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Effect of *Artemisia annua* on anticoccidial action, intestinal microbiota and metabolites of Hu lambs

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Abstract

Background Coccidia are among the primary pathogens causing diarrhea and even fatalities in lambs. With the increasing use of chemical drugs to treat coccidiosis, the problem of drug resistance is becoming more and more threatening. Therefore, there is an urgent need to identify novel alternative drugs for the treatment of the lamb coccidia. In this study, the effect of different doses and extraction methods of *Artemisia annua* (*A. annua*) on anticoccidial activity and growth performance was assessed by oocysts output (OPG), fecal index, average daily gain (ADG) and the new production value of experimental lambs. High-throughput sequencing technology was employed to investigate the effect of *A. annua* on the intestinal microbiota and metabolites of lambs afflicted with coccidiosis.

Results The results revealed that all *A. annua* treatment groups exhibited good anticoccidial effects. According to the soft stool index and ADG analysis, the Low-dose *A. annua* (AL) and *A. annua* alcohol extract (AA) groups demonstrated a better overall effect. The microbiota and metabolites of lambs changed after *A. annua* was administered. *Unclassified_Muribaculaceae* exhibited a significant positive correlation with ADG ($P < 0.05$) and a negative correlation with OPG, although the latter was not statistically significant ($P > 0.05$). *Alistipes* displayed a significant negative correlation with ADG ($P < 0.05$), and a positive correlation with OPG ($P > 0.05$). Additionally, *UCG 005* exhibited a highly significant negative correlation with OPG ($P < 0.01$).

Conclusion The above results demonstrated that AL and AA groups had more effective anticoccidial action. *Unclassified_Muribaculaceae* could be employed as a suitable probiotic to enhance weight gain in lambs, while *UCG-005* could inhibit intestinal *Eimeria* colonization in lambs. *Alistipes* may serve as a biomarker for predicting the risk of intestinal coccidia outbreaks in lambs. *A. annua* induced significant changes in gut microbiota, accompanied by corresponding changes in metabolites. These differences in gut microbiota and metabolites provide valuable insights for subsequent research on the mechanisms underlying anticoccidial action.

Keywords *Artemisia annua*, Anticoccidial effects, Hu lambs, Intestinal microbiota, Metabolites

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Background

With the development of the animal husbandry economy in China, the lamb industry has gradually evolved from grazing and free-range breeding to large-scale captive breeding. Coccidia is an obligate parasite with no intermediate hosts and mainly relies on fecal-oral transmission. Lambs are the most susceptible. Adult lambs show insignificant symptoms but are long-term

carriers and disseminators of the parasite [1, 2]. Therefore, captive breeding and fecal-oral transmission provide favorable conditions for the mass transmission of coccidia. Coccidioides infection can lead to stunted growth and decreased production performance of lambs, affecting meat quality and yield, increasing susceptibility to other diseases, and even leading to death in severe cases, resulting in huge economic losses every year [3]. Therefore, the prevention and control of coccidiosis are serious concerns [4]. Currently, the primary method to control coccidiosis relies on chemical drugs. However, when the use of chemical drugs is discontinued, the coccidiosis infections tend to rebound sharply. Moreover, the long-term use of chemical drugs not only leads to drug residues, toxic side effects, and drug resistance in meat, eggs, milk, and other animal products but also poses risks to human health [5–7]. Hence, there is an urgent need to find a safe, low-toxicity, environmentally friendly alternative to replace chemical drugs. In recent years, numerous studies have revealed that various Chinese herbs or their extracts have shown promising anticoccidial effects. These herbs can enhance animal immunity and increase intestinal flora abundance, thus improving animal production performance. Chinese herbs are also readily available, cost-effective, and less prone to drug resistance, making them a viable option for modern animal coccidia control. *Artemisia annua (A. annua)*, *a* Chinese herbal medicine, belongs to the genus *Artemisia* in the Asteraceae family. It contains rich nutrients, including flavonoids, terpenoids, polysaccharides, sterols, coumarins, and other active substances [8–10]. *A. annua* has anti-malaria, anti-fungal, anti-cancer, and anti-virus effects, and it also helps in clearing deficiency heat, relieving heat, and improving resistance. Its efficacy extends to parasitic infections unrelated to phylogeny, such as schistosomiasis [11, 12]. Fatemi et al. were the first to use *A. annua* alcohol extract to explore its effect on coccidiosis in broilers, finding that *A. annua* alcohol extract had preventive and therapeutic effects on coccidiosis in chickens [13]. Pop et al. confirmed that different concentrations of *A. annua* could affect the sporulation process of coccidia, reduce the disease score of chickens, and limit the discharge of co-infected oocysts [14]. However, a high dose of *A. annua* may have negative effects on animals. Relevant studies have proved that high doses of *A. annua* could reduce food intake and hinder weight gain of broilers, and even cause neurotoxicity, cardiotoxicity, and nephrotoxicity in severe cases [14, 15]. Different treatments of *A. annua* such as alcohol extract and water extract have been proved to have good anti-parasitic effects [13, 16]. However, no studies have been conducted on the content of the active ingredient of *A. annua*, the activity of different treatments against coccidia, or the range of safe dose.

Intestinal flora plays an important role in regulating the absorption and metabolism of nutrients, the physiological and immune activities of the animal organism, and the elimination of pathogens. The intestinal mucous membrane barrier is destroyed after the coccidia invades the lamb intestine, causing a disorder of the intestinal flora, and leading to a series of inflammatory diseases [17, 18]. Related studies have reported that harmful bacteria, such as enterococcus and Streptococcus, were increasing in the intestine during infection of *Eimeria* spp., rather than decreasing the number of non-pathogenic bacteria, such as Lactobacillus, coprolite, Ruminococcus UCG-013 and Clostridium. Therefore, coccidia may also increase host damage by decreasing the gut proper microflora [19]. Recently, Lijie Wang pointed out in a review that there are interactions between Chinese herbs and gut microbiota. On the one hand, Chinese herbs can regulate the composition of intestinal flora, such as increasing the abundance of *Akkermansia* and *Blautia* and decreasing the abundance of *Escherichia-Shigella* to protect the intestinal barrier and reduce intestinal inflammatory response. On the other hand, Chinese herbs can also regulate microbial metabolites (short-chain fatty acids and bile acids). In contrast, gut microbes can metabolize herbal compounds, thereby increasing their biological activity and availability, and reducing their toxicity [20]. Li showed that the extract of Changshan and artemisinin could improve the diversity of intestinal flora of lambs infected with coccidia, recover and stabilize the intestinal flora of lambs [21].

It is well established that gut microbes and their metabolic products play a critical role in regulating host metabolism. As the basis of an organism's phenotype, metabolites help us understand biological processes and their mechanisms more directly and effectively. As feces are the final product of intestinal digestion, the fecal metabolome provides valuable information for the discovery of potential biomarkers related to intestinal disorders [22]. Metabonomics has been widely used in parasitology to study the metabolic response of the host to parasitic infection and treatment [23]. For instance, 33 significantly correlated metabolites were identified in the hippocampus following *Toxoplasma gondii* infection, with 30 of them considered potential biomarkers [24]. Alterations in fatty acid metabolism and beta-oxidation were identified as the main metabolic characteristics associated with *Eimeria acervulina* infection [25]. Therefore, metabolomics can provide new insights into the occurrence and development of lamb coccidiosis, and help us clearly understand the differential changes

of metabolites during the treatment of lamb coccidiosis with Chinese herbs, to discover potential metabolic biomarkers that are beneficial to early diagnosis and treatment.

Based on these premises, we hypothesize that *A. annua* can control coccidia infection by regulating the intestinal flora of lambs. In this study, we employed different dosage groups of *A. annua* raw material (low, medium, and high dose groups) and different extraction groups (alcohol or water extraction) to identify more effective *A. annua* formulations with anti-coccidia and weight gain effects on lambs. We comprehensively explored the changes in intestinal flora and metabolites in lambs after *A. annua* treatment using 16 S rRNA gene sequencing and fecal metabolomics analysis, and screened out the differential flora and metabolites, providing a theoretical foundation for the treatment and detection of lambs coccidiosis.

Materials and methods

Preparation of *A. annua* extract

We took 1,680 g of A. annua, added 6 times its volume of solution (distilled water and 70% ethanol), soaked it for one week, and then filtered it with 6 layers of gauze. The filtrate was centrifuged at a rotating speed of 3,000 r/min for 10 min to collect the supernatant, which was concentrated in a boiling water bath to expand the water. The final constant volume concentration was 1 g/mL (1,680 mL). Both extracts were then sent to Biomeiker Technologies (BMK) for metabolite composition determination.

Animals, experimental design and diets

Forty-two 45-day-old female Hu sheep naturally infected with coccidia were provided by Henan Xinning Animal Husbandry Co., LTD. The lambs were fed under standard conditions, receiving two meals a day, once in the morning and once in the evening (concentrates + roughage). The primary formulations and ingredients of concentrate feed are shown in Table S1. Additionally, to confirm that the amounts of coccidia infection in lambs were basically equal in each group, fecal samples were collected from the rectum to assess the number of oocysts per gram (OPG) by salt flotation and light microscopy. Depending on the level of coccidia oocysts in the stool (ranging from 19,000 to 25,000), lambs were divided into 7 equal groups with 6 replicates per group: (1) Low-dose *A. annua* group (AL): Controlled addition of 120 g of *A. annua* to the diet; (2) Medium-dose *A. annua* group (AM): Controlled addition of 240 g of *A. annua* to the diet; (3) High-dose *A. annua* group (AH): Controlled addition of 480 g of *A. annua* to the diet; (4) *A. annua* water extract group (AW): Controlled addition of 30 mL water extract of *A. annua* to the diet; (5) *A. annua* alcohol extract group (AA): Controlled the addition of 30 mL alcohol extract of *A. annua* to the diet; (6) Diclazuril group (DI): Controlled addition of 1 mg/kg diclazuril to the diet, administered orally for 2 days; (7) Control group (CON): Normal diet. The groups were fed once a day for 14 days, except for the DI and CON groups. The flowchart of the test design is shown in Fig. 1.

Data collection

Measurement of fecal oocyst enumeration and fecal lesion

Rectal fecal samples were collected from lambs on days 0, 7, 14, 21, 28, 35, 42 of the experiment, respectively. The number of coccidium oocysts was calculated by the saturated saline floating method and microscopic examination. Subsequently, the relative reduction rate and negative conversion rate of oocysts were calculated. Additionally, based on our predefined fecal trait scoring table, the soft fecal rate and soft fecal index for each group in each period were calculated. Diarrhea rate in each group (%) = t/TD, where t is the number of diarrhea lambs in each cycle (the number of lambs with diarrhea in one cycle includes those with new diarrhea in this cycle and those with diarrhea in the previous cycle); T is the number of experimental lambs; D is the number of cycles detected. (Relative reduction rate of oocysts = (number of oocysts in the medication group - number of oocysts in the control group)/number of oocysts in the control group ×100%; Negative conversion rate of oocysts = number of lambs turning negative/number of positive lambs before medication ×100%)

Measurement of Grow Performance

Lambs were weighed on days 0, 14, and 42 of the experiment to calculate the average daily weight gain of the 14 days before the medication period. The average daily weight gain of the 42 days during the whole experiment were also calculated. The average weight gain and relative value increase of lambs in each group during the experiment were calculated according to the medication cost. (Average daily gain = (average final weight - average initial weight) / test days.

Microbial genomic DNA extraction and 16s rRNA gene sequencing

On the 14th day, about 1 g of fresh stool collected from the rectum was placed in a 2 mL EP tube, store in the refrigerator at −80℃ in the laboratory for future use. Then, according to the results of fecal oocyst shedding, fecal lesion and growth performance of each group. A total of 36 fecal samples were selected in 6 groups (AL, AH, AW, AA, DI, CON) with 6 replicates, and sent to BMK for microbial genomic DNA extraction and 16 S rRNA gene sequencing. Amplicon information: 16s

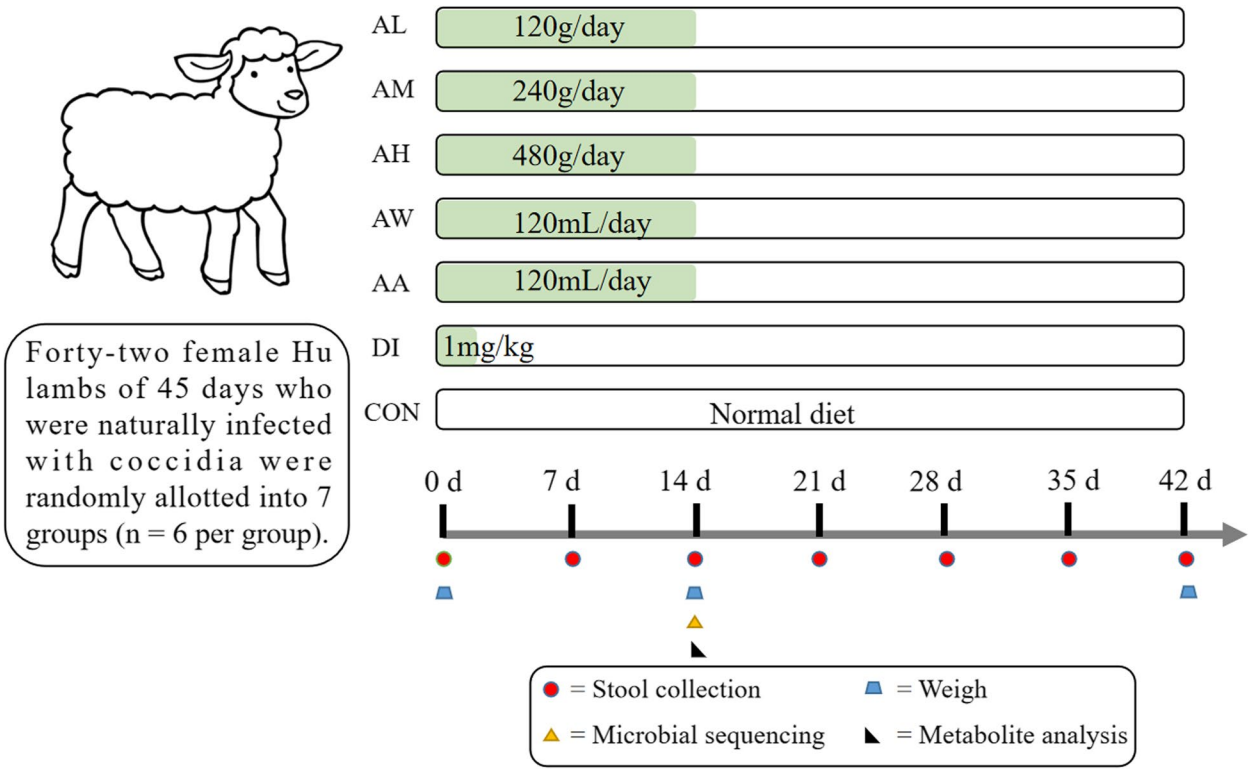

Fig. 1 Flowchart of the experimental design

v3 + v4_b. Primer information: F: ACTCCTACGGGAGGCAGCA; R: GGACTACHVGGGTWTCTAAT.

Fecal metabolomics analysis

According to the results of intestinal flora analysis, we selected the samples of 14 d here, with a total of 4 groups (AL, AA, DI, CON), 6 replicates in each group, and a total of 24 fecal samples.

Statistical analysis

After combing the original data with Excel, the Kruskal-Wallis test was used to compare the OPG reduction between the control group and the experimental group by using the non-parametric test in IBM SPSS Statistics 26 software to compare multiple independent samples. The results were expressed in the format of "median ± (upper quartile - lower quartile)". Utilize the General Linear Model (GLM) approach to examine the impact of various drug regimens and treatment and non-treatment periods on the daily weight gain of lambs. Assuming no interactions among the considered factors during computation. Employ Duncan test to compare and demonstrate statistically significant differences in daily weight gain among distinct treatment groups. The threshold of statistical significance was $P \leq 0.05$. OPG of oocysts in lambs were mapped using GraphPad Prism 8.0.2.

Results

Phenotypic and metabolomic analysis of two extracts

Metabolomic analyses of the two extracts are shown in Table 1. The abundance of Xanthoxylol and Zizaberanal acid in the water extract of *Artemisia annua* was more than that in alcohol extract, while the abundance of

Table 1 Statistical table of metabolite quantity of *A. Annua* extract

Component name	Relative abundance of AA	Relative abundance of AW
Caprolactam	2617593.689	3503491.366
Caprolactone	3530005.922	1739872.414
Clausenamide	102.733477	136.0658678
4-Methylene-2-pyrrolidinecarboxylic acid	6269133.589	5561352.568
Linolenic Acid	392353.1443	222856.5553
Zizyberanalic acid	305280.3767	823856.0045
Zanthodioline	137044.1404	1817777.317
Xanthoxylol	102.733477	136.0658678
Xanthoxylin	10828659.93	13630039.26
Xanthoxin	1,542,262,563	1,499,381,923

The first column shows the names of metabolically determined substances; the second and third columns to the end show the metabolite abundance of each sample

Linolenic Acid and 4-methylene Rolidinecarboxylic Acid was relatively less.

Effects of *A. annua* with different doses and different treatments on fecal oocyst shedding and fecal lesion of *Eimeria*-infected lambs

Based on the line plot of OPG at different periods (Fig. 2), the result showed that all drug groups had a repellent effect on coccidia. OPG in the AL group remained at the lowest level throughout the entire period. The water extract was more effective than the alcohol extract of *A. annua*. However, it's worth noting that DI treatment resulted in lower OPG levels in the first two weeks of the experiment, but the OPG of the DI group rebounded rapidly after drug withdrawal, with no significant difference in OPG compared to the CON group at the end of the experiment ($P > 0.05$). The OPG of coccidia tested in the control group also decreased to some extent initially, and then stabilized. Therefore, compared to the control group, the reduction of coccidian oocysts in the medication group could more effectively represent the reduction of coccidian in different periods, namely, the relative reduction rate (Table 2). AL and AW exhibited a high relative reduction rate of coccidioides oocysts in the initial tests, while the DI group had the lowest relative reduction rate. The negative conversion rate of coccidiosis (Table 3). The AL group had a higher level, while the other groups had a lower or zero negative conversion rate. The negative conversion rate in the DI group was higher in the first week of medication but decreased in the second week of medication.

Additionally, the fecal samples collected from each group in each cycle were scored based on the fecal trait levels we defined. From the soft fecal rate and average soft fecal index, it can be seen that the lambs in the AH and AW groups have more instances of soft feces, while those with normal feces include AL, AM, AA, DI, and CON (Figure supplement-Fig. S1, Table S2, Table 4).

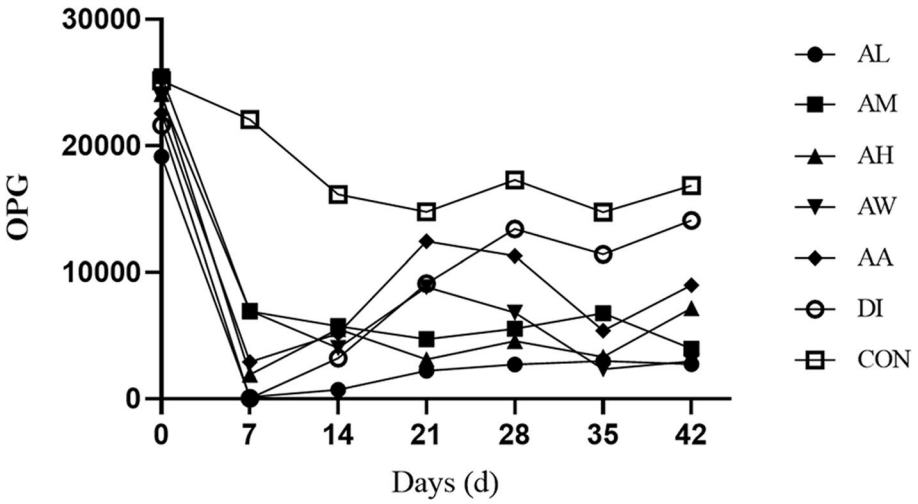

Fig. 2 The level of coccidia infection in each experimental group at different time periods

Table 2 Relative reduction rate of oocysts in different groups after treatment

Groups	Relative reduction rate of oocysts after treatment [a]					
	7d	14d	21d	28d	35d	42d
AL	99.40%	95.66%	84.99%	84.28%	79.80%	83.76%
AM	68.71%	64.46%	68.06%	67.98%	54.07%	76.44%
AH	91.38%	65.91%	79.01%	73.58%	77.49%	57.42%
AW	68.48%	75.21%	40.18%	60.56%	84.16%	82.38%
AA	86.85%	68.08%	15.86%	34.72%	63.46%	46.63%
DI	99.85%	80.06%	38.26%	22.37%	22.51%	16.34%
CON	0	0	0	0	0	0

[a] Relative reduction rate of oocysts = [(number of oocysts in the medication group - number of oocysts in the control group)/number of oocysts in the control group] × 100%

Table 3 Negative conversion rate of oocysts in each medication group

Groups	Negative conversion rate of oocysts after treatment [a]					
	7d	14d	21d	28d	35d	42d
AL	83.33%	50.00%	0.00%	0.00%	0.00%	0.00%
AM	33.33%	0.00%	0.00%	0.00%	0.00%	0.00%
AH	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
AW	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
AA	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DI	66.67%	33.33%	0.00%	0.00%	0.00%	0.00%
CON	0.00%	0.00%	16.67%	0.00%	0.00%	0.00%

[a] Oocysts turning negative rate = number of lambs turning negative/number of positive lambs before medication × 100%

Table 4 Overall defecation rate and defecation index of each group

Treatments	Soft bowel rate in each group (%)	Average soft fecal index
AL	2.08	2
AM	0.00	0
AH	12.50	12
AW	6.25	6
AA	4.17	2
DI	0.00	0
CON	2.08	2

Diarrhea rate in each group (%) = t/TD, where t is the number of diarrhea lambs in each cycle (the number of lambs with diarrhea in one cycle includes those with new diarrhea in this cycle and those with diarrhea in the previous cycle); T is the number of experimental lambs; D is the number of cycles detected

Effects of *A. annua* with different doses and treatments on growth performance of lambs

As can be seen from Table 5, GLM was used to analyze the effects of different factors on the daily weight gain of lambs. The two factors of different administration groups, administration and non-administration periods had extremely significant effects on the daily weight gain of lambs ($P<0.01$). There was no significant difference between Group and Time Period interaction ($P=0.385$). Eta-squared is Group, Time Period, Group * Time Period in descending order. Among them, eta-squared of Group was the largest, indicating that the daily weight gain of lambs in groups treated with different drugs had the greatest impact. The effects of different drug groups on the growth performance of lambs were shown in Table 6. The ADG of AW group was the lowest at 14d and 42d,

Table 5 Analysis of variance on the influence of different factors on daily weight gain of lambs

Factor	Three Sums of Squares	Degrees of Freedom	Mean Square	F Value	Significance	eta- squared
Group	51296.026	6	8549.338	3.993	0.002	0.255
Time Period	20340.743	1	20340.743	9.501	0.003	0.12
Group * Time Period	13823.561	6	2303.927	1.076	0.385	0.084

Group means different drug treatment groups. Time Period indicates the medication period and non-medication period

Table 6 Effects of each medication group on growth performance of lambs

Treatments	Initial BW(kg)	14d BW(kg)	42d BW(kg)	14dADG(g)	42dADG(g)
AL	12.43 ± 1.62	15.32 ± 2.12	17.62 ± 2.47	206 ± 62^{abA}	123 ± 41^{abcB}
AM	13.97 ± 0.54	15.95 ± 1.18	19.12 ± 1.58	142 ± 71^{abcA}	123 ± 35^{abcA}
AH	13.6 ± 0.68	15.73 ± 1.33	19.43 ± 0.75	152 ± 55^{abcA}	139 ± 18^{abA}
AW	13.6 ± 0.68	15.12 ± 0.91	17.75 ± 1.08	108 ± 40^{cA}	99 ± 16^{cA}
AA	12.83 ± 0.85	15.77 ± 0.84	19.25 ± 0.65	210 ± 96^{aA}	153 ± 33^{aA}
DI	11.73 ± 1.98	13.67 ± 1.89	16.98 ± 2.32	138 ± 37^{bcA}	125 ± 14^{abcA}
CON	12.72 ± 1.9	14.53 ± 2.24	17.18 ± 2.26	130 ± 29^{bcA}	106 ± 18^{bcA}

Different lowercase letters indicate significant difference in weight gain among different groups at the same time ($P<0.05$). Different capital letters indicate significant difference in weight gain in the same group at different times ($P<0.05$)

and was significantly lower than that of AA group ($P < 0.05$). After 14 days of administration, ADG of lambs in AA group was significantly increased compared with AW, DI and CON groups ($P < 0.05$). After 42d, ADG of lambs in AA group was significantly higher than that in AW and CON groups ($P < 0.05$). The results indicated that group AA could be used as a feed additive to increase the average daily gain of lambs. In addition, ADG of lambs in AL group at 14 days was significantly higher than that in 42d group ($P < 0.05$), but there was no significant difference in daily gain of lambs in other groups at these two periods ($P > 0.05$). The results showed that the drug effect could not be maintained for a long time after the drug was discontinued in the AL group, which may be the reason for the low dose in the AL group.

The new output value per lamb was calculated based on average weight gain and drug cost (Table 7). The results showed that the average weight gain of lambs after different doses of *A. annua* treatment was dose-dependent. Based on the analysis of relative weight gain and medication cost, AH has the highest added output value. From the perspective of different treatment methods (AL, AW, AA), AA showed the best weight gain effect. This indicates that among the different treatment methods of *A. annua*, alcohol extraction has the best weight gain effect on lambs, but the cost of alcohol extraction is relatively high. Based on comprehensive analysis, AA has the highest yield increase effect. We can further optimize the alcohol extraction process to reduce drug costs in the future, which will yield significant economic benefits.

Effects of *A. annua* with different doses and treatments on intestinal flora of lambs

To further explore the effect of *A. annua* on the control of microbial diversity of *Eimeria* spp. infection, we conducted an intestinal flora analysis of lambs. This test mainly involved six experimental groups: AL, AH, AA, AW, DI and CON. According to the Shannon-Wiener curve and dilution curve, when the curve flattens or reaches a plateau, it can be considered that the sequencing depth has covered all species in the sample, indicating that the sequencing data volume is large enough for data analysis (Figure supplement-Fig. S2 A-B).

Table 7 The new output value of lambs during the experiment

Treatments	Relative weight gain (kg)	Drug cost (Yuan / piece /day)	New output value (Yuan / piece)[A]
AL	0.73	0.16	14.112
AM	0.69	0.32	10.976
AH	1.37	0.64	21.728
AW	−0.31	0.16	−9.184
AA	1.96	1.12	28.224
DI	0.79	0.8	6.496

[A] New output value = (average weight gain in the medication group - average weight gain in the control group) × Unit Yield Reduction (0.7) × Unit price of live weight of lamb - drug cost (yuan / piece / day) × Medication days [21]. Lamb live weight unit price reference until December 2022

Alpha diversity index analysis

The Alpha diversity index analysis reflects the species richness (Chao1 index and ACE index) and species diversity (Shannon index and Simpson index) of a single sample, we calculated and analyzed the ACE index, chao1 index, Simpson index and Shannon index for each group on 14d, respectively (Fig. 3A). ACE index and Chao1 index of AH14, AA14, and DI14 were increased in different degrees compared with CON14 group, indicating that these three groups of drugs can improve the species richness of intestinal microbiota of lambs ($P > 0.05$). The Simpson index and Shannon index of each group had little change, and DI14 was lower than the CON14 group ($P > 0.05$).

Beta diversity index analysis

To clarify the distance matrix of intestine microbiomes between the test groups, we calculated β diversity (Fig. 3B and F). Principal coordinates analysis (PCoA) was used to demonstrate species diversity differences among samples. The figure shows the PCoA of CON group and each medication group respectively on the 14th day of the trial. There was no significant separation of PCoA points between the CON14 group and the DI14 group. Still, there was a significant separation between all *A. annua* treatment groups and the CON14 group, indicating that the Chinese herbal medicine of *A. annua* could change the structure of the intestinal microbiota of lambs, consistent with the anti-coccidioid effect of each medication group analyzed above. However, some groups have discrete PCoA points, which may be due to the particular physiology of the individual lamb.

Community composition analysis

To clarify the effects of *A. annua* on intestinal microbiota composition, the relative abundance of microbial taxa of the integral microbiome was analyzed. The species distribution histogram is shown in Fig. 4, which shows the proportion of different species at the phyla and genus levels on the 14th day of the experiment. At the phylum level, Firmicutes and Bacteroidetes were dominant in the intestinal microbiota of the different groups. At the genus level, *unclassified_Lachnospiraceae* and *UCG_005* were dominant. Figure 5(A-B) shows the top 3 abundant bacteria at phylum and genus levels. There was no significant difference between CON14 and DI14 in the proportion of

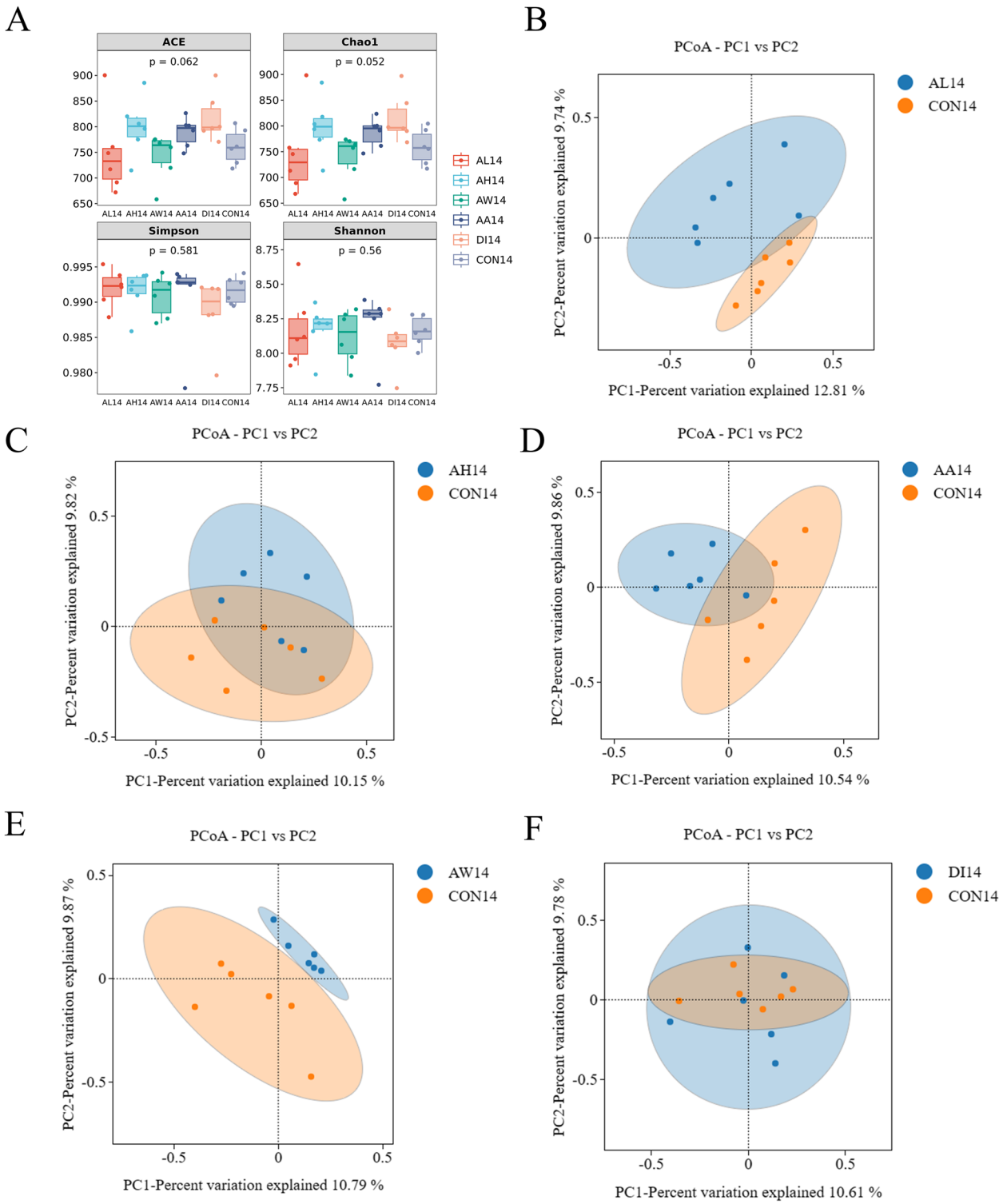

Fig. 3 α and β diversity analysis

Firmicutes and Bacteroidetes, and the abundance of Firmicutes in AH14 (85.89%) and AA14(73.95%) was higher than CON14(68.03%). Compared with the control group, *unclassified_Lachnospiraceae* and *UCG_005* were generally higher in AL14, AH14, AW14 and AA14, but there was no significant difference ($P > 0.05$).

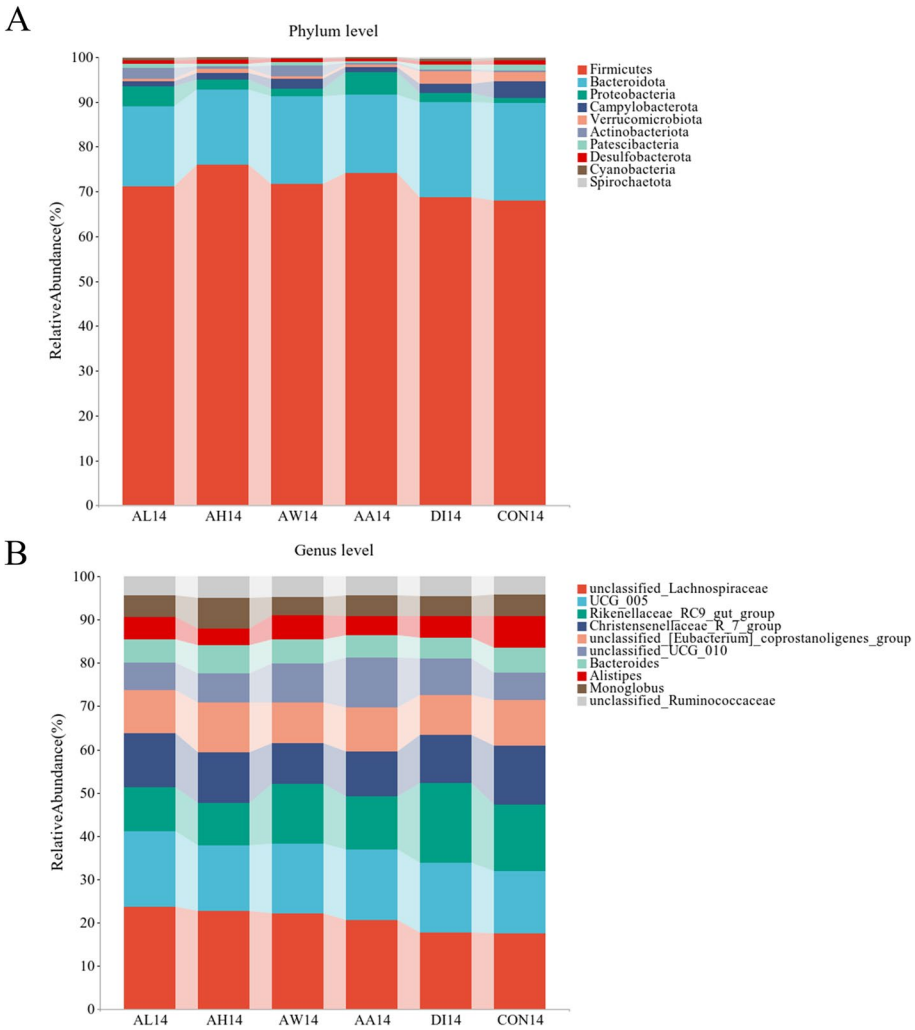

Fig. 4 The community is composed of columnar bodies. **A** phylum level. **B** genus level

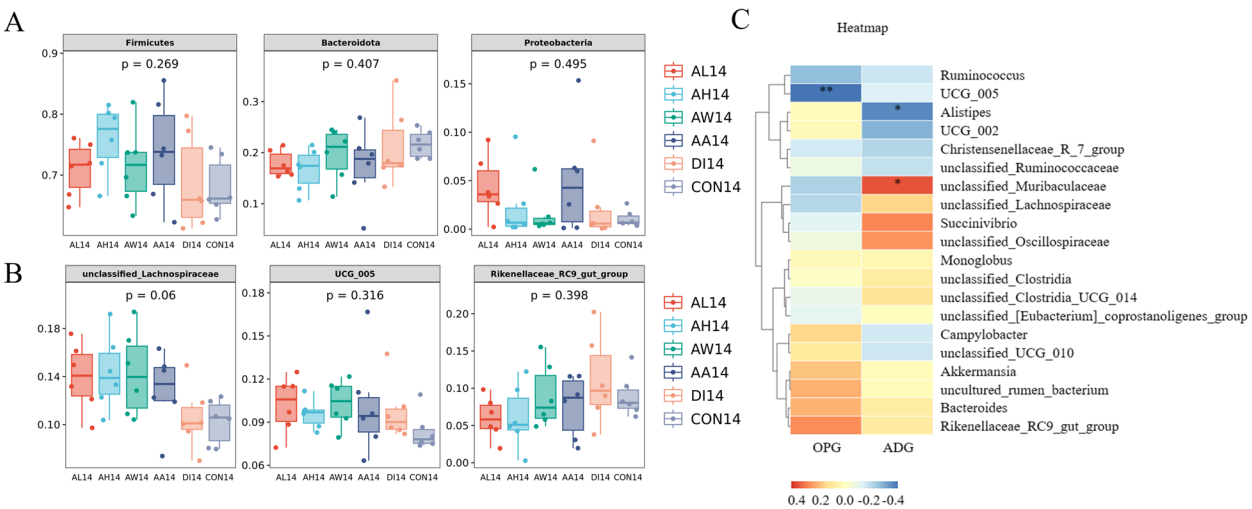

Fig. 5 Intestinal microbiota analysis. **A**-**B** Abundance analysis of intestinal microbiota at the phylum level and the genus level (top 3). **C** Heat map of correlation analysis of microflora with ADG and OPG at the genus level

Figure 5C shows a heat map of the correlation analysis of microflora with ADG and OPG at the genus level. *Unclassified_Muribaculaceae* was significantly positively correlated with ADG ($P<0.05$) and negatively correlated with OPG, but no significant difference was found ($P>0.05$). *Alistipes* exhibited a negative correlation with ADG and had significant differences ($P<0.05$), and it was positively correlated with OPG but has no significant difference ($P>0.05$). Additionally, *UCG_005* was negatively correlated with OPG, and the difference was extremely significant ($P<0.01$).

Through analysis of the dominant taxa, the differences in microbiota among groups could not be clearly explained. We further analyzed the impact of *A. annua* on the intestinal microbiota of experimental animals using LEfSe analysis. On the 14th day of the experiment, the level of Gut microbiota in six treatment groups was measured with LEfSe (LEfSe LDA > 3.0, $P<0.05$). A total of 15 genera with LDA scores > 3 were identified, as shown in Figure supplement-Fig. S3. *Odoribacter*, *uncultured_rumen_bacterium*, *Solobacterium*, *uncultured_Erysipelotrichaceae_bacterium* have the greatest difference in CON. Anaerovibrio, Oscillibacter, *unclassified_Clostridia_UCG_014*, and *Succinivibrio* are some of the biggest differences in AL. AA, DI and AW respectively *unclassified_UCG_010*, *Anaeroplasma*, *Bifidobacterium* abundance differences. The differences in the abundance of these different genera between groups are shown in Fig. 6.

Effects of *A. annua* on Metabolites of lamb feces

Based on the LC-QTOF platform, the metabolome of 24 samples was analyzed qualitatively and quantitatively. A total of 2831 metabolites were detected in positive and negative ion modes. Fatty Acyls, Carboxylic acids and derivatives, Organooxygen compounds are the main metabolites. Metabolism of terpenoids (13.32%) and polyketides, Biosynthesis of other secondary metabolites (12.59%), Lipid metabolism (8.74%) are the main metabolic pathways. The experimental group and the control group could be divided into different clusters, as shown in R2 Y and Q2 Y in the OPLS-DA Figure supplement-Fig. S4. The more stable and reliable the model should be used to screen differential metabolites.

The differential metabolites in AL and AA were higher than those in DI, and the up-regulated and down-regulated differential metabolites in AL were the highest (Table S3). The difference multiples of AL, AA, DI and CON were analyzed respectively, as shown in Fig. 7(A-B). CON vs. AL 12(13) − EpOME, Casticin, Histamine and other metabolites were up-regulated. Nicotinic acid mononucleotide, Staphyloferrin B, and other substances were down-regulated. CON vs. AA Avermectin B1b aglycone, Mupirocin, N-Methyltyramine and other metabolites were up-regulated. Staphyloferrin B, betatocotrienol and other metabolites were down-regulated.

The score of differential metabolite abundance is shown in Fig. 7(C-D). Compared with CON, the metabolic pathway of Nonribosomal peptide structures in AL was up-regulated. Polyketide sugar unit biosynthesis pathways were down-regulated. The metabolic pathway of Peptidoglycan biosynthesis was up-regulated in AA. The Polyketide sugar unit biosynthesis pathway was down-regulated.

Relationships between different bacterial genera and metabolites

The correlation between different bacterial genera and metabolites was analyzed based on the Spearman correlation coefficient calculation method (Fig. 8). Three bacterial genera were identified in AL to be significantly associated with 34 metabolites. Anaerofilum, Pygmaiobacter, and unclassified_Muribaculaceae had positive and negative relations to the metabolites (17 positives vs. 17 negatives). Five bacterial genera were identified in AA to be significantly associated with 35 metabolites. Treponema was significantly positively correlated with PG (14:0/16:1(9Z) and Oleandomycin, and negatively correlated with Butylidenephthalide, 16-Hydroxypalmitate and 14-Hydroxypergolide. Lachnospiraceae_AC2044_group were negatively related to most of the metabolites, whereas Candidatus_Stoquefichus (18 positives vs. 9 negatives) and Pygmaiobacter (15 positives vs. 6 negatives) were positively associated with most of the metabolites. Turicibacter had positive and negative relations to the metabolites (15 positives vs. 12 negatives). In summary, there is a strong correlation between gut flora and metabolites. Our results show that there is a significant change in the variety and abundance of gut microbes, resulting in a marked impact on metabolites in lambs.

Discussion

Lambs are the small ruminant animal and lamb coccidiosis is rarely treated with Chinese herbal medicine. The effects of different dosages and extraction methods of *A. annua* on OPG, growth performance, intestinal flora and fecal metabolites of Hu lambs were evaluated. Liviu Drăgan et al. studied the anticoccidiosis effect of *A. annua* and foeniculum vulgare on chickens infected with *Eimeria tenella* and found that *A. annua* significantly reduced oocysts production in broilers, which was similar to our results [26]. In this study, at the beginning of the experiment, all *A. annua* groups had an outstanding repellent effect on oocysts, among which the low-dose group (AL) had the best effect ($P<0.05$), and the

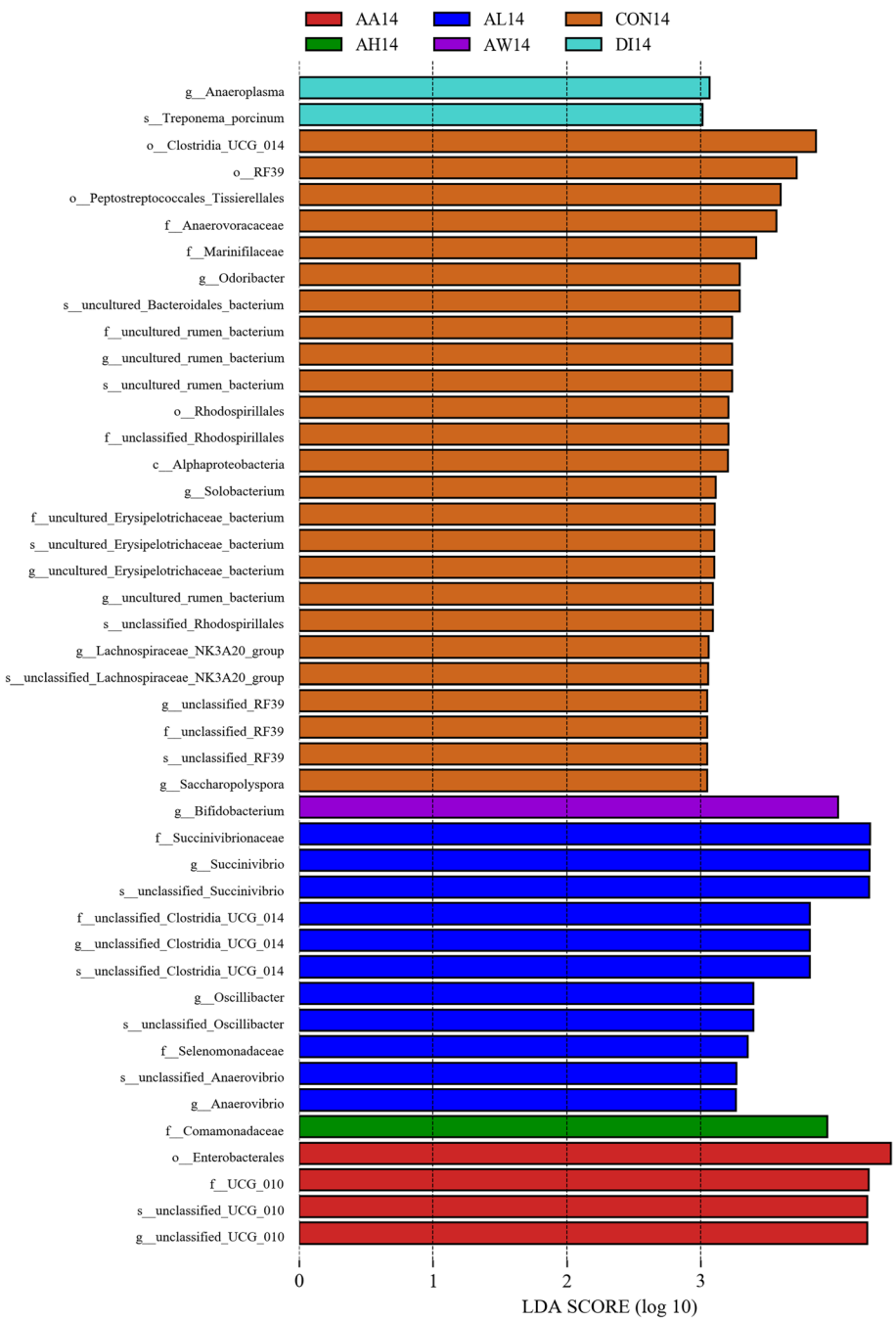

Fig. 6 The LDA score obtained through LEfSe analysis of different groups of gut microbiota. (LDA values greater than 3 are used as thresholds for LEfSe analysis)

negative conversion rate was the highest (83.33%) on the 7th day. In the comparison of different treatments, AW and AA had a similar oocyst-repellant effect, but in terms of efficacy durability, AW was superior to AA. This may be due to the higher levels of metabolites Xanthoxylol and Zizaberanal acid in AW compared to AA. Related research reports, Xanthoxylol is a lignan in the bark of Zanthoxylum piperitum (Rutaceae), which has a certain repellant effect on mosquito larvae [27]. Zizyberanalic acid has anti-inflammatory activity, which can relieve intestinal inflammation caused by coccidia [28]. In the initial stage of the experiment, the coccidia OPG value of CON in the line graph also decreased to a certain extent and then tended to be flat, which may be because in the

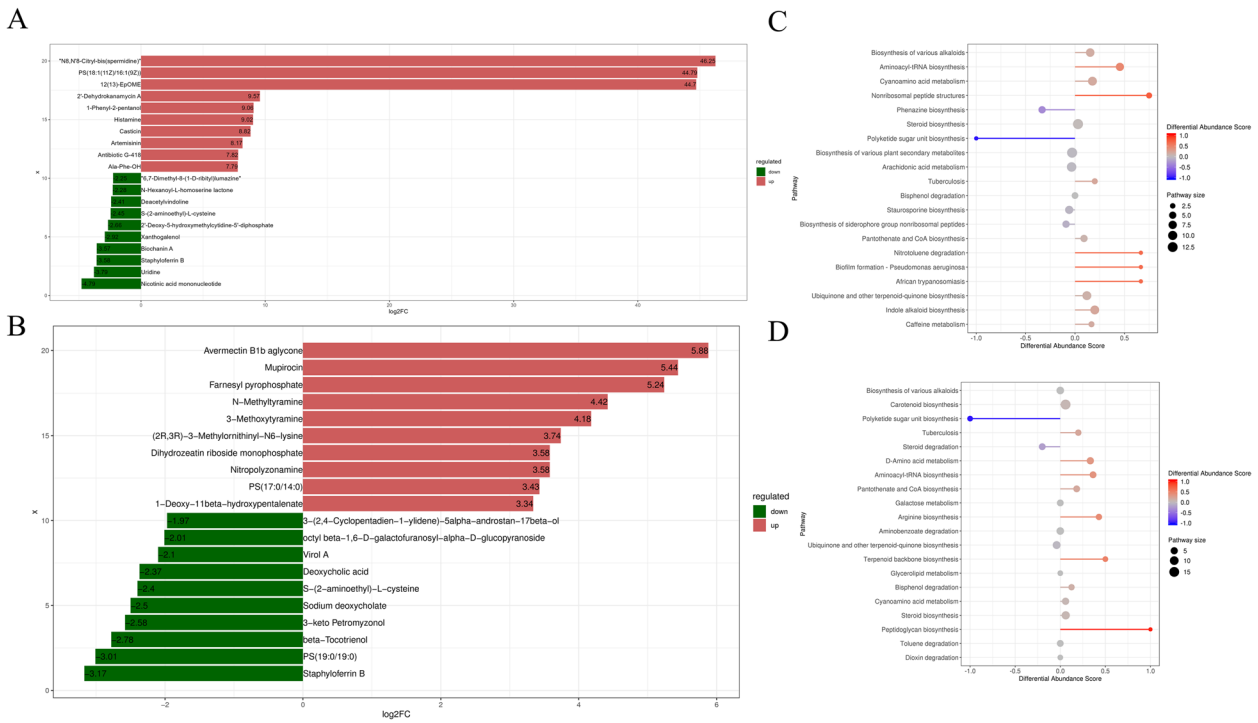

Fig. 7 Analysis of differential metabolites and differential metabolic pathways. A and B show the difference multiple bar charts. C and D show the difference abundance scores of different metabolic pathways. A, C CON vs. AL. B, D CON vs. AA.)

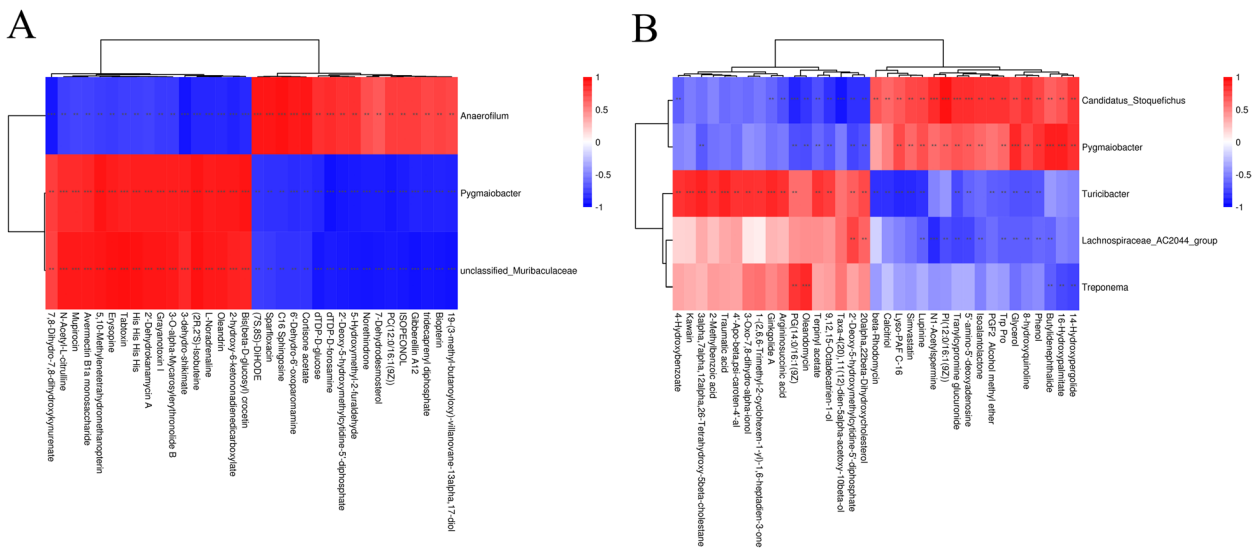

Fig. 8 Heat maps of significantly altered correlations between genus and metabolites. **A** Heat map of the correlation between the three genera of bacteria and differential metabolites in the AL group. **B** Heat map of the correlation between the four genera of bacteria and differential metabolites in the AA group. Red indicates a positive correlation, while blue indicates a negative correlation, and the darker the color, the stronger the correlation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

process of selecting lambs, we tried our best to select the one with the largest coccidia infection amount. According to the growth and development cycle of coccidia, the coccidia in lambs may be at its peak. Based on this, we calculated the reduction rate of oocysts relative to that of CON according to the coccidia OPG value in each group

at each stage. It can be seen that the relative reduction rate of DI was at a higher level on the 7d and 14d, which were 99.85% and 80.06% respectively, and then the relative reduction rate gradually decreased to 9.56% at the end of the experiment (42d). All medication groups of *A. annua* had better effects than DI. Artemisinin, the active component of *A. annua*, may act on the development stage of coccidia through a direct mechanism. The study of Emilio et al. found that artemisinin inhibited the expression of SERCA in the sarcoplasmic endoplasmic reticulum of coccidioides macrogametes, thus causing the Ca^{2+} homeostasis of coccidia cells to be out of balance, thus inhibiting the formation and sporialization of the oocyst wall of coccidia [29]. Jiao et al. demonstrated that artemisinin promoted apoptosis of the second generation of schizozoites in coccidia mainly by inhibiting the expression of transcription factor NF-κB and anti-apoptotic factor Bcl-xL [30]. Additionally, due to different coccidia hosts and species, sporulated oocysts time is also different, and the mechanism of action is not completely clear. On the other hand, according to our negative conversion rate of coccidia, although *A. annua* could not completely kill coccidia in the intestinal tract of lambs, a low level of infection might make the body of lambs in a coccidia immune state. Moreover, *A. annua* is rich in flavonoids, terpenoids, organic acids and polysaccharides, which can help to enhance the resistance to pathogens and reduce the risk of secondary bacterial infection [31].

In this study, the average daily gain (ADG) effect of AW was lower than that of CON no matter in the medication period or non-medication period, and the ADG effect of lambs in other medication groups was better than that of the control group, among which AA group showed the best effect. Therefore, the ADG effect of alcohol extract is better than that of water extract. This may be due to the higher levels of Linolenic Acid and 4-methylene Rolidinecarboxylic Acid in AA group metabolites compared to the AW group. Relevant studies have proved that Linolenic Acid is a beneficial nutritional supplement. 4-methylene 2-pyrrolidinecarboxylic acid can stimulate protein synthesis, promote muscle growth and repair, glucose metabolism and energy supply and improve endurance and resistance to fatigue [32]. Shamlan reported that the content of active substances in the alcohol extract of avocado was higher than that in the water extract, which was similar to the results of our study [33]. Additionally, the pH value of the two extracts may also affect this result. It is worth noting that in different dose groups, the lambs in AL had the best weight gain effect during the medication period (0-14d), which was consistent with the coccidia repellency effect. However, in the non-medication period (14-42d), the lambs in the AH gained the best weight. These results indicated that in the long run after drug withdrawal, the high dose of *A. annua* maintained the efficacy longer, and the long-term digestion, absorption and weight gain of lambs were more significant. However, the effect of short-term high dose of Artemisia's initial drug on lamb weight gain was insignificant, and the soft stools rate and soft stools index of lamb feces in the AH group were the highest. In conclusion, these results suggested that high dose of *A. annua* may cause some side effects, which may concern the safety of using *A. annu*a. Shahbazfar et al. explored the use of Artemisinin in the treatment of leishmaniasis in dogs in 2012, and found that it had obvious toxic and side effects. The authors suggested that artemisinin should be used cautiously in dogs [34]. However, in 2011, the effects of different doses of artemisinin on long-term use of broilers were studied, and it was found that the severity of brain lesions, low hematopoietic volume, and red blood cell count were dose-dependent, but the side effects were not serious when coccidiosis of broilers was controlled at the therapeutic dose [35]. Ramos analyzed the data on human administration of *A. annua* drugs, and the results showed that *A. annua* had no potential neurotoxicity [36]. Therefore, the species difference of *A. annua*, the class of *A. annua* derivatives, and the duration and frequency of administration will affect the absorption, distribution, metabolism and excretion of *A. annua* in the control of animal coccidiosis. According to our experiment, we can explore the effects of *A. annua* on coccidia killing and lamb weight gain by reducing the dosage and prolonging the duration of medication in the future. Additionally, *Eimeria spp.* infection results in cecal microbiome disturbance and intestinal dysfunction. However, functional additives can reduce the effect of *Eimeria spp.* on the gut microbes of animals [37]. The addition of *artemisia annua* extract significantly increased the number of intestinal villi, regulated the composition and abundance of intestinal flora, and thus played a good role in disease resistance [38, 39].

We also analyzed the effects of *A. annua* on the intestinal microbiota of lambs by 16s rRNA sequencing. On the 14 d, the diversity of bacteria in the *A. annua* treatment group was higher than that in the CON, which might be because *A. annua* improved the abundance of beneficial bacteria in the intestinal tract of lambs, and coccidial infection resulted in a decrease in intestinal microbial diversity, which was consistent with previous research reports [23]. At the phylum level, Firmicutes, Bacteroidota and Proteobacteria were the dominant phylum in the intestinal tract of lambs, which was consistent with previous reports [40–42]. Firmicutes and Bacteroidota are the most important microflora in the intestinal tract of animals [43]. Firmicutes can promote the effective absorption of nutrients and heat to maintain

health [44]. In this experiment, the proportion of Firmicutes in AH (75.89%) and AA (73.95%) was higher than that in CON (68.02%), which was consistent with the results of lamb weight gain, which might be one of the reasons for promoting lamb weight gain. The content of *unclassified_Lachnospiraceae* in *A. annua* medication group is also higher than that in CON. *Lachnospiraceae* are producers of butyrate, which can enhance the integrity of the epithelial barrier and inhibit inflammation [45]. Therefore, *A. annua* may promote the elevation of *unclassified_Lachnospiraceae*, making butyrate secreted by them play an important role in the resistance to coccidian infection. The increased abundance of *unclassified_Muribaculaceae* plays an important role in improving inflammation and intestinal structure [46, 47]. It has been reported that *unclassified_Muribaculaceae* is positively correlated with butyrate, which improves intestinal barrier function. Butyric acid can improve intestinal barrier function [48]. In our study, *unclassified_Muribaculaceae* was positively correlated with ADG ($P<0.05$) and negatively correlated with OPG. Therefore, it can be concluded that *A. annua* can improve intestinal damage and inflammation caused by coccidia infection in lambs by increasing the abundance of *unclassified_Muribaculaceae*, thereby promoting better intestinal absorption of nutrients, increasing ADG and decreasing OPG. Studies have shown that *Alistipes* dysbiosis might be harmful or beneficial. In this experiment, *Alistipes* was negatively correlated with ADG ($P<0.05$) and positively correlated with OPG, so *Alistipes* was harmful in this study. It has been reported that *Alistipes* is pathogenic in rectal cancer, and the imbalance of intestinal flora may be related to the abundance of *Alistipes* [49]. Additionally, *Bacteroidetes* are often associated with chronic intestinal inflammation, and *Alistipes* is a branch genus of Bacteroidetes, so we infered that *Alistipes* might further promote intestinal inflammation under the influence of intestinal coccidiosis in lambs. *Eimeria* invaded the intestinal tract of lambs, causing intestinal inflammation and thereby causing diarrhea. *Ruminococcaceae_UCG-005* had a significant effect on the prevention of calf diarrhea and can could be used as a probiotic to reduce or prevent calf diarrhea [50]. This is consistent with the results of our study, in which *UCG-005* showed a significant negative correlation with OPG. These results indicate that *unclassified_Muribaculaceae* can be used as a suitable probiotic to promote weight gain in lambs, *UCG-005* can be used as a suitable probiotic to inhibit intestinal Eimeria colonization in lambs, and *Alistipes* may be a biomarker of intestinal coccidia outbreak risk in lambs.

Fecal metabolomics has been used to evaluate drug efficacy and disease development, and to clarify the metabolic process and change mechanism of organisms through the screening of relevant differential metabolites and the analysis of metabolic pathways. In this study, non-targeted metabolomics results showed that artemisinin has a broad effect on metabolites for the treatment of lamb coccidiosis. Compared with CON, 12(13)−EpOME, Casticin, Histamine in AL and Avermectin B1b aglycone, Mupirocin, N-Methyltyramine in AA were up-regulated. According to relevant literature reports, EpOMEs mainly mediate the related inflammatory response after pathogen infection, and play an immunosuppressive role in insects [51]. Chan EWC's review clarified that Casticin is effective against cancer cell lines through different molecular mechanisms and confirmed that Casticin has anti-inflammatory properties [52]. Additionally, as an immunomodulator, Histamine is essential for local immune responses, regulation of intestinal gastric acid secretion, and neurotransmission in the central nervous system [53]. *Eimeria* spp. infection causes damage to the host intestinal mucosa and intestinal cells, resulting in intestinal inflammation. Therefore, we can assume that an increase in these metabolites can improve intestinal inflammation caused by *Eimeria* infection. Avermectin is commonly used as an insecticide to treat pests and parasites, and Avermectin B1b aglycone in AA may be a derivative of Avermectin, used to repel Eimeria [54]. Mupirocin is used to control outbreaks of Staphylococcus aureus and is used in infections of soft tissue or skin [55]. However, the increase of N-methyltyramine is likely to be the cause of the weight gain in AA lambs. Because N-methyltyramine has been shown to enhance appetite and food digestion by stimulating gastrin and pancreatic secretions [56]. In contrast, Staphyloferrin B was down-regulated in both AL and AA. Staphyloferrin B acts as an iron carrier for Staphylococcus aureus and may increase the likelihood of opportunistic infections, such as endocarditis, meningitis and osteomyelitis [57]. Among the differential metabolic pathways, Nonribosomal peptide structures were up-regulated in AL, and Peptidoglycan biosynthesis was up-regulated in AA. Nonribosomal peptide has a very wide range of clinical applications, such as their use as antifungal agents of last resort, antibiotics or immunosuppressants [58]. Additionally, it is worth noting that the Polyketide sugar unit biosynthesis pathway is down-regulated in both AL and AA. Liang D studied specific microorganisms which were conducive to the improvement of postoperative obesity and found that Polyketide sugar unit biosynthesis pathway was significantly negatively correlated with body weight and fast blood glucose. This may have a beneficial effect on mediating bariatric surgery [59]. This is consistent with our results. In this study, the body weight of lambs in AL and AA increased compared with CON, which may be achieved by *A. annua*

through down-regulating the Polyketide sugar unit biosynthesis pathway.

There are several limitations to this study. Firstly, the utilization of naturally infected lambs with coccidiosis, while providing valuable insights for coccidiosis treatment in primary farms, introduces a certain level of experimental error. Future research could consider employing artificial coccidiosis infection methods to establish a more controlled lamb coccidiosis model. Furthermore, our study solely conducted quantitative analysis of coccidium oocysts in lamb feces. To enhance the depth of investigation, a more focused approach on specific coccidia species could elucidate the repellent effects of *A.annua* across different coccidia strains. Lastly, while we screened intestinal microbes and metabolites linked to coccidia OPG and lamb weight gain, these associations were not validated. Subsequent studies will delve into the validation and elucidation of the roles played by these identified markers.

Conclusion

This study suggests that *A.annua* has the potential to improve the performance of lambs infected with coccidiosis and the number of coccidiosis oocysts in feces by regulating intestinal flora and fecal metabolite composition. Furthermore, the identification of potential probiotics (e.g., *Unclassified_Muribaculaceae* and *UCG-005*) and biomarkers (e.g., *Alistipes*) provides valuable targets for future research on lamb gut health and coccidiosis management. Spearman correlation analysis further revealed the significant correlation between 182 bacteria genera and metabolites in AL and AA groups, providing a new perspective for studying the interaction between the host, *A.annua* and *Eimeria*.

Abbreviations

A. Annua	*Artemisia annua*
OPG	Oocysts per gram
ADG	Average daily gain
AL	Low-dose *A. annua* group
AM	Medium-dose *A. annua* group
AH	High-dose *A. annua* group
AW	*A. annua* water extract group
AA	*A. annua* alcohol extract group
DI	Diclazuril group
CON	Control group
PCoA	Principal Co-ordinates Analysis
LEfSe	Linear discriminant analysis Effect Size
LDA	Linear Discriminant Analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04493-6.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Authors' contributions

Fuchun Jian, Longxian Zhang and Xiaoying Li were involved in the design and conceptualization of the experiment. Shuaiqi Liu, Shiheng Li and Shuqi Cheng conducted the animal tests. Jing Li assisted with sample collection. Senyang Li and Manyu Liu helped interpret the results and draft the manuscript. All authors read and approved the final version of the manuscript.

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Data availability

The datasets during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Research Ethics Committee of Henan Agricultural University reviewed and approved the experimental protocol. The collection of samples was performed following the Guide for the Care and Use of Laboratory Animals (Ministry of Health, China). All samples tested in this experiment were collected with permission from herders, while no special permission was required for sampling locations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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