Effects of Alfalfa Saponins on Growth Performance, Intestinal Microbiota, Tissue Antioxidant Capacity and Related Enzyme mRNA Expression in Weaned Piglets (Postprint)

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Abstract

This experiment was conducted to investigate the effects of alfalfa saponins on growth performance, intestinal microbiota, tissue antioxidant capacity, and mRNA expression of related enzymes in weaned piglets. Twenty-four Large × Landrace crossbred weaned piglets with an average body weight of 8 kg were randomly divided into 2 groups, with 3 replicates per group and 4 piglets per replicate. The control group was fed a basal diet, while the alfalfa saponin group was fed the basal diet supplemented with 0.25% alfalfa saponins. The pre-trial period was 10 d, and the formal trial period was 30 d. The results showed: 1) Compared with the control group, dietary supplementation with alfalfa saponins significantly increased average daily gain ($P<0.05$) and significantly decreased feed-to-gain ratio ($P<0.05$) in weaned piglets. 2) Dietary alfalfa saponin supplementation significantly decreased pH in the duodenum and cecum ($P<0.05$), and significantly increased the number of lactic acid bacteria in the duodenum, jejunum, and ileum ($P<0.05$). 3) Dietary alfalfa saponin supplementation significantly increased the activities of glutathione peroxidase (GSH-Px) and catalase (CAT) in the liver and kidney ($P<0.05$), and significantly increased GSH-Px mRNA expression in the liver and jejunum and CAT mRNA expression in the duodenum and ileum ($P<0.05$). In conclusion, alfalfa saponins can improve growth performance, enhance tissue antioxidant capacity, and effectively improve intestinal microbiota in weaned piglets.

Full Text

Effects of Alfalfa Saponin on Growth Performance, Intestinal Microflora, Antioxidant Ability and Related Enzyme mRNA Expression in Tissues of Weaned Piglets

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Abstract: This experiment was conducted to investigate the effects of alfalfa saponin on growth performance, intestinal microflora, antioxidant ability and related enzyme mRNA expression in tissues of weaned piglets. A total of 24 crossbred (Landrace × Large White) weaned piglets with an average body weight of 8 kg were randomly assigned to 2 groups, each with 3 replicates of 4 piglets. The control group was fed a basal diet, while the alfalfa saponin group received the basal diet supplemented with 0.25% alfalfa saponin. The experiment consisted of a 10-day pre-trial period followed by a 30-day formal trial period. The results showed that: (1) Compared with the control group, dietary alfalfa saponin significantly increased average daily gain ($P<0.05$) and significantly decreased feed-to-gain ratio ($P<0.05$) in weaned piglets. (2) Dietary alfalfa saponin significantly reduced pH values in the duodenum and cecum ($P<0.05$), and significantly increased Lactobacillus counts in the duodenum, jejunum and ileum ($P<0.05$). (3) Compared with the control group, dietary alfalfa saponin significantly enhanced glutathione peroxidase (GSH-Px) and catalase (CAT) activities in the liver and kidney ($P<0.05$), and significantly upregulated GSH-Px mRNA expression in the liver and jejunum as well as CAT mRNA expression in the duodenum and ileum ($P<0.05$). In summary, alfalfa saponin can improve growth performance, enhance tissue antioxidant capacity, and effectively modulate intestinal microflora in weaned piglets.

Key words: alfalfa saponin; weaned piglet; growth performance; intestinal microflora; antioxidant ability; mRNA expression level

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1.1 Experimental Materials

The alfalfa saponin used in this experiment was provided by Hebei Baoen Biotechnology Co., Ltd., with the following composition: 62.00% saponins, 10.97% flavonoids, 8.12% polysaccharides, 7.11% moisture, and 11.80% unknown factors. Saponins are generally hygroscopic, bitter in taste, and can irritate mucous membranes. They are typically water-soluble, readily dissolving in hot water, hot methanol, and hot ethanol, but insoluble in low-polarity organic solvents such as diethyl ether. Most steroidal saponins are neutral, while most triterpenoid saponins are acidic. The alfalfa saponin product used in this study

was neutral with a pH of 7.04.

1.2 Experimental Design and Diets

A single-factor completely randomized design was employed. Twenty-four healthy crossbred (Landrace × Large White) piglets with an average body weight of approximately 8.0 kg were selected based on similar age, parity, and body weight, and randomly divided into 2 groups with 3 replicates each and 4 piglets per replicate. The control group was fed a basal diet, while the alfalfa saponin group received the basal diet supplemented with 0.25% alfalfa saponin. The composition and nutrient levels of the basal diet are presented in Table 1 .

Table 1 Composition and nutrient levels of the basal diet (air-dry basis) (%)

1) The premix provided the following per kg of diet: VA 5,500 IU, VD 500 IU, VE 66.1 IU, VB 28.2 μg, VB 5.1 mg, VB 12.6 mg, VB 29.8 mg, choline 540 mg, Mn 40 mg, Zn 120 mg, Fe 130 mg, Cu 150 mg, Co 1 mg, Se 0.25 mg, I 4.5 mg.

2) Nutrient levels were calculated values.

1.3 Feeding Conditions and Management

The experiment consisted of a 10-day pre-trial period followed by a 30-day formal trial period. Piglets had free access to feed and water, with feeding times at 06:00, 10:00, 14:00, and 18:00 daily. During the pre-trial period, all groups received the same basal diet. During the formal trial period, the control and alfalfa saponin groups were fed their respective diets, with daily feed intake and health status recorded. The pig house temperature was maintained at approximately 25 °C with humidity between 65% and 75%. Pens were cleaned once daily, and routine immunization and disinfection procedures were followed according to standard pig farm protocols. At the end of the experiment, piglets were fasted (with free access to water) for 12 hours before weighing to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F/G). Diarrhea rate was calculated as: Diarrhea rate (%) = 100 × [(number of diarrheal piglets × diarrheal days) / (total number of piglets × trial days)].

1.4 Sample Collection

Six piglets from each group (2 per replicate) were selected and euthanized by jugular venipuncture. The abdominal cavity was opened to isolate the intestinal tract. The same segments of duodenum, jejunum, ileum, and cecum were collected and ligated. Intestinal contents were collected for microbial enumeration (Escherichia coli and Lactobacillus) and pH measurement. Liver, spleen, and kidney samples were collected from the same anatomical locations, along with duodenal, jejunal, ileal, and cecal tissues. All samples were rinsed with physio-

logical saline, blotted dry with gauze, wrapped in aluminum foil, immediately frozen in liquid nitrogen, and stored at -80 °C.

1.5 Measurements

1.5.1 pH Measurement The pH of duodenal, jejunal, ileal, and cecal contents was measured directly using a pH meter.

1.5.2 Enumeration of Escherichia coli and Lactobacillus in Intestinal Contents Intestinal content dilutions were prepared by homogenizing the samples, then aseptically weighing 0.5 g into 4.5 mL sterile physiological saline (Tube 1) and vortexing for 20 minutes. Five additional sterilized tubes each containing 4.5 mL sterile physiological saline were prepared. A 0.5 mL aliquot from Tube 1 was transferred to Tube 2, vortexed for 5 minutes, and serially diluted to 10 .

For microbial culture, Eosin Methylene Blue (EMB) agar was used for Escherichia coli and MRS agar for Lactobacillus, with enumeration by colony counting. Three appropriate dilutions were selected with two replicates each. Starting from the highest dilution, 100 µL of diluted sample was dropped onto each selective medium from a height of 2–3 cm and spread with a glass rod. Escherichia coli plates were incubated aerobically at 37 °C for 24 hours before colony counting. Lactobacillus plates were incubated anaerobically at 37 °C in a CO incubator for 48 hours before counting. Results were expressed as log colony-forming units per gram (lg(CFU/g)) of intestinal content.

1.5.3 Tissue Antioxidant Capacity Measurement Liver, kidney, and spleen samples (0.3–0.5 g) were placed in small beakers on ice. Ice-cold 0.9% physiological saline was added at a tissue-to-saline ratio of 1:9 (w/v). Tissues were rapidly minced with scissors, transferred to a glass homogenizer, and ground for 6–10 minutes in an ice-water mixture to ensure complete homogenization. The homogenate was centrifuged at 3,500 r/min for 15 minutes, and the supernatant was carefully collected and stored at low temperature. Superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity, catalase (CAT) activity, total antioxidant capacity (T-AOC), and malondialdehyde (MDA) content were measured using kits from Nanjing Jiancheng Bioengineering Institute according to the manufacturer' s instructions.

1.6 Related Enzyme mRNA Expression Analysis

1.6.1 Primer Design Using porcine SOD, GSH-Px, and CAT DNA sequences from GenBank as templates and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene, primers were designed using Primer 5.0 software and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Primer powder was centrifuged at 12,000 r/min for 1 minute before use, then reconstituted with ddH O to a working concentration of 100 µmol/L

according to the labeled concentration and stored at -20 °C. Primer sequences and parameters are shown in Table 2 .

Table 2 Sequences and parameters of primers

Gene	GenBank Accession No.	Sequences (5 –3)
GAPDH	NM_001206359.1	F: GTCGGTTGTGGATCTGACCTR: AGCTTGACGAAGTGGTCGTT
SOD	NM_001190422.1	F: GAGACCTGGGCAATGTGACTR: CCAAACGACTTCCAGCATTT
GSH-Px	NM_214201.1	F: AGAAGTGTGAGGTGAATGGCR: CCCGAGAGTAGCACTGTAAC
CAT	NM_214301.2	F: GAGCACGTTGGAAAGAGGACR: GGCTGTGGATAAAGGATGGA

1.6.2 Total RNA Extraction and Detection Appropriate amounts of tissue were ground in liquid nitrogen, and total RNA was extracted using the Trizol method. RNA concentration and purity were measured, with all samples showing absorbance ratios between 1.8 and 2.0, indicating sufficient purity for molecular biology experiments. Total RNA was reverse transcribed in a 10 μL reaction system, and the cDNA products were stored at -20 °C.

1.6.3 RT-PCR Amplification SOD, GSH-Px, and CAT mRNA expression was analyzed by RT-PCR using a fluorescent dye method in a 20 μL reaction system according to the kit instructions, with three replicates per sample. Cycling conditions were: 95 °C pre-denaturation for 60 s, followed by 40 cycles of 95 °C denaturation for 10 s, 58 °C annealing for 30 s, and 72 °C extension for 20 s. Relative gene expression was calculated using the 2 ΔΔCt method with GAPDH as the internal reference.

1.7 Data Processing

Experimental data were analyzed by t-test using SPSS 19.0 statistical software. Differences were considered significant at $P<0.05$. Results are expressed as means ± standard deviation.

2.1 Effects of Alfalfa Saponin on Growth Performance and Diarrhea Rate in Weaned Piglets

As shown in Table 3 , dietary alfalfa saponin increased ADFI and reduced diarrhea rate in weaned piglets, though these differences were not significant (P>0.05). However, ADG was significantly higher (P<0.05) and F/G was significantly lower (P<0.05) in the alfalfa saponin group compared with the control group.

Table 3 Effects of alfalfa saponin on growth performance and diarrhea rate of weaned piglets

Item	Control Group	Alfalfa Saponin Group
ADG (g)	492.97±22.54	545.92±13.71
ADFI (g)	769.24±46.06	803.95±33.82
F/G	1.56±0.04	1.47±0.02
Diarrhea rate (%)	9.62±6.53	3.06±1.92

In the same row, values with different small letter superscripts indicate significant difference (P<0.05). The same applies below.

2.2 Effects of Alfalfa Saponin on Intestinal pH and Microflora in Weaned Piglets

As shown in Table 4 , dietary alfalfa saponin significantly reduced pH values in the duodenum and cecum (P<0.05) but had no significant effect on jejunal or ileal pH (P>0.05). Alfalfa saponin decreased Escherichia coli counts in the duodenum, jejunum, ileum, and cecum, though not significantly (P>0.05). Compared with the control group, Lactobacillus counts in the duodenum, jejunum, and ileum were significantly increased (P<0.05), while cecal Lactobacillus counts showed an increasing trend without reaching significance (P>0.05).

Table 4 Effects of alfalfa saponin on intestinal pH and microflora of weaned piglets

Item	Control Group	Alfalfa Saponin Group
pH		
Duodenum	6.05±0.04	5.93±0.04
Jejunum	6.49±0.11	6.36±0.19
Ileum	6.48±0.10	6.62±0.16
Cecum	6.58±0.12	5.97±0.29
Escherichia coli [lg(CFU/g)]		
Duodenum	7.15±0.64	6.41±0.58
Jejunum	7.24±0.72	6.36±0.79
Ileum	7.36±0.51	6.66±0.29

Item	Control Group	Alfalfa Saponin Group
Cecum Lactobacillus [lg(CFU/g)]	6.37±0.43	6.28±0.91
Duodenum	8.39±0.12	8.87±0.09
Jejunum	8.46±0.19	8.94±0.05
Ileum	8.43±0.07	8.92±0.06
Cecum	8.43±0.34	8.79±0.16

2.3 Effects of Alfalfa Saponin on Tissue Antioxidant Capacity in Weaned Piglets

As shown in Table 5 , dietary alfalfa saponin reduced MDA content and increased SOD activity and T-AOC in the liver, kidney, and spleen, though these changes were not significant (P>0.05). GSH-Px activity in the liver and kidney was significantly higher in the alfalfa saponin group compared with the control group (P<0.05), while splenic GSH-Px activity showed a decreasing trend without significance (P>0.05). CAT activity in the liver, kidney, and spleen was higher in the alfalfa saponin group, with significant increases observed in the liver and kidney (P<0.05).

Table 5 Effects of alfalfa saponin on antioxidant ability in tissues of weaned piglets

Item	Control Group	Alfalfa Saponin Group
Liver		
MDA (nmol/mg prot)	3.17±0.53	3.02±0.49
SOD (U/mg prot)	138.28±5.21	147.70±14.21
GSH-Px (U/mg prot)	79.71±8.65	102.25±4.38
CAT (U/mg prot)	658.23±44.55	863.02±53.29
T-AOC (U/mg prot)	3.59±0.01	3.82±0.57
Kidney		
MDA (nmol/mg prot)	1.80±0.13	1.62±0.34
SOD (U/mg prot)	166.44±9.60	175.09±16.01
GSH-Px (U/mg prot)	96.39±7.36	128.12±9.45
CAT (U/mg prot)	441.63±50.43	658.42±31.20
T-AOC (U/mg prot)	2.66±0.47	2.82±0.33
Spleen		
MDA (nmol/mg prot)	2.00±0.10	1.94±0.27
SOD (U/mg prot)	23.03±12.02	43.76±23.34
GSH-Px (U/mg prot)	38.23±9.90	37.10±1.26
CAT (U/mg prot)	36.38±15.62	44.96±8.06
T-AOC (U/mg prot)	0.28±0.05	0.29±0.01

2.4 Effects of Alfalfa Saponin on SOD, GSH-Px and CAT mRNA Expression in Tissues of Weaned Piglets

As shown in Table 6 , dietary alfalfa saponin had no significant effect on SOD mRNA expression in the liver, duodenum, jejunum, or ileum (P>0.05). GSH-Px mRNA expression was significantly increased in the liver and jejunum (P<0.05) but not in the duodenum or ileum. CAT mRNA expression was significantly higher in the duodenum and ileum of the alfalfa saponin group compared with the control group (P<0.05), while hepatic and jejunal CAT mRNA expression showed increasing trends without reaching significance (P>0.05).

Table 6 Effects of alfalfa saponin on mRNA expression of SOD, GSH-Px and CAT in tissues of weaned piglets

Item	Control Group	Alfalfa Saponin Group
Liver		
SOD	1.00±0.27	1.08±0.23
GSH-Px	1.00±0.03	1.07±0.02
CAT	1.00±0.16	1.22±0.16
Duodenum		
SOD	1.00±0.14	1.01±0.18
GSH-Px	1.00±0.34	1.03±0.07
CAT	1.00±0.01	1.50±0.16
Jejunum		
SOD	1.00±0.15	0.97±0.19
GSH-Px	1.00±0.05	1.50±0.05
CAT	1.00±0.26	1.13±0.30
Ileum		
SOD	1.00±0.06	1.01±0.13
GSH-Px	1.00±0.26	0.97±0.16
CAT	1.00±0.04	1.34±0.09

3.1 Effects of Alfalfa Saponin on Growth Performance and Diarrhea Rate

Research findings on the effects of alfalfa saponin on livestock growth performance have been inconsistent, possibly due to differences in animal species, physiological status, and extraction sources of alfalfa saponin. Xu [3] reported that dietary alfalfa saponin increased ADFI and ADG in weaned piglets. Cao [4] found that adding alfalfa meal to piglet diets increased ADG. Hou et al. [5] observed that supplementing laying hen diets with 30, 60, and 90 mg/kg alfalfa saponin improved egg production rate, egg weight, and reduced F/G to some extent. Wang et al. [6] demonstrated that appropriate supplementation levels of alfalfa saponin (0.25%–0.50%) could enhance piglet growth performance. The present results showed that dietary alfalfa saponin increased ADFI and reduced

diarrhea rate in weaned piglets, with significant improvements in ADG and F/G compared with the control group. These findings indicate that appropriate dietary supplementation of alfalfa saponin can improve growth performance and reduce diarrhea incidence in weaned piglets.

3.2 Effects of Alfalfa Saponin on Intestinal pH and Microflora

Intestinal pH is a critical factor affecting microbial survival and proliferation, and it maintains digestive enzyme secretion and activity. Therefore, a stable and appropriate intestinal pH is essential for maintaining digestive health and normal digestive-absorptive function. Previous studies reported that high intestinal pH in weaned piglets not only adversely affects small intestinal digestive enzyme activity but also promotes pathogen proliferation, ultimately causing diarrhea. Lupton and Jacobs [7] and Kashiwagura et al. [8] reported that pH influences cell development, with changes in intracellular pH inducing cell division and promoting DNA synthesis, while lower pH helps maintain intact intestinal mucosal morphology and promotes mucosal cell proliferation. In this study, alfalfa saponin significantly reduced duodenal and cecal pH, possibly by promoting gastric acid and digestive juice secretion or by enhancing Lactobacillus populations, which lower intestinal pH.

The balance of the intestinal microecosystem significantly influences normal intestinal physiology and whole-body metabolism. Reduced Escherichia coli populations can decrease diarrhea and other gastrointestinal diseases in piglets [9], while beneficial bacteria such as Lactobacillus contribute to defense against pathogen invasion through their metabolites and antimicrobial substances [10]. Previous studies reported that plant saponins possess antimicrobial activity [11,12]. Jin et al. [13] found that Sapindus saponins exhibited strong antibacterial activity against Escherichia coli, while rose root saponins also showed inhibitory effects [14]. Research demonstrated that pentacyclic triterpenoid saponin monomer Bp3 from Bupleurum had antifungal activity against fluconazole-resistant Candida albicans [15]. Li et al. [16] reported that alfalfa saponin extract inhibited the growth of Escherichia coli and Bacillus subtilis. Wu [17] showed that dietary alfalfa saponin significantly inhibited Escherichia coli and Salmonella in weaned piglets, reducing diarrhea rate. The present results indicate that alfalfa saponin slightly inhibited Escherichia coli proliferation while promoting Lactobacillus growth, suggesting that alfalfa saponin may help maintain intestinal microecological balance.

3.3 Effects of Alfalfa Saponin on Tissue Antioxidant Capacity and Related Enzyme mRNA Expression

Antioxidant capacity is a self-protective mechanism against free radical damage. Antioxidant enzymes enhance defense and immune function, with their activity reflecting the oxidative-antioxidative status of the organism [18]. Appropriate

reactive oxygen species levels are essential for resisting harmful bacteria and many metabolic processes including cell signaling [19]. Under normal physiological conditions, nutritional status and free radical content maintain dynamic balance; disruption of this balance increases free radical production and causes oxidative cellular damage [20]. MDA is a lipid peroxidation product, GSH-Px reflects the ability to scavenge oxygen free radicals and is a major component of the antioxidant defense system, SOD protects cell membrane structure and function [21], and CAT promotes hydrogen peroxide (H O) decomposition to protect cells from free radical damage [22]. T-AOC serves as a comprehensive indicator of antioxidant system function, reflecting the combined effects of multiple antioxidant enzymes [23]. Increasing exogenous antioxidants or enhancing endogenous antioxidant production can reduce free radical damage to cell membranes and effectively scavenge free radicals [24].

Numerous studies have demonstrated that most saponins possess antioxidant capacity [25]. Liu et al. [26] reported that ginsenosides enhanced antioxidant enzyme activity and alleviated induced lung oxidative damage in mice. Shi et al. [27] found that alfalfa saponin significantly increased GSH-Px and SOD activities while reducing MDA content in piglet tissues. Sun et al. [28] demonstrated that jujube fruit triterpenoid saponins scavenged DPPH radicals in vitro, showing significant antioxidant effects. Guan et al. [29] found that a pentacyclic triterpenoid saponin compound exhibited strong scavenging capacity against DPPH and hydroxyl radicals. Huang et al. [30] reported that daidzein induced SOD expression in rat heart, brain, and liver tissues, enhancing free radical scavenging activity and significantly reducing MDA content. In this study, alfalfa saponin reduced MDA content and increased T-AOC and SOD activity in liver, kidney, and spleen. GSH-Px and CAT activities in liver and kidney were significantly higher in the alfalfa saponin group, and CAT mRNA expression in duodenum and ileum and GSH-Px mRNA expression in liver and jejunum were significantly upregulated. These results demonstrate that alfalfa saponin can enhance free radical scavenging capacity, reduce free radical production, and improve tissue antioxidant capacity.

In conclusion, alfalfa saponin can improve growth performance and tissue antioxidant capacity, modulate intestinal microflora, and promote intestinal health in weaned piglets.

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Note: Figure translations are in progress. See original paper for figures.