

Eucommia Ulmoides Flavones as Antibiotic Alternatives in a Low-protein Diet Improve Growth Performance and Intestinal Health in Weaning Piglets

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Abstract

Background

Eucommia ulmoides flavones (EUF), have been demonstrated to attenuate the inflammation and oxidative stress of piglets. This study was designed to test whether EUF could be as an antibiotic alternative to support growth performance and maintain intestinal health in weanling-piglets. Weaned-piglets (n = 480) were assigned into 3 groups and fed with a low-protein basal diet (NC), or supplementation with antibiotics (PC) or 0.1‰ EUF (EUF). Blood, intestinal contents and intestine were collected on d-15 and d-35, respectively.

Results

The results showed that the body weight on d-35, average daily gain and gain : feed from d 15 to d 35 and d 0 to d 35 in piglets of PC and EUF treatments were higher than ($p < 0.05$) these in NC treatment, whereas PC and EUF treatments decreased ($p < 0.05$) the diarrhea index of weanling piglets. Dietary supplementation of EUF significantly enhanced ($p < 0.05$) the serum concentrations of total protein, alanine transaminase and the immunoglobulin G on d-15 of piglets compared to piglets in NC treatment. EUF supplementation increased ($p < 0.05$) the jejunal and ileal villus height and the population of ileal lactic acid bacteria on d-15 but remarkable decreased ($p < 0.05$) the population of ileal coliform bacteria on d-15 and d-35.

Conclusion

These findings indicated the antibiotic alternative capacity of EUF in piglets exhibiting by improving growth performance and intestinal morphology, decreasing colonization of *coliform bacteria* and diarrhea index in weanling piglets.

Background

Announcement No. 194 of the Ministry of Agriculture and Rural Affairs of the People's Republic of China stipulate that medicated feed additives will be prohibited for using in animal feed in 2020 [1]. Antibiotics free diets have become a necessity in livestock and poultry industry. The withdrawal of antibiotic use will lead to lower quality and yield of animals or death, more serious disease outbreaks, then result in greater use of antibiotics for therapeutic purposes [2]. Therefore, new antibiotic alternative strategies are needed to guarantee animal health and yield growth. It has been well documented that some antibiotic alternatives and feed additives, such as plant extracts, organic acids, microecologics, antimicrobial peptides, could promote the animal development and enhance the intestinal health [3–6].

Of the various alternative, plant extract is one of the most readily available and safe choice that being investigated [7, 8]. The *Eucommia ulmoides* flavones (EUF), which are bioactive phytochemicals derived from the leaves of *Eucommia ulmoides*, have been demonstrated to improve the antioxidative function by reacting with free radicals [9], inhibit the growth of bacteria and reduce the response of inflammation in previous studies [10–12]. Our group has showed that EUF alleviate the oxidative stress induced by diquat in piglets by reducing the growth performance impairment, pro-inflammatory cytokines secretion and intestinal barrier dysfunction [3, 13]. Meanwhile, we recently reported the NF-E2-related factor 2 (Nrf2) signaling pathway played an important role in EUF-regulating oxidative stress in the intestine of piglets [14]. The Nrf2 pathway not only involved in antioxidant by regulating the mRNA levels of antioxidant enzymes, but also enhancing the intestinal barrier integrity through increasing the expression of tight junction proteins [15]. The improved functional gut immunity and integrity were vital to reduce the permeability for viable pathogens and pathogen colonization in the gut. These benefit effects may enable it to be an effective antibiotic alternative to promote animal growth in animal husbandry.

Therefore, the present study was conducted to investigate the effect of EUF as antibiotic alternative in a low-protein diet on growth performance and intestinal health of weaning piglets. The low-protein diet was used in this study because it was benefit to relieve the nutritional burden of excess dietary protein and alleviate intestinal dysfunction and diseases [16]. Growth performance, serum biochemical parameters, intestinal morphology and microbiota composition were monitored so as to provide the scientific basis for the application of EUF in antibiotics-free diets in swine production.

Methods

The animal experiments were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Hunan, China.

Animal Protocol and Dietary Treatment

A total of 480 piglets (Duroc × Landrace × Large Yorkshire) weaned at 25-d of age were randomly assigned to 3 groups (8 pens per group and 20 piglets per pen) as follows: (1) Negative Control (NC), low-protein basal diet no antibiotics included; (2) Positive control (PC), low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); (3) EUF treatment (EUF), low-protein Basal diet + 0.1‰ EUF. The low-protein basal diet was formulated in two phases (phase 1, days 0-15; phase 2, days 15-35) according to the nutrient requirements for weanling piglets (NRC, 2012) and the previous studies [17] (Table 1). EUF powder contained 83.61% total flavones was prepared at the department of medicine, Jishou University (Jishou, Hunan, China), which has been used in the previous study by Yuan et al [13]. Additives were added to the negative control diet at the expense of corn.

The piglets were housed in an environmentally controlled nursery with hard plastic slatted flooring, and had free access to drinking water. Piglets were fed their respective diets 3 times per day at 8:00, 13:00 and 18:00 for a 35-d period. On the morning at d 15 and 35, 24 piglets (1 piglet per pen) were randomly selected and blood samples were obtained aseptically from the jugular vein at 2 h after feeding. Serum

samples were obtained by centrifugation at $2000 \times g$ for 10 min at 4 °C and then immediately stored at -80 °C for further analysis. Piglets were anesthetized with sodium pentobarbital and killed by jugular puncture. And the intestine samples were collected after rinsing thoroughly with ice-cold physiological saline solution. About 2 cm segments of the jejunum and ileum were cut and fixed in 4% formaldehyde for observation of the morphology of intestinal mucosa. Ileal and colonic contents were collected for bacteria counting and DNA extraction.

Growth Performance, Diarrhea Index

Body weight and feed intake were measured at day 15 and 35. Average daily gain, average daily feed intake and gain : feed ratio were calculated. The number of pigs with diarrhea was recorded every day. Diarrhea index (%) was calculated as the number of diarrhea piglets \times diarrhea days / the total number of piglets \times experiment days.

Intestinal Morphology Evaluation

The jejunal and ileal morphology were analyzed using hemotoxylin eosin staining according to Xiao et al. [18]. Villous height and crypt depth were measured with computer-assisted microscopy (Micrometrics TM; Nikon ECLIPSE E200, Tokyo, Japan).

Serum Biochemical Parameters Determination

Total protein (TP), Albumin (ALB), blood urea nitrogen (BUN), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), immunoglobulin G (IgG) and IgM in the serum were measured using Biochemical Analytical Instrument (Cobas c311, F. Hoffmann-La Roche Ltd., Basel, Switzerland) and commercial kits (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

Microbiota Composition Analysis

Bacteria counting were performed according to the previous studies [5, 19]. 0.2 g of ileal and colonic contents were collected and immediately diluted with 9 mL of 0.9% sterilizing saline and homogenized. Then, 10-fold dilutions of homogenate were performed (ranging from 10^{-1} to 10^{-8}) and then cultivated onto MacConkey Agar Medium for the enumeration of *Escherichia coli*, and GM17 Medium for the enumeration of Lactic acid bacteria. The GM17 medium were then incubated for 48 h at 30 °C under anaerobic conditions, while the MacConkey agar plates were incubated for 24 h at 37 °C. The coliform bacteria and lactic acid bacteria colonies were counted immediately after removal from the incubator. Values were reported as log₁₀ colony-forming units per gram.

DNA was extracted from ileal and colonic contents with the Tiangen stool mini kit (TianGen) according to the instructions of the manufacturer. DNA concentration was determined by spectrophotometry (Nanodrop). The DNA obtained from the intestinal luminal content was used as the template to analyze intestinal bacteria by qRT-PCR. Primers (*Lactobacillus spp.* (F) 5'-CACCGCTACACATGGAG-3' (R) 5'-TGGAAGATTCCCTACTGCT-3', *Escherichia coli* (F) 5'-CATGCCGCGTGTATGAAGAA-3' (R) 5'-

TTTGCTCATTGACGTTACCCG-3', total bacteria (F) 5'-ACTCCTACGGGAGGCAGCAG-3' (R) 5'-ATTACCGCGGCTGCTGG-3') were synthesized according to the previous study [20]. Relative expression of genes in the treatment group was normalized to the values for the NC.

Statistical Analysis

All data were subjected to ANOVA analysis using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The differences among treatments were evaluated using Tukey's test. Probability values $p < 0.05$ were taken to indicate statistical significance.

Results

Growth Performance and Diarrhea Index

The average daily gain, average daily feed intake, and gain : feed were showed in Figure 1. The body weight on d 0 and 15 were not different among all treatments ($p > 0.05$). The body weight of piglets in PC and EUF treatments were less ($p < 0.05$) than those in the NC treatment on d 35. Compared with the piglets of NC treatment, PC and EUF treatments increased average daily gain and gain:feed from d 15 to 35 and d 0 to 35 ($p < 0.05$). However, there was no difference in average daily feed intake of all experimental periods among all treatments ($p > 0.05$). The diarrhea index in the piglets of PC and EUF treatments from d 0 to 15 and d 0 to 35 were significantly lower than those of NC treatment ($p < 0.05$) (Figure 2).

Serum Biochemical Parameters

Dietary supplementation with EUF increased ($p < 0.05$) the serum concentrations of TP and IgG, as well as ALT activity on d 15 and serum TP content on d 35 compared to NC treatment. But in comparison to PC treatment, EUF addition only promoted ($p < 0.05$) serum TP content and ALP activity on d 15 and there was no difference ($p > 0.05$) in other determined serum biochemical parameters on d 15 or 35 (Table 2).

Microbiota Composition in Ileum and Colon

In the ileum, EUF addition increased the number of Lactic acid bacteria on d 15 but decreased the number of Coliform bacteria on d 15 and 35 compared with NC treatment ($p < 0.05$). And no significant difference was observed in the numbers of Lactic acid bacteria and Coliform bacteria between PC and EUF treatments on d15 and 35 ($p > 0.05$) (Table 3). For the relative abundance of selected microbiota, both PC and EUF treatments significantly decreased the population of *Escherichia coli* on d 15 and 35 but increased the population of *Lactobacillus spp.* on d 15 in the ileum compared to those of NC treatment ($p < 0.05$) (Figure 3). There was no difference in determined microbiota in the colon among three treatments on d 15 and 35 ($p > 0.05$).

Intestinal Morphology

Dietary supplementation with EUF increased ($p < 0.05$) the jejunal and ileal villus height on d 15 compared to these in NC treatment. No difference was observed on villus height and crypt depth in jejunum and ileum between PC and EUF treatments on d 15 and 35 ($p > 0.05$) (Table 4).

Discussion

The present study demonstrated that dietary supplementation with EUF in a low-protein and antibiotic-free diet improved growth performance and intestinal morphology, decreased the colonization of *coliform bacteria* and diarrhea index in weaning piglets. A low-protein diet was used as basal diet, which could improve intestinal health. Growing evidences have showed that feeding a low-protein in post-weaning period decreased the cost of feed while effectively relieving the nutritional burden of excess dietary protein by decreasing hindgut microbial protein fermentation [16, 21]. Although reducing the dietary protein content and removing antibiotics influence the growth performance and intestinal microbial structure [22, 23], the low-protein diets is an efficient way for antibiotic alternative.

Ideal antibiotic alternatives should have the same beneficial effects of antibiotic growth promoters, ensuring optimum animal performance and nutrient availability [8]. Similar increases in body weight, average daily gain and feed efficacy between the piglets of antibiotic positive control and EUF treatment were obtained in our present study. In addition, EUF supplementation promoted serum TP content and ALP activity, which in a certain extent showing the increased nutrient efficiency [24]. It is important to develop an alternative strategy to stimulate innate immune response and limit the infections in livestock, and subsequently decrease the use of antibiotics [25, 26]. IgG is a feature of immune cell maturation and plays a critical role in defending against infection via the direct neutralization of toxins and microbes [27–29]. Dietary supplementation with EUF was shown to enhance the serum IgG concentration in current study. Results from our previous published report also demonstrated that EUF exerts immunomodulatory activities by modifying the production of cytokines *in vivo* [13] and regulating the NF- κ B pathway *in vitro* [30].

The proposed mechanism of promoting growth effects of a practical alternative may be involved in modulating the gut microbiome [7, 8, 31]. Weaning stress induces the population of pathogenic *Escherichia coli* to proliferate to exceed those of other bacterial populations, which is associated with many diseases after weaning [32]. Removing antibiotics from the diet will inevitably lead to a further increase in the number of microorganisms [33]. In current study, antibiotic free dietary supplementation with EUF significantly increased the population of lactic acid bacteria and decreased coliform bacteria in ileal content. Various stress factors exposed to piglets could lead to microbial imbalance due to the increased *Enterobacteriaceae* and decreased lactic acid bacteria counts resulting in infectious diarrhea [34, 35]. Lactic acid bacteria is most commonly used probiotic in livestock, and predominant at the early stage of pig gut microflora construction [36]. Increased lactic acid bacteria can reduce fecal pH and ammonia nitrogen levels [34, 37], as well as prevent colonization of pathogenic organism colonization,

therefore improve the natural microbiome and gut health [31]. Lots of bioactive antimicrobial chemical forms, including phenolic acids, quinones, flavonoids, tannins and alkaloids, have been identified and been used in animal nutrition [7, 8]. However, due to their complex compositions and the potential for multiple sites of action, the mechanisms of antibacterial activity are not fully understood. One of the mechanisms of inhibitory action is involved that hydroxyl groups in phenolic compounds interact with the cell membrane of bacteria to disrupt membrane structures and cause the leakage of cellular components [38].

Recent studies have explored that antibiotic exposure early in life has long-term consequences on intestinal homeostasis and epithelial barrier function [39, 40]. In addition to antibacterial activity, the effect of EUF on the intestinal barrier function was investigated in present study. Although we did not detect the tight junction protein expression and intestinal permeability, the significantly increased villus height in jejunal and ileal mucosa were observed in EUF supplemented group. In our previous study, EUF improved the morphology structure and barrier function of intestine in piglets challenged by diquat exhibiting higher intestinal villus height and lower serum concentrations of D-lactate and diamine oxidase [13]. The intestinal barrier is the first line of defense against pathogen attachment to and invasion of epithelial cells [41]. The effect of EUF on intestinal barrier function may benefit its ability to enhance host defense against microbial infections [8, 42].

Conclusion

In summary, flavones extracted from *Eucommia ulmoides* leaf have shown the health-promoting properties and antibiotic alternative capacities in weanling piglets fed a low-protein diet. The results indicated that EUF improved the growth performance, maintained the intestinal barrier morphology, enhanced the serum immunoglobulin G level, as well as reduced the colonization of *coliform bacteria* and diarrhea index in weaning piglets. These findings can contribute to exploration of EUF as a potential alternative antibiotic to against the microbe infection in swine production although further studies are needed to further explain the mechanism.

Abbreviations

EUF: *Eucommia ulmoides* flavones; NC: basal diet; PC: diet supplementation with antibiotics; Nrf2: NF-E2-related factor 2; TP: total protein; ALB: Albumin; BUN: blood urea nitrogen; GLU: glucose; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; IgG: immunoglobulin G.

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Author contributions

Conceptualization, J.W., X.D. and B.T.; Methodology, X.D. and D.Y.; Formal Analysis, D.Y. and J.L.; Data Curation, D.Y. and J.L.; Writing – Original Draft Preparation, D.Y.; Writing – Review & Editing, J.W. and Y.L.; Supervision, X.D. and B.T.

Ethics approval and consent to participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed standards for the protection of animals used for scientific purposes. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Hunan, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table1.Composition of Basal Diets (as-fed basis)

ingredients, %	phase 1	phase 2	chemical composition	phase 1	phase 2
corn	57	60	crude protein	17.4	16.3
expended maize	5	5	calculated DE, kcal/kg	3466	3420
soybean meal (43%CP)	22	22			
rice bran meal	5	5			
broken rice	5	5			
fish meal	2	/			
sucrose	1	/			
calcium lactate	0.3	0.3			
calcium hydrogen phosphate	1	1			
limestone powder	0.1	0.1			
premix ^a	1	1			
98% lysine	0.4	0.4			
threonine	0.1	0.1			
methionine	0.1	0.1			

^a Providing the following amounts of vitamins and minerals per kilogram on an as-fed basis: Zn (ZnO), 50 mg; Cu (CuSO₄), 20 mg; Mn (MnO), 55 mg; Fe (FeSO₄), 100 mg; I (KI), 1 mg; Co (CoSO₄), 2 mg; Se (Na₂SeO₃), 0.3 mg; vitamin A, 8,255 IU; vitamin D3, 2,000 IU; vitamin E, 40 IU; vitamin B1, 2 mg; vitamin B2, 4 mg; pantothenic acid, 15 mg; vitamin B6, 10 mg; vitamin B12, 0.05 mg; nicotinic acid, 30 mg; folic acid, 2 mg; vitamin K3, 1.5 mg; biotin, 0.2 mg; choline chloride, 800 mg; and vitamin C, 100 mg. The premix did not contain additional copper, zinc, antibiotics, or probiotics.

Table2. Serum biochemical parameters of piglets

Items	NC	PC	EUf	SEM	P-value
Day 15					
Total protein, g/L	48.29 ^b	50.21 ^b	59.82 ^a	2.30	0.006
Albumin, g/L	32.15	35.94	37.45	3.04	0.552
Blood urea nitrogen, mmol/L	4.21	4.53	4.78	0.16	0.075
Glucose, mmol/L	6.78	6.49	6.53	0.20	0.629
Alanine transaminase, U/L	32.38 ^b	38.59 ^{ab}	47.37 ^a	2.73	0.006
Aspartate aminotransferase, U/L	30.24	34.27	29.57	3.18	0.608
Alkaline phosphatase, U/L	214.24 ^{ab}	199.27 ^b	237.57 ^a	9.20	0.029
Immunoglobulin G, g/L	1.36 ^b	1.76 ^{ab}	1.97 ^a	0.15	0.036
Immunoglobulin M, g/L	0.36	0.45	0.48	0.04	0.121
Day 35					
Total protein, g/L	45.26 ^b	49.67 ^{ab}	52.98 ^a	1.95	0.046
Albumin, g/L	28.56	29.79	32.97	3.21	0.631
Blood urea nitrogen, mmol/L	4.39	4.06	3.78	0.18	0.083
Glucose, mmol/L	6.87	6.79	6.94	0.16	0.838
Alanine transaminase, U/L	35.19	39.46	41.38	2.81	0.306
Aspartate aminotransferase, U/L	28.49	31.05	29.87	1.84	0.642
Alkaline phosphatase, U/L	197.56	214.56	227.69	9.33	0.104
Immunoglobulin G, g/L	1.27	1.58	1.87	0.18	0.088
Immunoglobulin M, g/L	0.29	0.32	0.28	0.03	0.710

NC = negative control, low-protein basal diet no antibiotics included; PC = positive control, low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); EUf = *Eucommia ulmoides* flavones, low-protein Basal diet + 0.1‰ EUf. Values are the mean \pm SEM, n = 8 per treatment group. ^{a-b}Mean values sharing different superscripts within a row differ ($p < 0.05$).

Table 3. Lactic acid bacteria and coliform bacteria in the ileum and colon of piglets^a

Log ₁₀ cfu/g		NC	PC	EUf	SEM	p-value
day 15						
Lactic acid bacteria	ileum	6.59 ^b	7.35 ^{ab}	7.49 ^a	0.13	<0.001
	colon	7.28	7.18	7.29	0.21	0.933
Coliform bacteria	ileum	5.59 ^a	4.58 ^b	4.76 ^b	0.19	0.003
	colon	4.54	4.28	4.32	0.22	0.687
day 35						
Lactic acid bacteria	ileum	6.27	5.98	6.18	0.12	0.240
	colon	7.15	6.87	7.09	0.25	0.721
Coliform bacteria	ileum	4.79 ^a	4.15 ^b	4.06 ^b	0.15	0.001
	colon	4.35	4.28	4.21	0.13	0.751

NC = negative control, low-protein basal diet no antibiotics included; PC = positive control, low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); EUf = *Eucommia ulmoides* flavones, low-protein Basal diet + 0.1% EUf. Values are the mean \pm SEM, n = 8 per treatment group.^{a-b} Mean values sharing different superscripts within a row differ ($p < 0.05$).

Table 4. Jejunal and Ileal Morphology in Piglets^a

item		NC	PC	EUf	SEM	p-value
day 15						
villous height, μ m	jejunum	246.47 ^b	274.32 ^a	276.35 ^a	7.80	0.023
	ileum	197.89 ^b	241.32 ^a	242.86 ^a	12.79	0.035
crypt depth, μ m	jejunum	105.62	89.57	102.45	7.85	0.348
	ileum	86.57	74.54	78.54	7.22	0.551
day 35						
villous height, μ m	jejunum	197.23	226.89	234.35	12.37	0.151
	ileum	168.54	187.43	198.48	14.91	0.362
crypt depth, μ m	jejunum	112.54	105.84	104.58	7.94	0.777
	ileum	98.57	99.45	97.58	4.13	0.951

NC = negative control, low-protein basal diet no antibiotics included; PC =positive control, low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); EUF = *Eucommia ulmoides* flavones, low-protein Basal diet + 0.1‰ EUF. Values are the mean \pm SEM, n = 8 per treatment group.^{a-b} Mean values sharing different superscripts within a row differ ($p < 0.05$).

Figures

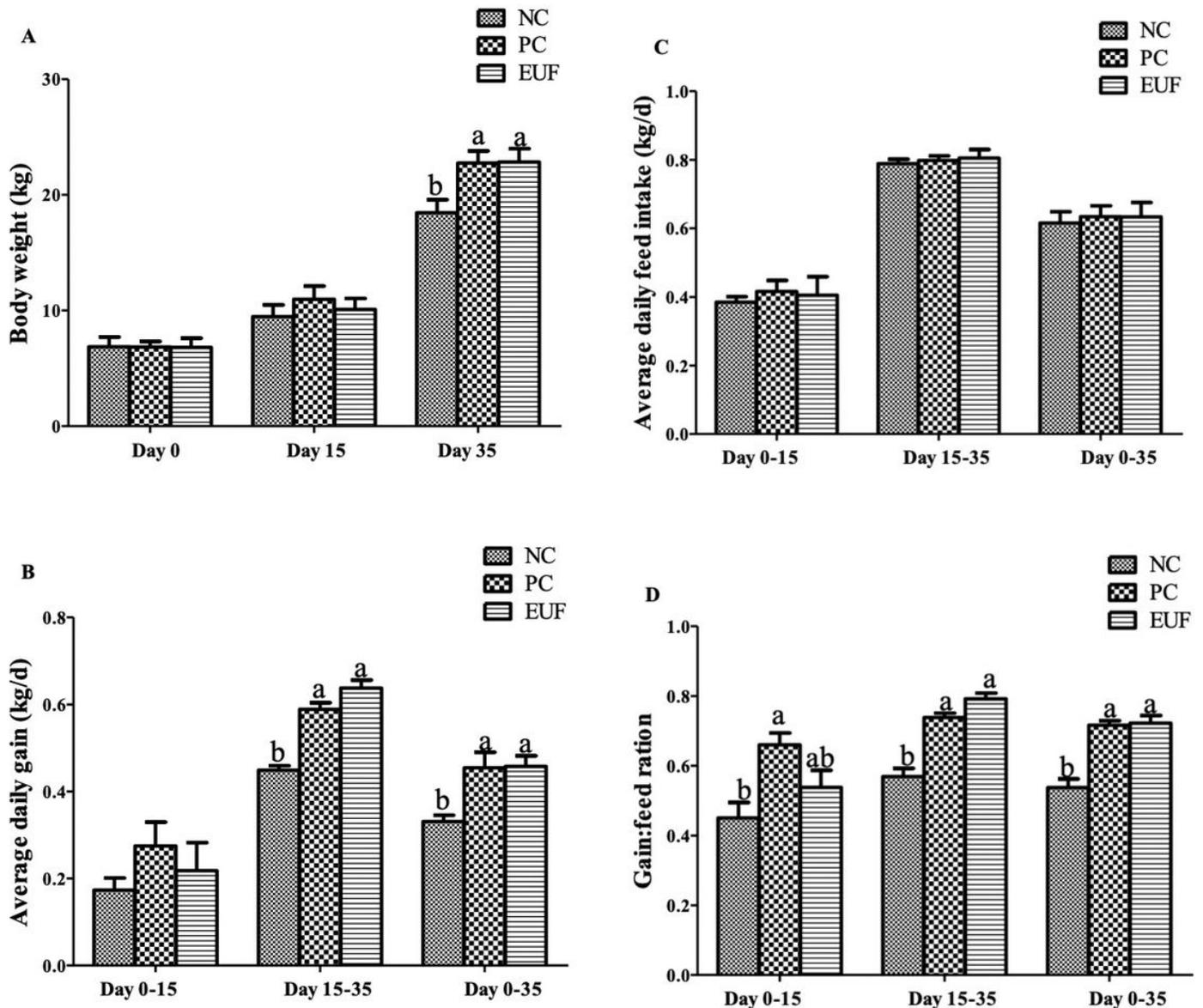


Figure 1

Growth Performance. A, body weight; B, average daily gain; C, average daily feed intake; D, Gain Z: feed ration; NC = negative control, low-protein basal diet no antibiotics included; PC =positive control, low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); EUF

= *Eucommia ulmoides* flavones, low-protein Basal diet + 0.1‰ EUF. Values are the mean ± SEM, n = 8 per treatment group. a-bValues with different letters are significantly different (p < 0.05).

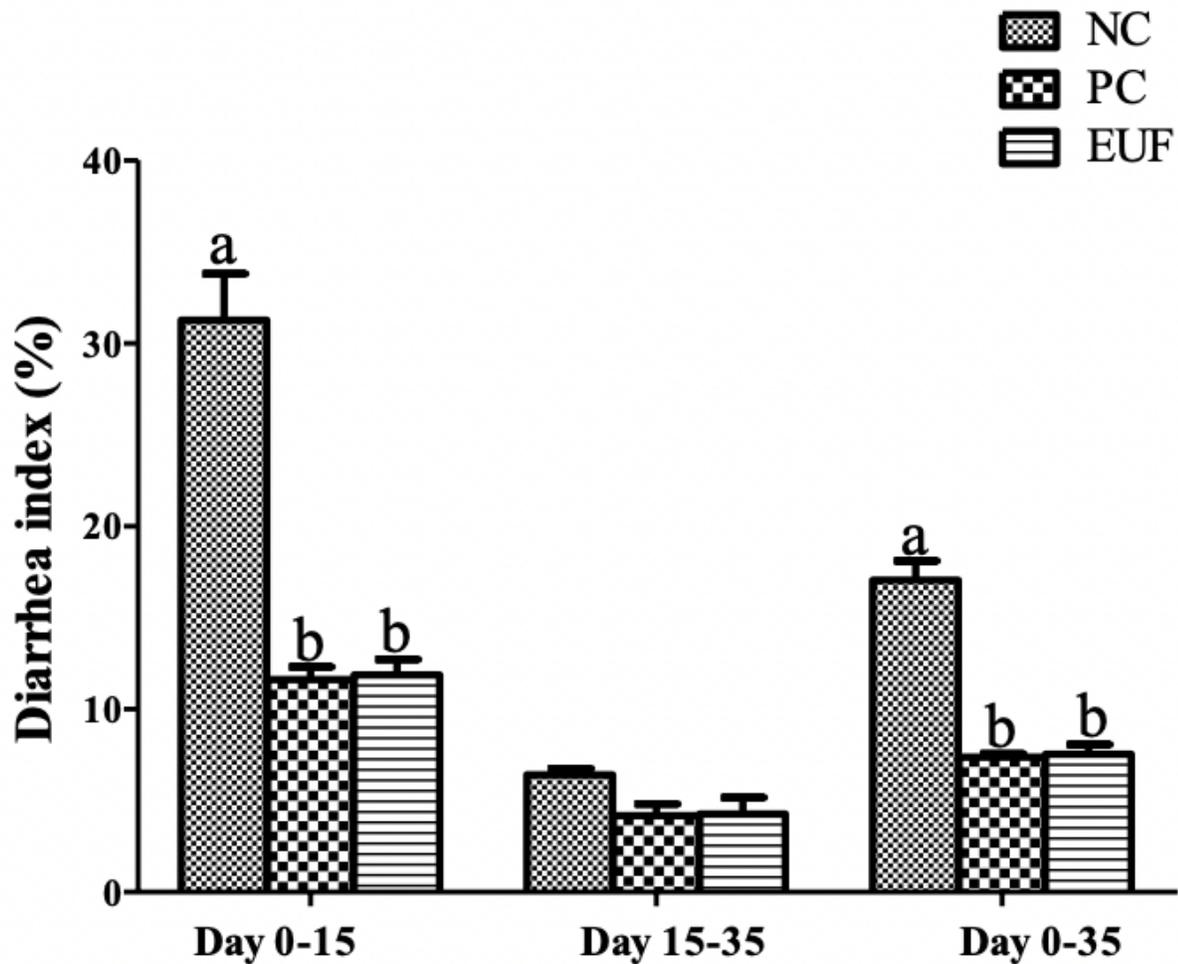


Figure 2

Diarrhea Index. NC = negative control, low-protein basal diet no antibiotics included; PC = positive control, low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); EUF = *Eucommia ulmoides* flavones, low-protein Basal diet + 0.1‰ EUF. Values are the mean ± SEM, n = 8 per treatment group. a-bValues with different letters are significantly different (p < 0.05).

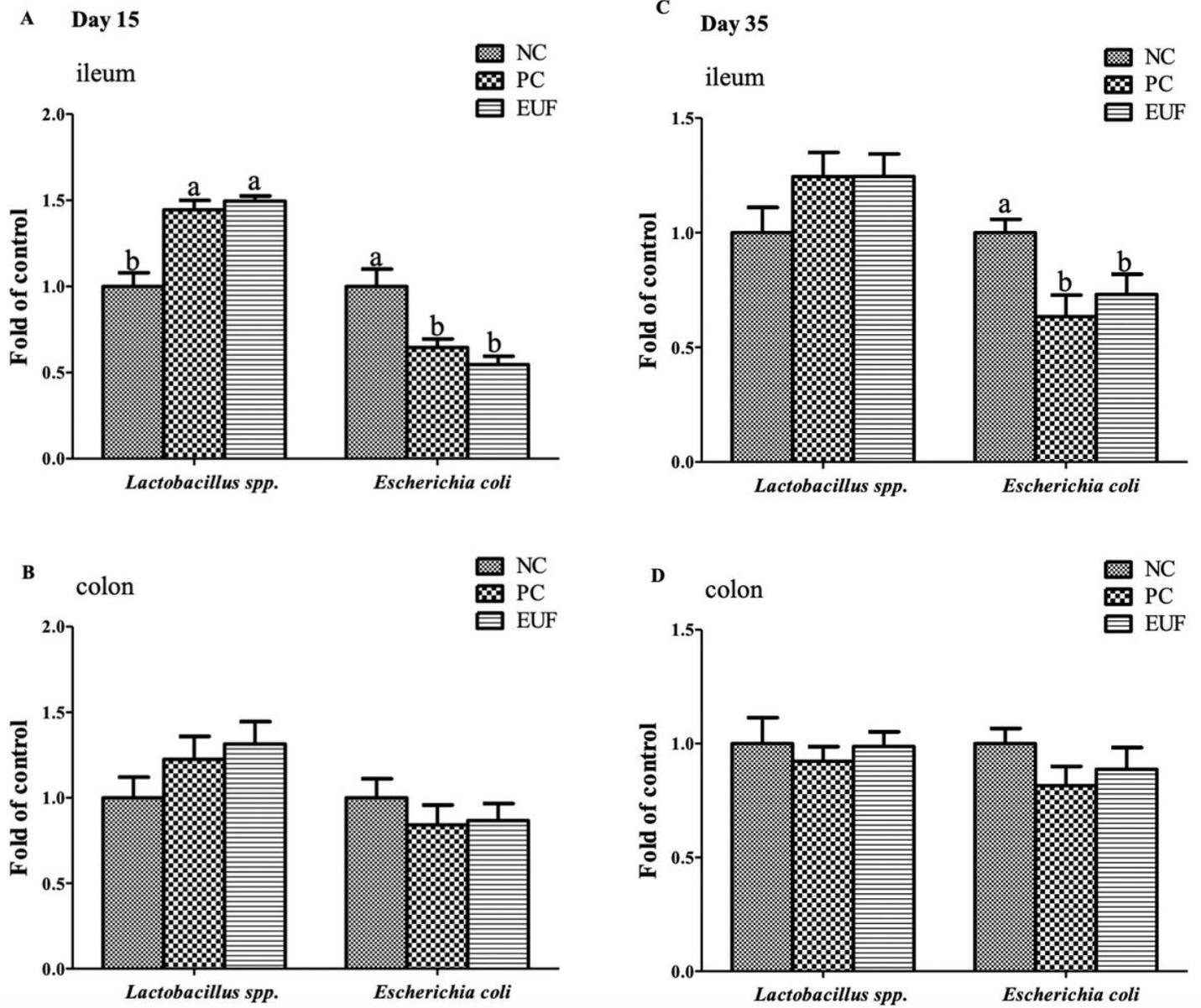


Figure 3

Relative abundance of *Lactobacillus spp.* and *Escherichia coli* in the ileum (A, B) and colon (C, D) of piglets. NC = negative control, low-protein basal diet no antibiotics included; PC = positive control, low-protein Basal diet + antibiotics (75 ppm quinocetone, 20 ppm virginomycin and 50 ppm aureomycin); EUF = *Eucommia ulmoides* flavones, low-protein Basal diet + 0.1‰ EUF. Values are the mean \pm SEM, n = 8 per treatment group. a-b Values with different letters are significantly different ($p < 0.05$).