polysaccharides, are mild immune enhancers that play a role in immune regulation by promoting the growth and development of immune organs and activating immune cells to secrete cytokines and immunoglobulins (Jiang et al. 2010; Wang et al. 2001). Zhang et al. (2016) showed that IRP can rapidly activate B cells in mouse lymph nodes, increase the number of T and B cells in the blood, induce the secretion of high levels of anti-inflammatory factors by spleen cells, and enhance host specific immunity. Zhao et al. (2020) treated pig small intestinal epithelial cells with hipthorn polysaccharide and reported that the levels of IgM, IgA and IgG increased with increasing hipthorn polysaccharide concentration. Weaning stress usually impairs the immune function of piglets, and the loss of maternal antibody intake, combined with incomplete development of the autoimmune system, eventually leads to a significant decrease in the immunoglobulin level in piglets (Tang et al. 2022). In our study, there were highly significant linear and quadratic increases in the serum IgA and IgG levels as the dietary IRP concentration increased, and there was a significant linear increase in the IgM level. Tang et al. (2019) reported that polysaccharides derived from purple sweet potato can increase the secretion of IgA, IgG, IgM, and sIgA in both normal and immunosuppressed mice, and Yin et al. (2021) also reported an increase in serum IgM and IgG in weanling piglets supplemented with Lycium barbarum polysaccharide, which was similar to the results of the present study. In the context of normal health conditions, an increase in immunoglobulin levels can potentially trigger an overactive immune response, resulting in inflammation and subsequent tissue damage (Chen et al. 2023). Consequently, our findings indicate that IRP can improve the immune state of weanling piglets.

Intestinal villus morphology and tight junction proteins in the intestinal upper mucosa serve as the primary defense against pathogens (Ma et al. 2018; Martens et al. 2018). Maintaining an intact intestinal barrier protects the host from infection and exposure to a potentially stimulating environment (Tang et al. 2022). The key indicators for evaluating animal intestinal morphology include the VH, CD and V/C. A larger VH increases the surface area of the intestinal mucosa, facilitating nutrient absorption and utilization. A shallower CD indicates an increased rate of cell production and enhanced absorption function (Yi et al. 2021). Weaning stress in piglets damages intestinal morphology, resulting in decreased villus height, increased crypt depth, and a reduced villus-to-crypt ratio (Cao et al. 2022; Wang et al. 2022). In this study, we observed that the villus height and the ratio of the villus height to the crypt depth increased linearly and quadratically in the jejunum and ileum, respectively, that the crypt depth decreased linearly and quadratically, and that the number of goblet cells in the jejunum and ileum villi increased linearly and quadratically. The quantity and function of goblet cells have a direct impact on the

proliferative capacity of intestinal stem cells. Adequate mucus secretion helps establish a favorable microenvironment for the gut, thus supporting stem cell activity and proliferation. As intestinal cells renew, goblet cells can enhance stem cell proliferation and help maintain their niche, which facilitates the repair and regeneration of intestinal tissue, ultimately improving conditions associated with diarrhea (Ma et al. 2022; Zhou et al. 2021). These findings are consistent with the results of a previous study conducted on plant polysaccharides (Qiao et al. 2022; Yang et al. 2019), which suggested that IRP can improve intestinal morphology and goblet cell proliferation in weanling piglets.

Tight junctions are crucial for maintaining the integrity of the intestinal epithelial barrier. These junctions consist of proteins such as occludin, claudin, and zonaoccludens (Modina et al. 2019; Upadhaya and Kim 2021). They close the intercellular space, forming a physical barrier against pathogen invasion, while also regulating the movement of water molecules, ions, and solutes (Moeser et al. 2017). Therefore, the integrity of the tight junction structure serves as an important marker for gut health. Early weaning stress can impair the intestinal barrier function of piglets. In our study, we found that IRP increased the relative mRNA expression of *ZO-1*, occludin, claudin-1, and mucin2 in the intestines of weaning piglets, which is similar to the findings of some previous studies (Hao et al. 2022; Qiao et al. 2022). Intestinal epithelial cells play a crucial role in nutrient absorption and digestion, and maintain the integrity of the physical and biochemical barrier of the intestinal mucosa (Okumura and Takeda 2017). In the present study, the impact of IRP on the intestine of early weaning piglets was verified *via* IPEC-J2 cells. The findings demonstrated a significant increase in the expression of *ZO-1* and occludin in the presence of 400 μg/mL IRP. In conclusion, these results indicate that IRP may improve the integrity of intestinal tight junctions in weanling piglets.

Early weaning stress often leads to excessive inflammation in the intestines of piglets, causing damage to the intestinal epithelium and impairing repair mechanisms (Cao et al. 2022). This situation significantly affects the health and performance of piglets. IL-10 is a pleiotropic cytokine with potent anti-inflammatory properties. It is primarily secreted by antigen-presenting cells, such as activated T cells, monocytes, B cells, and macrophages. IL-10 inhibits the expression of inflammatory cytokines such as TNF-α, IL-6, and IL-1 by activating macrophages (Ouyang and O'Garra 2019; Wei et al. 2020). In our study, IRP increased the relative mRNA expression of *IL-10* and decreased the relative mRNA expression of *IL-1β* in the jejunum and ileum. Furthermore, we found a highly significant linear increase in sIgA levels in the ileal contents of piglets with increasing dietary IRP levels. SIgA is the main immunoglobulin in the intestinal mucosa. It plays a crucial role in eliminating harmful bacteria, neutralizing viruses, and protecting against potential harm from pathogens (Corthésy 2013; Pabst 2012). Our findings suggest that IRP may improve intestinal immune function and inhibit excessive inflammation in the intestines of weanling piglets.

The gut microbiota is an essential component of the gastrointestinal tract, and is involved in maintaining intestinal morphology, nutrient absorption, and various physiological activities (Shi et al. 2017; Vos et al. 2022). Previous research has demonstrated the beneficial effects of plant polysaccharides on intestinal diseases through modulation of the composition and functionality of the gut microbiota (Song et al. 2021). For instance, Chen et al. (2020) conducted a study in which they reported that dietary supplementation with 4,000 mg/kg lycium barbarum polysaccharide resulted in an increase in the *Lactobacillus* population and a reduction in *Escherichia coli* numbers in the ileum and cecum of weaned piglets, consequently decreasing the occurrence of diarrhea. In our study, we used 16S rDNA sequencing to analyze the alpha diversity of the microbiota in the anterior segment of the colon in weanling piglets. We observed that IRP increased colonic microbial α diversity in weanling piglets. Furthermore, PCoA based on bray_curti revealed a significant difference in the microbial composition between the 1.0% IRP group and the control group. An increased abundance of probiotics in the gut microbiota generally benefits host health (Adak and Khan 2019). At the phylum level, there was a linear increase in the relative abundance of Firmicutes in piglets fed with IRP, and a linear decrease the relative abundance of Campylobacterota and Deferribacteres. Campylobacterota is a zoonotic pathogen known to cause various diseases, particularly bacterial diarrhea, in humans and animals. At the species level, the relative abundance of *Roseburia inulinivorans* increased linearly and quadratically with the dietary IRP concentration, while that of *Escherichia coli*, *Ralstonia pickettii*, *Porphyromonadaceae bacterium_DJF_B175* and *Lactobacillus murinus* decreased linearly and quadratically with increasing dietary IRP concentration. The relative abundance of *Ruminococcus_sp_N15.MGS-57* and *Desulfovibrio fairfieldensis* decreased linearly ($p = 0.030$ and $p = 0.002$, respectively). We also observed an inhibitory effect of IRP on *E. coli K88 in vitro*, similar to some previous results (Yao et al. 2022; Zhu et al. 2018). *Roseburia inulinivorans*, a member of the *Roseburia* genus, is a crucial gut bacterium that utilizes various metabolic substrates to produce SCFAs, such as acetic acid, propionic acid, and butyric acid, which are important for controlling intestinal inflammation (Nie et al. 2021). These SCFAs serve as energy sources for intestinal cells and play essential roles in intestinal health (van der Hee and Wells 2021). Most strains of *Escherichia coli*, *Actinobacillus minor*, *Ralstonia pickettii*, and *Desulfovibrio fairfieldensis* are pathogenic and can cause diarrhea in animals (Ren et al. 2022; Zheng et al. 2023). Correlation analysis verified that IRP improved diarrhea in weanling piglets by reducing the relative abundances of *Escherichia coli*, *Actinobacillus minor*, and *Ralstonia pickettii*. Overall, our findings indicate that appropriate supplementation with IRP may

increase the abundance of intestinal probiotics and inhibit the proliferation of pathogenic bacteria, thereby promoting intestinal health in piglets.

Conclusion

In conclusion, under experimental feeding conditions, we found that dietary IRP supplementation has the potential to alleviate diarrhea and intestinal injury induced by early weaning in piglets. The observed effects may be attributed to the enhancement of liver function, improvement in intestinal morphology and immune state, promotion of goblet cell proliferation, preservation of intestinal barrier integrity, increase in intestinal probiotic levels, and reduction in harmful bacteria. Importantly, this study represents an initial investigation; therefore, further research involving larger participant groups and long-term trials is needed to validate these findings. Additionally, a more comprehensive investigation is necessary to elucidate the specific mechanism through which IRP improves diarrhea and intestinal injury in young animals.

Ethics approval and consent to participate

All experimental procedures were performed according to the Institutional Animal Care and Use Committee at Hunan Agricultural University, Changsha, P. R. China (Approval No. 202105).

Authors' contributions

Conceptualization, M.Z., Z.H.Y and Y.L.Y; Methodology, M.Z. and Z.H.Y; Software, D.Q.W., M.Z. and Z.H.Y; Validation, L.L.W; Formal analysis, M.Z. and Z.H.Y; Investigation, R.L; Resources, C.Y.L. and Y.L.Y; Data Curation, D.Q.W; Writing-Original Draft, M.Z. and Z.H.Y; Writing-Review& editing, J.Y., C.Y.L. and Y.L.Y; Visualization, R.L. and J.Y; Supervision, C.Y.L. and Y.L.Y. Project administration, M.Z. and Z.H.Y.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix A

Table A1. Ingredient composition and nutrient levels of the basal diet (as-fed basis).

Ingredients	Content	Nutrient levels[a]	Content
Corn, %	55.00	Digestible energy, kcal/kg	3543.30
Fermented soybean meal, %	31.00	Crude protein, %	18.51
Soybean oil, %	3.00	Calcium, %	0.80
Glucose, %	2.00	Total phosphorus, %	0.60
Sucrose, %	3.00	Available phosphorus, %	0.38
Limestone, %	1.00	SID lysine, %	1.40
$CaHPO_4$, %	1.50	SID methionine, %	0.53
NaCl, %	0.30	SID methionine + cystine, %	0.73
Citric acid, %	0.90	SID threonine, %	0.77
Choline chloride, %	0.10	SID tryptophan, %	0.19
L-Lysine HCl, %	0.60		
DL-Methionine, %	0.30		
L-Threonine, %	0.12		
Premix[b], %	1.18		
Total, %	100.00		

[a]The digestible energy and ileal standard digestible amino acids (SID) were calculated according to NRC (2012), and the analysis of crude protein, calcium, total phosphorus, and available phosphorus was conducted using the reference method provided by AOAC (2006).

[b]Premix provided the following per kilogram of diets for weaned piglets: Cu 126.00 mg/kg; Fe 102.00 mg; Zn 106.50 mg; Mn 17.70 mg; I 0.18 mg; Se 0.14 mg; VA 8000 U; VB_1 1.8 mg; VB_2 4.4 mg; VB_6 4.4 mg; VB_{12} 0.025 mg; VC 150.00 mg; VD_3 1000.00 U; 25-OH-D_3 0.025 mg; VE 120.00 mg; Pantothenic acid 12.40 mg; Niacinamide 25.00 mg; Folic acid 0.88 mg; Biotin 132.00 mg.

Table A2. Primers used for gene expression analysis *via* real-time qPCR.

Gene	Primer sequence (5′–3′)	Product length, bp
IL-1β	F: CCTGGACCTTGGTTCTCT	123
	R: GGATTCTTCATCGGCTTCT	
IL-10	F: TCGGCCCAGTGAAGAGTTTC	127
	R: GGAGTTCACGTGCTCCTTGA	
ZO-1	F: TTGATAGTGGCGTTGACA	126
	R: CCTCATCTTCATCATCTTCTAC	
Claudin-1	F: GCATCATTTCCTCCCTGTT	97
	R: TCTTGGCTTTGGGTGGTT	
Occludin	F: CAGTGGTAACTTGGAGGCGTCTTC	103
	R: CGTCGTGTAGTCTGTCTCGTAATGG	
Mucin2	F: CTGTGTGGGGCCTGACAA	65
	R: AGTGCTTGCAGTCGAACTCA	
Beta-actin	F: CTGCGGCATCCACGAAACT	147
	R: AGGGCCGTGATCTCCTTCTG	

IL-1β = interleukin-1β; *IL-10* = interleukin-10; *ZO-1* = tight junction protein 1.

Table A3. Isatis root polysaccharide on the inhibitory of *Escherichia coli* K88 *in vitro*.

Isatis root polysaccharides addition levels, g/mL	Antibacterial circle diameter
0.1	12.75 mm
0.2	15.46 mm