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Effects of Dietary Alfalfa Saponin on Digestive Physiology in Weaned Piglets

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ABSTRACT

Background: Saponins have been considered to affect the digestion of animal because of its relatively complex compound. Regarding the effect of saponins on animal production performance, different animals or the same animals with different physiological periods are different. The purpose of this experiment was to evaluate the effect of dietary alfalfa saponins (AS) on the digestive physiology of piglets.

Methods: 72 weaned piglets (Duroc × Landrace × Large white pigs) were weaned at 28 days of age with an average weight of 7.5 kg and assigned randomly to 4 treatment groups. Diets containing 0, 0.5%, 1%, 2% alfalfa saponins were fed to piglets.

Result: The results showed that: Alfalfa saponins increased daily gain and decreased diarrhea in piglets to some extent. It tended to decrease the pH value of the gastrointestinal tract and increase the pH value of the jejunum (P<0.05), ileum and cecum (P<0.05). AS had no obvious inhibitory effect on Escherichia coli, but had an increasing trend on the number of intestine Lactobacillus, especially Lactobacillus in jejunum increased significantly by adding 2% AS (P<0.05). Adding 2% alfalfa saponins can improve the digestive physiology of piglets.

Key words: Alfalfa saponin, Diarrhea, Digestive physiology, Weaned piglets.

INTRODUCTION

Saponins have been considered a chemical substance that affects the digestion of animal bodies and ruminant abdominal distension (Calvert *et al*., 1985; Broderick, 1995; Lin *et al*., 2020). Saponin is a relatively complex compound, with its aqueous solution producing a large number of long-lasting honeycomb foam when shaking, which is similar to soap. Saponin is made up of saponin (sapogenins) and sugar, ironic acid, and other organic acids (Li *et al*., 2023). The constituents of saponins are different in different regions and different seasons. According to its chemical structure, it can be divided into triterpenoid saponins and steroidal saponins.

Alfalfa can produce saponin during its growth, which comes from its fresh leaves, stems, crowns, residual plants, rotten roots and seeds. Alfalfa saponin (AS), extracted from alfalfa with unique biological properties and formed by dehydrating the hydroxyl of triterpenoid homologs and the hydroxyl of glycosyl hemiacetals an active substance with unique biological properties extracted from alfalfa, which belongs to pentacyclic triterpenoid saponin. Triterpene saponin ligands, sugars and glucuronic acids were obtained from the hydrolysis of alfalfa saponins, which were identified as glucose, arabinose, xylose and a small amount of galactose and rhamnose. Alfalfa saponin has a series of biological activities, such as enhancing the immune function, regulating the metabolism of substances, promoting the synthesis of nucleic acids and proteins, antioxidation, eliminating free radicals, antibacterial, antiviral, anti-inflammatory and anti-mutagenic. The studies found that alfalfa saponin inhibited the growth of monogastric animals, mainly due to the bitter taste of alfalfa saponin and its stimulating effect on the mouth and digestive tract, as well as the reduction of palatability and intake (Calvert *et al*., 1985; Broderick, 1995). The use of alfalfa products has been proven to improve the animals' growth and reproductive performance, enhance the quality of birds and livestock products, as well as feed utilization (Wildeus *et al*., 2007; Landers *et al*., 2008; Landers *et al*., 2008). These researches provide a basis for alfalfa product extract for further utilization in livestock and poultry production.

Some early studies have shown that some kinds of saponins were related to ruminant abdominal distension (Lindahl *et al*., 1957; Cheeke, 1989; Cheeke, 1989); however, recent research has shown that saponins are composed of one additional factor in the pathogenesis of herbage inflation (Hristov *et al*., 1999; Santoso *et al*., 2004; Lin *et al*., 2020; Lu *et al*., 2023). Yucca saponin and Panax quinquefolium saponin have several current and potential applications in animal nutrition and their effects on pig performance are

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well-conducted (Makkar *et al*., 1998; Hristov *et al*., 1999; Santoso *et al*., 2004). It was reported that adding 20% alfalfa powder to the diet of pigs, had no adverse effect on pigs (Wang *et al*., 2008).

Saponins is a kind of chemical substance that affects the digestion of animals. Adding alfalfa saponins may affect the growth and digestion of weaned piglets with incomplete intestinal development. In this experiment, we studied the effects of adding alfalfa saponins on digestive physiology of weaned piglets.

MATERIALS AND METHODS

Experimental animals, grouping and materials

Piglets (n = 72, Duroc × Landrace × Large white pigs) were weaned at 28 days of age with an average weight of 7.5 kg and assigned randomly to 4 treatment groups accounting for weight and gender (three replicates of six pigs per treatment, 3 sows and 3 castrated boars). Piglets were provided with water and feed for 30 days under the standard feeding conditions and management. The diets contained sufficient nutrients based on the recommendation of NRC (1998). Four experimental treatments were studied: 0, 0.5%, 1%, 2% alfalfa saponins. The nutritional composition of our experimental diet is shown in Table 1.

AS was got from Hebei Bao'en (a Biotechnology corporation in Shijiazhuang, China). The purity of AS was 61.64% and included alfalfa flavonoids (10.97%), alfalfa polysaccharides (8.12%), moisture (7.11%) and some unknown components (12.16%).

Sample collection and determination

Weighed weaned piglets on the experiment first day, during the entire period of the experiment, we recorded the amount of feed and diarrhea every day. Weighed the weaned piglets and calculated the average daily weight gain at the end of the experiment.

Thirty days after the experiment, two pigs (one female and one male) were selected randomly from each repetition for blood. The blood samples were anterior vena cava collected for the preparation of serum and plasma and then centrifuged at 4,000 × g for 10 min at 4°C.The serum was stored at -80°C refrigerator for biochemical indexes. The piglets were then killed by intravenous injection of 200 mg pentobarbital/mL solutions and jugular vein exsanguinations according to (Animalcare Ltd., York, U.K.) and signal ((Arnolds Veterinary products, Shrops, U.K.). The abdominal cavity was opened immediately and the pancreas, duodenum, jejunum, ileum and cecum were cut off respectively. Some intestine samples were sent to the laboratory for microbiological determination immediately. And some intestine samples were put into a plastic bag and immediately put into the liquid nitrogen quick freezing and then transferred to the -80°C refrigerator for preservation for the determination of digestive tract enzymes.

Determination of Escherichia coli and Lactobacillus in the intestine: Escherichia coli was cultured on MacConkey agar medium, while Lactobacillus was cultured on MRS agar medium. The logarithm of the number of bacteria per gram of intestinal contents using Log_{10}cfu/g. Determination of digestive enzyme activity in the intestine and pancreas: Use the reagent kit produced by Nanjing Jiancheng Biotechnology Research Institute. Determination of pH value: Measure the pH value using a SenTix pH meter while collecting intestinal samples and keeping records.

Statistical analysis

Data were analyzed using the software of SPSS 19.0 with one-factor variance analysis.

The significance of the statistical analysis of weaned piglets in different groups was determined at the level of $P<0.05$.

Average daily gain: the difference final weigh and initial weigh, divided by measured days; Diarrhea rate: the value of the number of diarrhea cases divided by the total number of cases; Diarrhea frequency: The value of the number of diarrhea cases divided by total number of pigs tested, multiply by test days; Diarrhea index: the value of the sum of fecal scores divided by total number of pigs tested; Fecal scoring criteria: 0 points normal, 1 point soft and formed

Table 1: Composition and nutrients of the basal diet.

Ingredients	%	Nutrient level	%
Corn	56.7	DE (MJ/kg)	14.02
Soybean meal	23.5	Crude protein	20.01
Fish meal	4.8	EE	5.27
Whey powder	7	Ca	0.83
Soybean oil	2.5	Available phosphorus	0.48
Dicalcium phosphate	0.8	Lysine	1.14
Brushite	1.5	Methionine	0.35
Limestone	1		
Premix[a]	1		
Saponins	0.5~2.0		

a Premix supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 5,000 IU; vitamin E, 40 IU; vitamin B12, 28.2 μg; vitamin B2, 5.1 mg; vitamin B3, 12.6 mg; vitamin B5, 29.8 mg; choline, 540 mg; Mn,50 mg; Zn, 150 mg; Fe, 150 mg; Cu, 150 mg; Co, 1 mg; Se, 0.5 mg; I, 0.5 mg; Thr, 400 mg; and zinc bacitracin, 100 mg.

feces, 2 points yellow liquid feces (moderate diarrhea) and 3 points watery spray feces (severe diarrhea). Apparent nutrient digestibility: One minus the value of the acid insoluble ash in feed multiply by Nutrient content in feces divided by the value of the content of a certain nutrient in feed multiply by acid insoluble ash content in feces.

RESULTS AND DISCUSSION

Alfalfa saponins on nutrient digestibility, daily gain and diarrhea

From the Table 2, we found that adding saponins had a certain effect on the digestibility of nutrients. The digestibility of crude protein in 1% AS group and 2% AS group was lower than the control group (*P*<0.05) significantly, but there was no significant difference between the 0.5% AS group and the control group. The digestibility of EE and calcium had no obvious regularity, but the digestibility of crude fiber decreased. There was a certain difference in the digestibility of phosphorus among the treatment groups, only the 2% AS group was significantly higher than the control group (*P*<0.05).

The piglets' average daily gain of the 1% AS group and the 2% AS group were significantly higher (*P*<0.05) than the control group, but there was no significant difference between the 0.5% AS group and the control group (Fig 1). Compared to the control group, 0.5%, 1%, 2% alfalfa saponins groups showed a downward trend consistently (Fig 1). Diarrhea rate, diarrhea frequency and diarrhea index have no significant difference (Fig 1).

Alfalfa saponins on the pH

The pH value in the stomach tended to decrease with no significant difference (Table 3). The change of pH value in the duodenum was similar to the stomach with decreased tendency. The pH value of the 2% AS group was the highest in the jejunum than those of other treatment groups and significantly higher than the control group (*P*<0.05). There was no significant difference in ileum pH between the control and the treatment groups, but with an increased tendency. In the cecum, the pH values of the 0.5% AS group and the 2% AS group were significantly higher than the control group (*P*<0.05).

Alfalfa saponins on intestinal microorganism

It can be seen from the Table 4 that adding saponins had an increasing trend in the jejunum, ileum and large intestine on the number of Lactobacillus. The number of Lactobacillus in 2% AS group was the highest in the jejunum than those of other treatment groups and significantly higher than the control group (*P*<0.05). The number of Lactobacilli in other intestinal segments showed an increasing trend with no

Table 2: Effects of alfalfa saponins on nutrient digestibility of weaned piglets.

Items	CP (%)	EE (%)	CF (%)	Ca (%)	P(%)
0%	69.37±1.25[a]	69.38±3.28	31.47±5.40	52.71±4.70	33.68±1.41[b]
0.5%	67.44±0.69[ab]	68.53±5.53	27.31±4.60	50.48±5.33	33.41±0.76[b]
1%	66.38±2.04[b]	69.96±4.76	26.50±3.54	48.94±6.16	35.36±3.97[ab]
2%	66.28±1.09[b]	71.10±1.62	26.25±4.38	51.20±1.62	39.23±1.77[a]

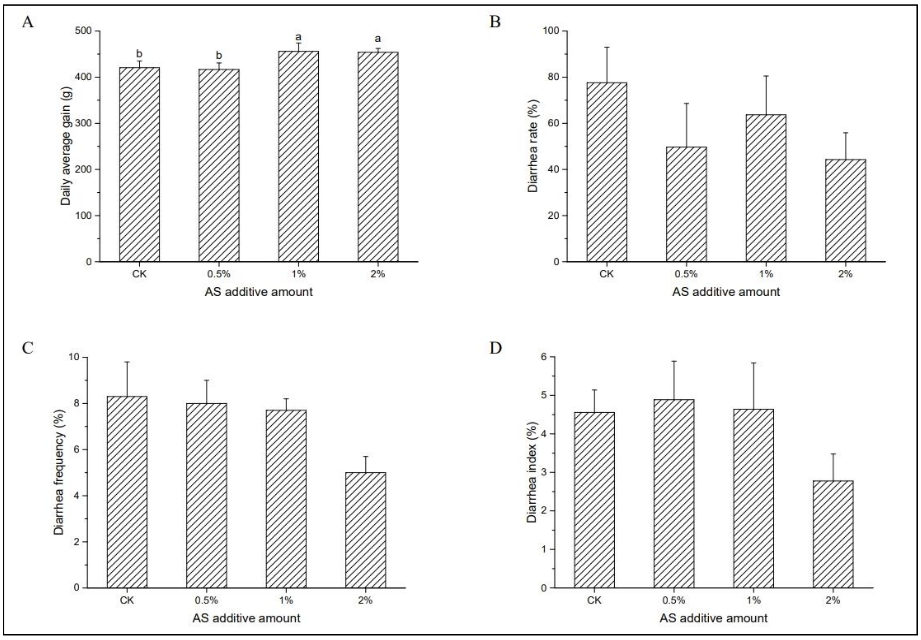

Fig 1: Alfalfa saponins on daily gain and diarrhea.

significant difference. On the contrary, the number of *E. coli* in the jejunum, ileum and large intestine decreased with no significant difference between the treatment groups and the control group.

Alfalfa saponins on enzyme activity

Between the treatment groups and the control group, it had no significant difference in the digestive enzyme activity of AS (Table 5), but it had a distinct trend. The activities of protease, lipase in duodenum, pancreas and serum were increased. The activities of amylase were opposite to the protease and lipase, the amylase content of duodenum, pancreas and serum showed a decreasing trend.

Alfalfa saponins on nutrient digestibility daily gain and diarrhea

Our results in this experiment showed that the digestibility of protein in feed decreased with the increase of AS, which showed the same trend as that of soybean saponin. Saponins do not affect the digestibility of calcium and fat; they can promote the digestion of phosphorus to some extent; however, the mechanism is not clear and needs further study.

The study about saponins on weaned piglets' digestibility are not very much. The effect of saponins on animal feed digestibility is not consistent with different structure. Different animals or the same animals with different physiological periods have different feed digestibility. Triterpenoid saponins play an important role in inhibiting digestive enzyme activity, regulating appetite, regulating lipid metabolism energy consumption and regulating intestinal microbiota (Long *et al*., 2022). The study done by Gu *et al*. (2021) showed that soy saponins might lead to SBM-induced enteritis, destroy tight junction structure and induce oxidative damage in juvenile turbot, which affected its digestion. The ability of saponins and glycoalkaloids to permeabilize the mammalian intestinal barrier has been previously demonstrated *in vitro*, leading to the hypothesis that membranolytic saponins may facilitate transfer to the tissues of otherwise excluded macromolecules. The decrease in the digestibility of crude fiber and crude protein may be related to the damage of villi on the surface of the intestine. This may lead to reduced feed efficiency and weight gain. (Gee *et al*., 1997). The addition of Anemoside B4 could significantly reduce diarrhea incidence in suckling calves at 14-28 days and promote the digestibility and metabolic rate of total energy and had no adverse effect on the rumen fermentation index of calves (Lu *et al*., 2023). The effects of bitter melon saponins diets have adverse effects on intestinal digestion with the supplemental level of 6,400 mg/kg in Songpu mirror carps (Fan *et al*., 2023).

Our results show that AS can increase the daily gain of piglets, but the effect of saponins on the growth performance

Table 3: The pH value of digesta in different gastrointestinal tracts of the piglets.

Items	Stomach	Duodenum	Jejunum	Ileum	Cecum
0%	3.20±1.40	7.27±1.38	6.01±0.33b	6.73±0.25	5.70±0.18b
0.5%	2.86±1.12	6.44±0.36	6.10±0.36ab	6.96±0.18	6.08±0.14a
1%	3.09±1.37	6.51±1.04	6.60±0.37ab	7.15±0.38	5.97±0.16ab
2%	2.47±1.13	6.73±1.49	6.71±0.27a	6.90±0.45	6.04±0.19a

Table 4: Bacterial populations of the digesta in different gastrointestinal tracts.

Items	Jejunum		Ileum		Cecum	
	Escherichia coli	*Lactobacillus*	*Escherichia coli*	*Lactobacillus*	*Escherichia coli*	*Lactobacillus*
0%	5.33±0.03	4.79±0.63^{b}	3.31±0.48	4.91±0.49	2.96±0.33	5.86±0.29
0.5%	5.16±0.32	5.68±0.67^{ab}	3.27±0.51	5.19±0.09	2.84±0.23	6.14±0.32
1%	5.05±0.13	5.64±0.31^{ab}	3.01±0.36	5.28±0.38	2.96±0.47	6.11±0.31
2%	5.13±0.22	6.08±0.20^{a}	3.35±0.20	5.41±0.19	2.98±0.14	6.31±0.33

Table 5: Effects of alfalfa saponins on digestive enzyme activity of weaned piglets.

	Item	0%	0.5%	1%	2%
Duodenum	Protease	6952.46±252.93	10014.39±183.31	10811.80±206.62	11165.98±241.51
	Lipase	1369.63±174.68	1723.25±252.22	1749.09±208.58	1655.67±156.49
	Amylase	82.58±13.53	122.27±14.22	106.52±16.06	138.65±16.47
Pancreas	Protease	1004.22±152.31	1028.68±154.23	1020.87±146.72	1029.01±130.48
	Lipase	444.84±90.88	788.57±103.85	852.68±113.74	678.24±114.31
	Amylase	14.50±0.13	9.96±0.92	11.92±1.09	10.25±0.71
Serum	Protease	146.66±1.62	166.22±3.76	164.66±4.46	183.11±2.36
	Lipase	38.32±3.08	38.35±3.76	44.22±3.82	41.27±2.52
	Amylase	113.78±12.46	96.69±11.19	90.15±17.33	94.92±11.778

of different animals is inconsistent. Regarding the effect of saponins on animal production performance, different animals or the same animals with different physiological periods are different on growth performance; And it is difficult to compare and study different types of saponins. Saponins have immunomodulatory effects and strong biological activity, which may be the key factor for reducing diarrhea in piglets (Wang *et al*., 2008). The results of our study also showed a decreasing trend in diarrhea frequency and diarrhea index, it indicated that adding alfalfa saponins prevents piglet diarrhea.

Alfalfa saponins on the pH

The change of intestinal pH value is affected by various factors, such as weaning time, physicochemical properties of feed and development of tissues and organs. The pH value in the animal digestive tract is an important factor affecting the survival and reproduction of microorganisms. Some pathogenic microorganisms, such as Escherichia coli, Salmonella, Staphylococcus and Clostridium, are easy to survive in a neutral environment with a pH value of 6.0-8.0, while beneficial bacteria, such as Lactobacillus, are suitable for an acidic environment. The increase of pH value (> 7.0) is easy to cause the proliferation of opportunistic pathogens, such as Escherichia coli It can cause diarrhea and other diseases (Wang *et al*., 2008). Oral administration of active yeast preparation in early weaned piglets can improve the production performance, reduce the diarrhea rate and reduce the pH value of intestinal contents.

Adding alfalfa saponin to the piglet diet could reduce the pH value in the duodenum and cecum of piglets significantly (Wang *et al*., 2017), the number of lactic acid bacteria in the duodenum, jejunum and ileum was significantly increased by adding alfalfa saponin. After weaning, the production of lactic acid in the gastrointestinal contents of piglets decreased significantly, the secretion of endogenous hydrochloric acid was affected and the pH value increased, which reduced the beneficial bacteria number, such as Lactobacillus. And it increased the number of harmful bacteria, such as Escherichia coli. In this way, the microflora and pH value of the gastrointestinal tract of piglets were mutually conditional. The results of this study showed that alfalfa saponins had a trend of decreasing pH value in the front of the digestive tract and increasing pH value in the back of the digestive tract and its mechanism needs to be further studied.

Alfalfa saponins on intestinal microorganism

Normal microbiota has many good effects on animal barriers, nutrition, immunity and so on. This effect is an important physiological function formed in the process of long-term evolution to protect oneself and eliminate aliens. Lin *et al*. (2020) reported that the abundance of intestinal flora and the community structure from phylum to genus was also effectively regulated, the relative abundance of beneficial flora was increased and the pathogenic flora was inhibited after intragastric administration of tea seed saponin in rats. It showed that tea seed saponin had a positive regulatory effect on the intestinal flora of hyperlipidemia rats induced by a high-fat diet (Lin *et al*., 2020). Normal intestinal flora has a barrier effect on animals to prevent the infection of harmful microorganisms and beneficial bacteria could compete with harmful bacteria for attachment sites. Probiotics and prebiotics are commonly used as intestinal microecological regulators (Guo and wang, 2002). Gao (1990) found that camellia saponins could inhibit the growth of unicellular fungi (Candida albicans, Cryptococcus neoformans, *etc*.) and multicellular filamentous fungi. The effect of saponin extracts from different species of plants on microorganisms is different (Gao, 1990). The number of lactic acid bacteria in the duodenum, jejunum and ileum was significantly increased by adding 0.25% alfalfa saponin to the basal diet (Wang *et al*., 2017). It had no effect on the digestibility of protein or fat, feeding the Atlantic salmon with AS. All dietary treatments did not cause significant morphological changes in the liver or middle and distal intestine, except for the positive control group (Mette *et al*., 2011). Saponins can be metabolized and transformed by gut microbiota and the secondary metabolites formed by the transformation of saponins in gut microbiota can increase the abundance of probiotics and reduce the abundance of pathogenic bacteria (Wang *et al*., 2021).

The results showed that AS had no obvious inhibitory effects on *Escherichia coli*, but had an increasing trend on the number in intestine *Lactobacillus*, especially Lactobacillus in jejunum increased significantly by adding 2% AS. It was speculated that the mechanism of different kinds of saponins on microorganisms was not the same. Some had antibacterial effect, but some had no effect. Difference of chemical essence leads to different effects on bacteria.

Alfalfa saponins on enzyme activity

The fastest development stage of the digestive tract of piglets after weaning is between 20 and 70 days old. During this period, the secretion and activity of digestive enzymes in the digestive tract of piglets are very low and the secretion of amylase and protease is insufficient. Moreover, due to too little gastric acid secretion and different development processes of digestive enzymes, it is not conducive to feed digestion.

The effects of ginsenosides can significantly reduce the total protein content of Mythimna separata and inhibit the activities of digestive enzymes (cellulase and α - amylase) in Mythimna separata (Tan *et al*., 2013). The study done by Zhang *et al*. (2002) of the inhibitory effect of total saponins of Panax quinquefolium on pancreatic showed that lipase activity in vitro was strong at the dose of 0.5mg/ml. Studies have shown that the growth and development of digestive enzymes are very similar to those of tissues secreting these enzymes, such as the small intestine and pancreas. The effects of different traditional Chinese medicines on the activities of related enzymes in piglets' intestines are inconsistent. Some traditional Chinese medicines have significant effects on the activities of amylase, lipase and chymotrypsin. However, due to the complexity of the components, it cannot be confirmed that a certain component plays a role on this basis (Wu *et al*., 1998). However, it was proved that ginsenosides can increase microvilli number, mitochondria, granules and endoplasmic reticulum on the surface of gastrointestinal epithelial cells in rats with spleen deficiency syndrome, protect gastrointestinal cells and improve the protease activity of normal animals (Peng and Lei, 1995).

The results of this experiment showed that AS tended to increase the activities of protease and lipase in the intestine, pancreas and serum, which may be consistent with the action mechanism of ginsenoside; however, the amylase enzyme activities had decreased. This may be due to the different regulation mechanisms of synthesis, release and activation of different enzymes and the action mechanism was different from different sources of saponins.

CONCLUSION

This experiment found that AS has increased daily gain with no obvious effect on the nutrient digestibility in piglets. AS has an impact on the pH value and microorganisms of the gastrointestinal tract, it could increase the number of Lactobacillus in the intestine and decrease diarrhea. In addition, AS tended to increase the activities of lipase and protease in the intestine and serum. Adding 2% alfalfa saponins can improve the digestive physiology of piglets.

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