Rectal microbiomes and serum metabolomics reveal the improved effect of *Artemisia ordosica* crude polysaccharides on the lactation performance, antioxidant and immune responses of lactating donkeys

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**ABSTRACT**

This study is aimed at investigating the effects of dietary supplementation with *Artemisia ordosica* crude polysaccharides (AOCP) on lactation performance, antioxidant status, and immune status of lactating donkeys and analyzing rectal microbiomes and serum metabolomes. Fourteen lactating Dezhou donkeys with similar age (6.16 ± 0.67 years of BW ± SD), weight (250.06 ± 25.18 kg), days in milk (39.11 ± 7.42 d), and averaged parity of 3 were randomly allocated into 2 treatments: a control group (CON, basal diet) and an AOCP group (AOCP, basal diet with 1.0 g/kg DM AOCP). Ten weeks were allotted for the experiment, 2 weeks for adaptation, and 8 weeks for collecting data and samples. The results showed that supplementation of donkey diets with AOCP increased lactation performance, including dry matter intake, milking yield, estimated milk yield, solids-corrected milk, energy-corrected milk, milk protein yield, milk lactose yield, milk total solids yield, and milk solid not fat yield. The digestibility of dry matter, crude protein, acid detergent fiber, and neutral detergent fiber was increased in the AOCP group compared with the CON group. The AOCP group increased the concentrations of immunoglobulin A, immunoglobulin G, and immunoglobulin M, the activities of the superoxide dismutase, catalase and total antioxidant capacity in the serum. AOCP decreased the concentrations of tumor necrosis factor-α, nitric oxide, reactive oxygen species, and malondialdehyde in the serum. Compared with the CON group, AOCP increased propionate, butyrate, isovalerate, and total VFA concentrations in rectal feces (*P* < 0.05). The addition of AOCP to increased diversity (Shannon index) and altered structure of the rectal microflora. As a result of AOCP supplementation, there has been a significant improvement in the colonization of beneficial bacteria, including Lactobacillus, Unclassified_f_Prevotellacea, Ruminococcus, and Fibrobacter genera. In contrast, a decrease in the colonization of the Clostridium_sensu_stricto_1 bacterial genus and other pathogenic bacteria was observed. Meanwhile, metabolomics analysis found that AOCP supplementation upregulated metabolites L-tyrosine content while downregulating 9(S)-HODE, choline, sucrose, LysoPC (18:0), LysoPC (18:1(9Z)), and LysoPC (20:2(11Z,14Z)) concentrations. These altered metabolites were involved in the PPAR signaling pathway, prolactin signaling pathway, glycerophospholipid metabolism, carbohydrate digestion and absorption, and tyrosine metabolism pathways, which were mainly related to antioxidant capacity, immune responses, and protein metabolism in the lactating donkeys. As a consequence of feeding AOCP diets, beneficial bacteria were abundant, and antioxidant and protein metabolism-related pathways were enriched, which may enhance lactation performance in donkeys. Therefore, supplementing AOCP diets is a desirable dietary strategy to improve donkey health and lactation performance.

**Keywords:** *Artemisia ordosica*, natural extracts, animal health, milk performance, microbiome, metabolome, lactating donkeys

**INTRODUCTION**

Donkey milk is gaining popularity as a dairy product with unique features and flavors (Perna et al., 2015). The characteristics of donkey milk include low cholesterol, low fat, high proportions of unsaturated fatty acids, and a high concentration of whey protein, with high nutritional levels and medicinal values such as antioxidant and anti-inflammatory (Martemucci et
al., 2012). Donkey milk is believed to be the closest substitute for human milk in terms of its nutritional composition (Martini et al., 2021). The nutritional supply of donkey herds is of great significance for improving their milk production performance and the quality of donkey milk. It is reported that a poorly developed mammary cistern structure is the primary cause of low lactation performance in donkeys (Liang et al., 2022). Further, high metabolism during lactation contributes to the development of oxidative stress (Salari et al., 2021). Female donkeys suffering from oxidative stress experience frequent diseases and suffer from a decline in milk production and quality (Burden et al., 2019). Consequently, improving lactating donkeys’ immune and antioxidant functions is essential to enhancing lactation performance (Ponnampalam et al., 2022).

The use of phytoextracts and active botanical ingredients in animal production has received considerable interest in recent years, mainly due to increased efforts to reduce both the environmental burden of drug use and antimicrobial resistance (Nannoni et al., 2023). Alternative feed additives, such as plant extracts, have been introduced as potential antibiotic replacements and nutritional strategies to prevent ruminant metabolic disorders (Giorgino et al., 2023). Artemisia ordosica crude polysaccharide (AOCP) is isolated from Artemisia ordosica Krasch (A. ordosica, Compositae family, Artemisia genus), a traditional Chinese/Mongolian medicine. A. ordosica is a prominent and representative plant in dry East Asian areas, especially in the north and northwest of China, such as Inner Mongolia, Ningxia, and the rest. Regardless, the capacity of A. ordosica to exert a variety of pharmacological effects is primarily due to the presence of its bioactive substances, such as polysaccharides, flavonoids, terpenoids, and sterols (Bisht et al., 2021). Studies have demonstrated that A. ordosica increased CAT activity in broilers, reduced serum MDA levels in weaned piglets in a linear or quadratic dose-dependent manner, increased milk yield and milk lactose yield in dairy cows, and promoted intestinal health in rats (Xing et al., 2023; Xing et al., 2019; Yu et al., 2021; Xing et al., 2020). However, its application to donkeys has yet to be reported. Consequently, it is necessary to investigate the effects of AOCP on lactation performance, antioxidant levels, and immunological functions and Mechanism in lactating donkeys. A study conducted by Li et al. (2023) found that probiotics can modulate gut microbial structure, increase metabolite-derived SCFAs, enhance antioxidant status, and reduce inflammation in short-haired domestic cats, thereby promoting gut health. Based on the findings of Wang et al. (2023) in dairy goats, lactation performance involves a variety of biological processes, including rectal microorganisms and a variety of metabolic processes that are regulated. As a result, the role of the rectal microbiome, driven by essential bacteria and key metabolites, in regulating phenotype-related metabolic pathways is vital when considering the microbiome-metabolome axis that influences the milk performance of lactating donkeys (Wang et al., 2023).

In our study, it is hypothesized that AOCP has a promotive effect on milk performance and antioxidant and immune functions in lactating donkeys, and that the mechanism may be related to changed bacteria and metabolites. Consequently, the present research was conducted to verify the role of AOCP in improving lactation performance and regulating the “microbiome-metabolome” axis by integrating multiple omics technologies, including rectal microbiomes and serum metabolomics, and to provide a basis for the comprehensive utilization of A. ordosica plant resources in the feed field and to provide novel ideas for nutritional studies of donkeys.

**MATERIALS AND METHODS**

**Animal Ethics Statement**

This study was conducted in Yulv Grassland, China (40.4°N and 111.6°E). A temperate continental monsoon climate characterizes the climate of the region. The Animal Ethics and Welfare Committee approved the experimental procedures at the Inner Mongolia Agricultural University (NND2022050), which were under the university’s guidelines for animal research.

**Preparation of Artemisia Ordosica Crude Polysaccharide.** Fresh A. ordosica (aerial part) was harvested from Erdos (40.4°N, 110.03°E, Inner Mongolia, China) in August. Raw materials were washed with distilled water and dried at room temperature. AOCP was prepared using the method described by Xing et al.(2020). This process can result in obtaining 55.6g of A. ordosica crude polysaccharide per kilogram of raw A. ordosica material. Di et al. (2017) reported that the total polysaccharide content of A. ordosica crude polysaccharide was 52.65%, based on the anthranilic sulfate method, with glucose as the reference standard. The gallic acid in 53% ethanol was used as a standard for determining the total phenolic content, which contained 0.11%. Gel permeation chromatography coupled to multi-angle laser light scattering, a refractive index detection system and ion-exchange chromatography were used to determine the characterization of AOCP. These results indicated that AOCP, with a molecular weight of 2.1 kDa (62.6%) and 1.5 kDa (37.4%), had narrow polydispersity and rod conformations and was composed of arabinose, galactose, glucose, xylose, man-
Experimental Design and Treatments

Based on a one-way, completely randomized design, a total of 14 lactating Dezhou donkeys (n = 14) with similar age (6.16 ± 0.67 years, mean ± SD), weight (250.06 ± 25.18 kg), days in milk (39.11 ± 7.42 d), and averaged parity of 3 were selected and randomly divided into 2 groups with 7 donkeys in each block. Blocks were randomly assigned into 2 treatments, basal diets without (control, CON) or with A. ordosica crude polysaccharide (AOCP) at 1.0 g/kg DM. The supplemented dose of AOCP was based on our previous study (Xing et al., 2021). Donkeys were fed libitum. Donkeys were fed with concentrate, corn silage, and alfalfa daily at 7:00 a.m. and 2:00 p.m. 1.0 g/kg DM AOCP was mixed with 200 g of corn silage, then top-dressed on the rest of the corn silage in the morning. Millet straw was fed 5 times a day. Table 1 shows the feed ingredients and nutrient composition (with a concentration-to-forage ratio of 30:70). Ten weeks were allotted for the experiment, 2 weeks for adaptation (pretrial period), and 8 weeks for collecting data and samples (experimental period). The donkeys were individually housed in a stall (3.5m x 2.0 m) with their foal (1.6m x 2.0 m) and fed twice daily at 7:00 a.m. and 2:00 p.m. The foals were isolated from their mothers from 7:00–10:00 a.m. and 2:00–5:00 p.m. every day, and the rest of the time they spent with the mother suckling naturally. Water was supplied ad libitum.

Sample Collection. The donkeys were milked 2 times daily (10:00 a.m. and 5:00 p.m.), and their milking yields were recorded during each milking. Milk samples were collected on the last day of the week, and the morning and afternoon samples were mixed in a 1:1 ratio. Preservatives (Bronopol Table, D & F Control System, San Rannon Inc., Dublin ON, Canada) were added and stored at 4°C. During the eighth week of the experiment, 200 g of rectal feces from donkeys were collected consistently over 6 consecutive days. As part of the evaluation of the nutrient digestibility of the product, it was dried at 65°C for 72 h, pulverized, and ground through a 1 mm screen. During the final day of the experiment, rectal feces specimens from lactating donkeys were collected using sterile gloves, stored in DNase and RNase-free tubes (Shanghai Jingke Chemical Technology Co., Ltd., Shanghai), and immediately frozen in liquid nitrogen (−196°C) and used for short-chain fatty acid (SCFAs) and 16S microbiomics. Blood samples were collected on the last day of the experiment. Following puncturing the jugular vein of the donkey, blood was collected using 10 mL non-anticoagulated vacuum tubes (Corning, Corning Inc., Costar, NY). Blood collection tubes were placed on ice, stood, and layered. Then, they were centrifuged at 4°C and 2054.3 × g for 15 min and separated into serum, and one portion was collected into 2 mL cryotubes (Corning, Corning Inc. Costar, NY). These cryotubes were snap-frozen and transferred to an ultra-low-temperature refrigerator to be analyzed using metabolomics at −80°C. The other portion of the sample was stored at −20°C for antioxidants, immunology, and biochemistry measurements.

Milk Performance. The feed offered and refused was weighed daily to calculate each group’s daily matter intake (DMI). The quantities of refused diet were approximately 5% to 10% of the total. From 7:00 a.m. to 10:00 a.m. and 2:00 p.m. to 5:00 p.m. daily, the donkeys were separated from their foals for 3 h each time, for a total of 6 h per day to allow for milk collection. Milking with an individual vacuum pumping machine (JuduH5402, Judu Technology, Hebei) was performed daily at 10:00 a.m. and 5:00 p.m., and the donkey milking yield (MY, kg/day) was recorded. In our study, foals were allowed to suckle freely for 3 h after each milking, so the amount of milk recorded twice a day was added together and assigned as the milking yield of lactating donkey for the day (Li et al., 2013). Milk samples were collected on the last day of each week. Morning and afternoon milk samples were blended and analyzed for protein, fat, lactose, solids-not-fat (SNF), and total solids (TS) using an automatic milk analyzer with mid-infrared waveband (Milkoscan FT +, Foss Analytical Co., Ltd., Hillerød, Denmark). The estimated milk yield (EMY, kg/day) was calculated from milking yield measurements. As the foals were allowed freely to suckle with their mothers after milking, the daily milk yield was estimated by dividing the milk yield by the proportion of milking isolation time (6 h) to 24 h (Liang et al., 2022). Solids-corrected milk (SCM, kg/day) = {(12.3 × milk fat (%) content of non-standard milk + 6.56 × solid-not-fat (%) content of non-standard milk − 0.0752) × Estimated milk yield (kg/day)} (Yue et al., 2012), energy-corrected milk (ECM, kg/day) = 0.327 × EMY (kg/day) + 12.95 × fat (kg/day) + 7.65 × protein (kg/day) (Zhou et al., 2015). The milk production efficiency was expressed as follows: EMY (kg/day)/DMI (kg/day), SCM (kg/d)/DMI (kg/day), and ECM (kg/day)/DMI (kg/day). Milk protein synthesis efficiency = {EMY (kg/day) × milk protein (%)}/DMI (kg/day) × CP level (%)(Leiber et al., 2015).

Nutrient Digestibility. Samples of feed and feces were analyzed in duplicate for dry matter (DM, NO.967.03), crude protein (CP, NO.954.01), and ether extract (EE, NO.920.39) based on the Association of Official Analytical Chemists (AOAC) methods (Hel-
Rectal microbiomes and serum... Li et al.

rich, 2006). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined based on the method described by Soest et al. (1991), which were analyzed using an ANKOM A2000i fiber analyzer (ANKOM Technology, New York, NY). The concentrations of calcium and phosphorous were analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (iCAP6300, Thermo Fisher, New York, NY) for feed samples. Acid-insoluble ash (AIA) was used to determine the apparent total-tract digestibility (ATTD) of a certain nutrient according to the description of Keulen et al. (1977) using the following formula:

\[
\text{ATTD} (%) = 100 - \frac{(A \times B)}{(A1 \times B1)} \times 100.
\]

Where A is the content of a given nutrient in the diet (%), A1 is the content of the same nutrient in the feces (%), B is the content of AIA in the diet (%), and B1 is the content of AIA in the feces (%).

**Serum Antioxidants, Immune Cytokines, and Biochemistry.** The serum contents of total antioxidant capacity (T-AOC, A015–1–2), glutathione peroxidase (GSH-Px, A005–1–2), superoxide dismutase (SOD, A001–1–2), catalase (CAT, A007–1–1), malondialdehyde (MDA, A003–1–2), nitric oxide (NO, A012–1–2), inducible nitric oxide synthase (iNOS, A014–1–2), albumin (ALB, A028–2–1), glucose (GLU, A154–1–1), total cholesterol (T-CHO, A111–1–1), urea (UREA, C013–2–1), and total protein (TP, A045–4–2) were measured using the standard commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing). The serum levels of interleukin –13 (IL-13), interleukin –4 (IL-4), interleukin –10 (IL-10), and tumor necrosis factor-α (TNF-α) were determined by ELISA test kits (Baoman Biological Technology Co., Ltd., Shanghai), following the manufacturer’s instructions. The serum concentrations of immunoglobin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin-2 (IL-2), interleukin-6 (IL-6), interferon-γ (IFN-γ), and reactive oxygen species (ROS) were measured using the standard commercial kits (Beijing sinouk institute of biological technology, Beijing).

**SCFAs Analysis of the feces.** SCFAs include acetate, propionate, iso-butyrate, butyrate, isovalerate and valerate. The measurement of these SCFAs followed a previously described method (Xie et al., 2023). In brief, 1.5 g of rectal feces was weighed, and 3 mL of saline solution was added, vortexed, and centrifuged at 2054.3 (× g) for 15 min. A sample of 1 mL of the supernatant was collected in an EP tube containing 25% metaphosphoric acid solution containing 2 g/L of the internal standard (2-ethylbutyric acid). This experiment’s solution was centrifuged at 11100 (× g), 4°C for 20 min. The supernatant was filtered through a 0.22-mm membrane into the injection vial for SCFA determination. For quantification of purified SCFAs, a Shimadzu 2014 gas chromatograph (Agilent Technologies, Santa Clara, CA) with helium as the carrier gas was used along with a DB-FFAP column (60 m x 0.25 mm x 0.5 m). The column, detector, and injector temperatures were 120°C, 250°C, and 220°C, respectively. Quantification of SCFAs was completed by comparison against known standards (Supelco Volatile Fatty Acid Standard Mix, Sigma-Aldrich, St. Louis, MO) containing acetate, propionate, iso-butyrate, butyrate, isovalerate, and valerate.

**Rectal Microbiome Analysis.** Rectal feces were collected from all lactating donkeys on the last day of the experiment. For DNA extraction, feces samples were thawed on ice. DNA was extracted using an E.Z.N.A.® soil DNA kit (Omega Bio-Tek Norcross, GA), which includes a bead-beating technique. A sample of 0.5 g of rectal feces was transferred to a 2 mL tube that contained 0.5 g of sterile 0.1 mm zirconia beads (BioSpec, OK) and 1 mL of SLX-Mlus Buffer. Samples were bead-beaten using a smashed grinder (TL-48R, Wan Bai Biotechnology Co., Ltd., Shanghai), operating at 45 Hz for 250 s. Following the manufacturer’s instructions, the sample was processed, and a DNA sample was obtained. Analyses were conducted using a 1% agarose gel to determine the concentration and purity of the

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

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millet straw</td>
<td>33.97</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>23.55</td>
</tr>
<tr>
<td>Corn silage</td>
<td>12.49</td>
</tr>
<tr>
<td>Corn</td>
<td>15.19</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>8.55</td>
</tr>
<tr>
<td>Corn germ meal</td>
<td>1.80</td>
</tr>
<tr>
<td>Distillers dried grains with solubles</td>
<td>1.80</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>0.90</td>
</tr>
<tr>
<td>Nac</td>
<td>0.39</td>
</tr>
<tr>
<td>CaCO3</td>
<td>0.21</td>
</tr>
<tr>
<td>CaHPO4</td>
<td>0.66</td>
</tr>
<tr>
<td>Premix1</td>
<td>0.50</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Nutrient levels

- Digestible energy (MJ/Kg) × 2
- Dry matter
- Crude protein
- Ether extract
- Neutral detergent fiber
- Acid detergent fiber
- Calcium
- Phosphorous

1Provided per kg of premix: vitamin A 1,200,000 IU, vitamin D 250,000 IU, vitamin E 3,000 IU, Fe 4.0 g, Cu 1.6 g, Zn 12 g, Mn 12 g, I 72 mg, Se 60 mg, Co 100 mg.

2 DE was calculated value according to the Chinese Feed Ingredients and Nutritional Value Table (30th edition).
DNA extracts. ABI GeneAmp® 9700 PCR thermal cycler (ABI, Waltham, MA) was used to amplify the 16S rRNA region of bacterial V3 and V4 using primers 338F (GGACTACHVGGGTWTCTAAT) and 806R (GGACTACHVGGGTWTCTAAT). It is important to note that the PCR products obtained were identified by electrophoresis on an agarose gel (2%), purified by a DNA gel extraction kit (Axygen Biosciences, Union City, CA), and quantified by a fluorometer (Quantis™ Fluorometer (Promega, Madison, WI)). Following OTU classification (similarity level of 97%) using UPARSE (version 7.0.1090, http://drive5.com/uparse/), the following analysis was performed. Alpha-diversity analysis (https://www.mothur.org/wiki/Download _mothur, (accessed on 24 September 2023)), Venn diagrams (R language, version 3.3.1, (accessed on 24 September 2023)), β-diversity analysis (http://qiime.org/install/index.html, (accessed on 24 September 2023)), Community composition analysis, Test of significant difference between groups(at the level of the phylum family, and genus, (accessed on 24 September 2023)), LEfSe analysis, and correlation coefficients between environmental factors and remarkable genera (Spearman rank correlation coefficient, (accessed on 30 September 2023)) were calculated, and the resulting numerical matrix was presented through heatmaps. Colors indicate the magnitude of the data values in a matrix or table. Both calculations and visualizations can be done using the vegan package in R. (version 3.3.1). The raw sequencing data are available at national center for Biotechnology Information database (Bio Project ID: PRJNA1054228).

**Untargeted Liquid Chromatography-MS Metabolomic analysis.** Metabolomic analysis of the plasma samples was performed by liquid chromatography (LC)-mass spectrometry (MS) (Thermo Scientific, Vanquish Horizon UHPLC System-Q Exactive HF-X) platform. In brief, 100 µL of serum samples were precisely transferred into a 1.5 mL centrifuge tube. A 0.02 mg/mL concentration of 2-chloro-l-phenylalanine solution was added as the internal standard, typically obtained by mixing equal volumes of methanol and acetonitrile. The prepared solution was vortex-mixed for 30 s and then subjected to low-temperature ultrasound extraction for 30 min (5°C, 40 KHz). In the following steps, the ultrasonic extract was allowed to stand at −20°C for 30 min, centrifuged at 4°C for 15 min at 13000 ( × g), and the supernatant was collected and dried under nitrogen. Upon nitrogen drying, a reconstitution solution was prepared by mixing 100 µL of acetonitrile and water at a ratio of 1:1, and the complex solution was aspirated for reconstitution after drying with nitrogen. An ultrasound probe was used for 5 min at 5°C, 40 KHz, followed by centrifugation for 10 min at 4°C, 13000 ( × g). After centrifugation, the supernatant was transferred to a lined injection bottle for the LC-MS analysis.

The experimental procedures were analyzed according to the standard protocol of Majorbio Bio-pharma Platform (Majorbio Bio-pharma Technology Co., Ltd., Shanghai). It employed an ACQUITY UPLC HSS T3 column (100 mm x 2.1 mm in diameter, 1.8 µm; Waters, Milford). Two solutions were used as the mobile solvent: solvent A consists of 95% water and 5% acetonitrile (containing 0.1% formic acid), and solvent B consists of 47.5% acetonitrile, 47.5% isopropanol, and 5% water (containing 0.1% formic acid). The serum injection volume was 2 µL, and the column temperature was 40°C. Mass spectrometer (MS) was carried out in positive - and negative-ion modes. The identification and screening of metabolites were obtained by matching MS and MS/MS information with reliable biochemical databases, such as the human metabolome database (http://www.hmdb.ca, (accessed on 25 September 2023)) and METLIN database (https://metlin.scripps.edu, (accessed on 25 September 2023)). Metabolites pathway analysis was performed on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/, accessed on 25 September 2023). Furthermore, the Majorbio Cloud Platform (https://cloud.majorbio.com, accessed on 25 September 2023) was applied as the data analysis tool.

**Statistical Analysis.**

All statistical analyses were performed using SAS software (version 9.2, SAS Institute Inc., Cary, NC). Treatment effects of DMI, yields of milk, milk production efficiency, milk protein synthesis efficiency, milk components, and yields of milk protein, fat, lactose, solid-not-fat, and total solids were analyzed using the PROC MIXED procedure as follows:

\[ Y_{ijkm} = \mu + C_i + W_j + C_i W_j + bX_{jk} + Sim + \varepsilon_{ijkm}, \]

Where \( Y_{ijkm} \) = the dependent variable; \( \mu \) = overall mean; \( C_i \) = fixed effect of dietary AOCP levels; \( W_j \) = fixed effect of lactation week (wk 1, 2, 3, 4, 5, 6, 7, and 8), \( C_i W_j \) = effect of the interaction between diet treatment and lactation week, \( bX_{jk} \) = effect of covariate (wk 0, the observations during the 2 weeks of pretrial period served as covariates for the corresponding experimental period), \( Sim \) = a random effect (individual donkey); \( \varepsilon_{ijkm} \) = residual error. Means were separated by using the PDIFF option in the LSMEANS statement. The nutrient digestibility, antioxidative indices,
inflammatory cytokines, and biochemical indicators in serum were analyzed using the T-Test procedure on normally distributed data, otherwise using the Kruskal-Wallis test. Kruskal-Wallis rank sums were utilized to analyze differences between phyla and families at the rectal bacteria level. Differences in the rectal microbial abundance of donkeys at the genus level were classified via LEfSe analysis if the LDA score of the rectal microbiota exceeded 3. Differentially expressed metabolites were screened based on a fold change ($FC > 1$ and $P < 0.05$. Significant differences were defined as $P$-value $<0.05$, while $0.05 \leq P$-value $<0.10$ were considered to have a trend toward significance.

RESULTS

Milk Performance

The effects of A. ordosica crude polysaccharides on the milk performance of lactating donkeys are shown in Table 2. In the AOCP group, DMI ($P < 0.001$), milking yield ($P = 0.002$), EMY ($P = 0.002$), SCM ($P < 0.001$), and ECM ($P < 0.001$) contents were higher than in the CON group. The AOCP group achieved a higher milk fat ($P = 0.018$), protein ($P < 0.001$), lactose ($P = 0.002$), SNF ($P = 0.001$), and TS ($P = 0.001$) yield than the CON group. It was found that there was a trend of increased milk lactose ($P = 0.075$) content in the AOCP group compared with the CON group. The efficiency of milk protein synthesis, milk production efficiency (EMY/DMI, SCM/DMI, and ECM/DMI), and the components of fat, protein, SNF, and TS showed no difference between the CON and AOCP groups ($P > 0.05$).

Nutrient Digestibility

Table 3 shows the effects of crude polysaccharides from A. ordosica on lactating donkeys’ nutrient digestibility. The apparent digestibility of DM ($P = 0.014$), CP ($P = 0.001$), NDF ($P = 0.018$), and ADF ($P = 0.024$) was increased in the AOCP group compared with the CON group ($P < 0.05$). There was no difference in EE apparent digestibility between the CON and AOCP groups ($P = 0.144$).

Serum Antioxidative Status, Inflammatory Cytokines, Immunoglobulin, and Biochemical Parameters

As shown in Table 4, SOD ($P = 0.014$) and CAT ($P = 0.021$) activities and T-AOC ($P = 0.002$) enzyme in serum were higher in the AOCP group than in the CON group. Compared with the CON group, the MDA level was lower in the AOCP group ($P = 0.004$). There was no significant difference in GSH-Px enzyme activity between the CON and AOCP groups ($P = 0.424$).

The effects of A. ordosica crude polysaccharides on serum inflammatory cytokines in lactating donkeys are shown in Table 4. The contents of IgA ($P = 0.029$), IgG ($P = 0.019$), and IgM ($P = 0.009$) were increased in the AOCP group compared with the CON group. The concentrations of TNF-α ($P = 0.008$), NO ($P < 0.001$), and ROS ($P = 0.016$) in the AOCP group were lower than in the CON group. There was a tendency for IL-1β ($P = 0.084$) and iNOS ($P = 0.093$) concentrations to decrease in the AOCP group compared with the CON group. The contents of IL-2, IL-4, IL-6, IFN-γ, and IL-10 showed no difference between the CON and AOCP groups ($P > 0.05$).

As shown in Table 4, ALB ($P = 0.003$) and TP ($P = 0.002$) levels are higher in the AOCP group than in the CON group. The GLU ($P = 0.034$) and T-CHO ($P < 0.001$) concentrations were lower in the AOCP group than in the CON group. There was no difference in UREA concentrations between the CON and AOCP groups ($P = 0.209$).

SCFAs in the Rectal Feces

As shown in Table 5, Propionate ($P = 0.007$), Butyrate ($P = 0.027$), Isovalerate ($P = 0.003$), and Total VFA ($P = 0.025$) levels are higher in the AOCP group than in the CON group. There was a tendency for Acetate ($P = 0.072$) concentrations to be increased in the AOCP group compared with the CON group. There was no difference in Isobutyrate ($P = 0.100$) and Valerate ($P = 0.369$) concentrations between the CON and AOCP groups.

Rectal Bacterial Community Richness, Diversity and Composition

According to the α-diversity analysis, community coverage reached 98% for all 2 test groups (Figure 1A), indicating that rectal microorganisms’ species and structural diversity could be accurately assessed. It can be concluded that the Shannon index of the AOCP group was higher than that of the CON group. In contrast, there were no significant differences in the richness indices among the 2 experimental groups, suggesting that AOCP increased the diversity of microbial communities in the rectal region of lactating donkeys. The number of shared and unique species across several groups or samples can be counted using Venn diagrams. In this experiment, non-repeat sequences were clustered using OTUs based on 97% similarity, which produced 2886 OTUs. Of these, 298 and 360 unique OTUs were found in the CON and AOCP groups of rations, re-
spectively, making a total of 2228 OTUs between the 2 experimental groups (Figure 1B). Principal coordinate analysis (PCoA) based on the Bry-Curtis distance matrix revealed that the AOCP group’s rectal microbial samples had more variation than the CON group’s, with PC1 and PC2 accounting for 22.54% and 26.51% of the total variation, respectively (Figure 1C), indicating that there were differences in rectal microbial species.

Significantly Different Rectal Bacteria Between the CON and AOCP groups

Rectal microorganisms were analyzed at the phylum and family levels. At the phylum level, Firmicutes and Bacteroidetes were the dominant species in the rectal flora, making up more than 98% of all species (Figure 1D). The relative abundance was 68% and 19% in the CON group and 63% and 22% in the AOCP group, respectively. Additionally, the AOCP group had a lower Firmicutes to Bacteroidetes ratio than the CON group.

Changes in microbial communities at the phylum, family, and genus levels in the CON and AOCP groups are shown in Figure 2. At the phylum level, Fibrobacterota relative abundance was higher in the AOCP group than in the CON group (Figure 2A). At the family level, the relative abundance of Ruminococcaceae, Lactobacillaceae, Fibrobacteraceae, and M2PB4-65_tera...
mite_group was higher in the AOCP group than in the CON group. However, the relative abundance of Ethanoligenenaceae and Burkholderiaceae was lower in the AOCP group than in the CON group (Figure 2B). At the genus level, the AOCP group had a higher relative abundance of Lactobacillus, Ruminococcus, unclassified_f_Prevotellaceae, Fibrobacter, Blautia, norank_f_Atopobiaceae, and norank_f_M2PB4--65_termite_group relative abundance compared with the CON group. In contrast, the AOCP group had a higher relative abundance of Clostridium_sensu_stricto_1, Mogibacterium, norank_f_Synergistaceae, Lachnospiraceae_ND3007_group, norank_f_Ethanoligenenaceae, Mucinivorans, unclassified_f_Rikenellaceae, and Burkholderia-Caballeronia-Parabukholderia compared with the CON group (Figure 2C).

A LEfSe analysis was performed to identify different bacterial compositional differences between the 2 treatments of rectal in lactating donkeys (Figure 2D). A total of 7 OTUs were obtained in 12 rectal fecal samples, with 2 OTUs in the CON group, including Clostridium_sensu_stricto_1, Mogibacterium; Within the AOCP group, Lactobacillus, Ruminococcus, unclassified_f_Prevotellaceae, Fibrobacter, Blautia, norank_f_Atopobiaceae, and norank_f_M2PB4--65_termite_group relative abundance compared with the CON group (Figure 2C).

Table 4. Effects of AOCP on antioxidant capacity, immune cytokines, and biochemical parameters of serum in lactating donkeys

<table>
<thead>
<tr>
<th>Item</th>
<th>CON1</th>
<th>AOCP1</th>
<th>SEM2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum antioxidative indicators</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>69.35b</td>
<td>73.32a</td>
<td>1.161</td>
<td>0.014</td>
</tr>
<tr>
<td>CAT (U/mL)</td>
<td>2.40b</td>
<td>2.98a</td>
<td>0.186</td>
<td>0.021</td>
</tr>
<tr>
<td>T-AOC (U/mL)</td>
<td>1.89b</td>
<td>2.08a</td>
<td>0.037</td>
<td>0.002</td>
</tr>
<tr>
<td>GSH-Px (U/mL)</td>
<td>507.64</td>
<td>552.86</td>
<td>29.379</td>
<td>0.424</td>
</tr>
<tr>
<td>MDA (mmol/mL)</td>
<td>0.92b</td>
<td>0.82a</td>
<td>0.023</td>
<td>0.004</td>
</tr>
<tr>
<td>Serum inflammatory cytokines and immunoglobulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>200.33</td>
<td>114.29</td>
<td>41.503</td>
<td>0.084</td>
</tr>
<tr>
<td>IL-2 (pg/mL)</td>
<td>175.93</td>
<td>180.82</td>
<td>2.623</td>
<td>0.112</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>2.423</td>
<td>2.860</td>
<td>0.273</td>
<td>0.161</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>126.44</td>
<td>118.14</td>
<td>6.940</td>
<td>0.277</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>37.90b</td>
<td>21.90a</td>
<td>4.150</td>
<td>0.008</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>1.840</td>
<td>2.067</td>
<td>0.424</td>
<td>0.611</td>
</tr>
<tr>
<td>INF-γ (pg/mL)</td>
<td>37.58</td>
<td>37.04</td>
<td>0.727</td>
<td>0.489</td>
</tr>
<tr>
<td>iNOS (U/mL)</td>
<td>14.63</td>
<td>13.80</td>
<td>0.419</td>
<td>0.093</td>
</tr>
<tr>
<td>NO (µmol/L)</td>
<td>221.62b</td>
<td>169.86a</td>
<td>6.350</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>3.00b</td>
<td>3.76a</td>
<td>0.294</td>
<td>0.029</td>
</tr>
<tr>
<td>IgG (g/L)</td>
<td>12.13b</td>
<td>15.64a</td>
<td>1.098</td>
<td>0.019</td>
</tr>
<tr>
<td>IgM (g/L)</td>
<td>1.34b</td>
<td>1.67a</td>
<td>0.092</td>
<td>0.711</td>
</tr>
<tr>
<td>ROS (Fluorescence intensity/mL)</td>
<td>300.29b</td>
<td>262.32a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum biochemical parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>28.42b</td>
<td>33.88a</td>
<td>1.148</td>
<td>0.003</td>
</tr>
<tr>
<td>GLU (mmol/L)</td>
<td>3.35b</td>
<td>3.43a</td>
<td>0.059</td>
<td>0.034</td>
</tr>
<tr>
<td>UREA (mmol/L)</td>
<td>6.04</td>
<td>5.73</td>
<td>0.222</td>
<td>0.209</td>
</tr>
<tr>
<td>T-CHO (mmol/L)</td>
<td>2.19b</td>
<td>1.65a</td>
<td>0.025</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>63.03b</td>
<td>71.58a</td>
<td>1.673</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Different superscript letters (a and b) within the same row indicate significant difference between experimental groups (P < 0.05).

1CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP).

2SEM = standard error of the mean.

3ALB = albumin; CAT = catalase; GLU = glucose; GSH-Px = glutathione peroxidase; INF-γ = Interferon-γ; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; IL-10 = interleukin –10; IL-1β = interleukin-1β; IL-2 = interleukin-2; IL-4 = interleukin-4; iNOS = inducible nitric oxide synthase; MDA = malondialdehyde; NO = nitric oxide; ROS = reactive oxygen species; SOD = superoxide dismutase; T-AOC = total antioxidant capacity; T-CHO = total cholesterol; TNF-α = tumor necrosis factor-α; TP = total protein; UREA = urea phosphate.

Table 5. Effects of AOCP on SCFAs of rectal feces in lactating donkeys

<table>
<thead>
<tr>
<th>Item</th>
<th>CON1</th>
<th>AOCP1</th>
<th>SEM2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (mmol/L)</td>
<td>6.516</td>
<td>7.941</td>
<td>0.654</td>
<td>0.072</td>
</tr>
<tr>
<td>Propionate (mmol/L)</td>
<td>3.141b</td>
<td>3.882a</td>
<td>0.182</td>
<td>0.007</td>
</tr>
<tr>
<td>Butyrate (mmol/L)</td>
<td>0.846b</td>
<td>1.080a</td>
<td>0.080</td>
<td>0.027</td>
</tr>
<tr>
<td>Isobutyrate (mmol/L)</td>
<td>0.195</td>
<td>0.225</td>
<td>0.015</td>
<td>0.100</td>
</tr>
<tr>
<td>Valerate (mmol/L)</td>
<td>0.247</td>
<td>0.258</td>
<td>0.012</td>
<td>0.369</td>
</tr>
<tr>
<td>Isovalerate (mmol/L)</td>
<td>0.225b</td>
<td>0.289a</td>
<td>0.013</td>
<td>0.003</td>
</tr>
<tr>
<td>Acetate: Propionate ratio</td>
<td>2.074</td>
<td>2.049</td>
<td>0.154</td>
<td>0.876</td>
</tr>
<tr>
<td>Total VFA (mmol/L)</td>
<td>11.170b</td>
<td>13.675a</td>
<td>0.847</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Different superscript letters (a and b) within the same row indicate significant difference between experimental groups (P < 0.05).

1CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP).

2SEM = standard error of the mean.
Factors with Rectal Microbiota Digestibility, Antioxidant and Anti-inflammation Biochemical Parameters, Milk Performance, Nutrient Correlations Between Serum Metabolites and Metabolomic Profiles in the Serum and Identification of Metabolites

As shown by OPLS-DA in Figure 3A and Figure 3B, the serum metabolome of the CON and AOCP groups was well separated. Software classification parameters were stable and correlated with fitness and prediction (Figure 3C and Figure 3D). Both positive and negative models contained 14 samples and 5 quality control samples. There were 867 positive peaks and 604 negative peaks (P_value < 0.05 & VIP_pred_OPLS-DA > 1). The positive model identified 43 peaks that discriminated CON from AOCP. There were 24 differential peaks between CON and AOCP in the negative model. There were 67 named differential metabolites (dm) between CON and AOCP, of which 44 dm were downregulated, and 23 dm were upregulated (P_value < 0.05 & VIP_pred_OPLS-DA > 1 and log2 fold change > 0) in the AOCP group compared with CON group (Figure 3E and Figure 3F).

The primary dm between the CON and AOCP groups were sucrose, choline, 9(S)-HODE, L-tyrosine, taurine, pantothenic acid, LysoPC (18:0), LysoPC (18:1(9Z)), and LysoPC (20:2(11Z,14Z)). Metabolome maps showed the pathways based on the dm identified between the CON and AOCP groups (Table 3A). Among the CON and AOCP groups, the PPAR signaling pathway, prolactin signaling pathway, and glycerophospholipid metabolism were significantly, and carbohydrate digestion and absorption, taurine and hypotaurine metabolism, pantetheine and CoA biosynthesis, and tyrosine metabolism were also enriched (Figure 3G).

Correlations Between Serum Metabolites and Biochemical Parameters, Milk Performance, Nutrient Digestibility, Antioxidant and Anti-inflammation Factors with Rectal Microbiota

A correlation heatmap was constructed using Spearman’s correlation coefficient to examine the relationship between milk composition, digestibility of nutrients, serum antioxidant status, immune cytokines, biochemical parameters, SCFAs, rectally differentiated bacteria, and serum metabolites. According to the results, beneficial bacterial abundance (unclassified_f__Prevotellaceae, norank_f__M2PB4–65_termite_group, and Fibrobacter) negatively correlated with the above indicators. Moreover, downregulated metabolites (Choine, LysoPC(18:1(9Z)), LysoPC(20:2(11Z,14Z)), LysoPC(18:0), Sucrose, and 9(S)-HODE) were negatively correlated with beneficial bacterial abundance (Figure 4D), immunity (Figure 4F), nutrient digestibility (Figure 4F), biochemical parameters (Figure 4F), SCFAs (Figure 4E), milk composition (Figure 4G), and positively correlated with potentially pathogenic bacteria. Conversely, upregulated metabolites (L-tyrosine and pantothenic acid) were negatively correlated with potentially pathogenic bacteria and positively correlated with beneficial bacteria and the above indicators. It remains unclear whether the rectal microflora directly metabolizes these metabolites and whether it affects the donkey’s antioxidant and immune status or milk composition. Nevertheless, our results suggest an interaction between the addition of AOCP to lactating donkeys’ diets, milk composition, nutrient digestibility, serum antioxidant status, immune cytokines, biochemical parameters, SCFAs, rectal microbes, and serum metabolites.

DISCUSSION

Increased milk production was found to be related to increased nutrient digestibility (Daniel et al., 2020). Adding Macleaya cordata extract to dairy goats improved milk production by increasing fiber digestibility and energy efficiency (Ling et al., 2023). The current study showed that AOCP supplementation increased milk performance and milk fat and protein production, which may be partly attributed to the increased digestibility of nutrients, including DM, CP, NDF, and ADF, and improved DMI in lactating donkeys.

As Barszcz et al. (2018) demonstrated, plasma concentrations of TP and ALB are closely linked to protein synthesis in the body. Our study showed that TP and ALB levels increased in serum after dietary supplementation with AOCP in lactating donkeys, partly explaining why AOCP promoted milk protein synthesis.

A stable intestinal microflora plays a crucial role in digestion and absorption, directly affecting a host’s health (Liu et al., 2018). Several butyrate-producing microorganisms, including Ruminococcus and Lactobacillus, are implicated in fiber degradation, modulating immunity, and playing a pivotal role in food digestion and absorption (Uddin et al., 2023; Taweechotipatr et al., 2009; Siddiqui et al., 2021). In our study, the Ruminococcus genus was positively correlated with NDF digestibility, and the Lactobacillus genus was positively correlated with NDF and ADF digestibility. The Fibrobacter genus was mainly involved in fiber degradation to generate SCFAs (Neumann, 2018). As members of...
Figure 1. Rectal microbiota compositional profiles of CON and AOCP donkeys. (A) A comparison of a-diversity indices between 2 dietary treatments. (B) Venn diagram of the OTUs in the rectal microbiota; (C) principal-coordinate analysis (PCoA). The relative abundances of rectal bacteria are at the (D) phylum and (E) family levels. ab Means that 2 groups that do not have a common marked letter differ significantly (P < 0.05). CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP). Error bars represent SEM.
Li et al.: Rectal microbiomes and serum…

Figure 2. Differential bacterial compositional functions between CON and AOCP donkeys. Differential bacterial compositions at the (A) phylum, (B) family, and (C) genus levels using 16S rRNA sequence data based on the Wilcoxon rank sum test. LefSE analysis of rectal microbiota among 2 dietary treatments. (D) Linear discriminant analysis (LDA) value distributed histogram, and the score ≥ 3 means significant. CON = control (basal diet); AOCP = *Artemisia ordosica* crude polysaccharide (basal diet with 1.0 g/kg DM AOCP).
Figure 3. Serum metabolome profiles of CON and AOCP donkeys. Orthogonal partial least squares discrimination analysis (OPLS-DA) following (A) positive ion electrospray ionization (ESI+) and (B) negative ion electrospray ionization (ESI-). OPLS-DA substitution test follows (C) positive ion electrospray ionization (ESI+) and (D) negative ion electrospray ionization (ESI-). Identification of the differentially abundant metabolites following (E) positive ion electrospray ionization (ESI+) and (F) negative ion electrospray ionization (ESI-). Red represents an upregulation, blue represents a downregulation, and gray represents no change. (G) Metabolic pathway enrichment analysis of the differentially presented compounds between CON versus AOCP groups. CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP).
the phylum Bacteroidetes, Unclassified_f_Prevotellaceae is not only responsible for the degradation of fibers and semi-fibers but is also capable of breaking down polysaccharides, producing SCFAs, and participating in immunomodulation (Wang et al., 2021). It was found that Unclassified_f_Prevotellaceae and Fibrobacter genus were positively correlated with CP digestibility. According to Chen et al. (2022), the distribution and expression of Clostridium_sensu_stricto_1 are closely related to the intestinal bacteria composition and SCFA concentrations in patients with diarrheal gastrointestinal disorders, which may be used as biomarkers of irritable bowel syndrome. In our study, the genus Clostridium_sensu_stricto_1 was negatively correlated with NDF digestibility. The current study showed that AOCP supplementation improved nutrient digestibility (CP, ADF, NDF, and DM) in lactating donkeys, as Xing et al. (2019) observed in piglets. It may be partly attributed to the change in gut microbiota composition, with AOCP promoting colonization by beneficial bacteria (Ruminococcus, Lactobacillus, Unclassified_f_Prevotellaceae, and Fibrobacter) and decreasing colonization by potentially pathogenic bacteria (Clostridium_sensu_stricto_1) colonization, as observed by Li et al. (2023) in goats. Therefore, AOCP improves donkeys' lactation performance by altering

![Figure 3 (Continued).](image)

Table 6. KEGG pathway enrichment and metabolites annotated to the pathway in metabolism between AOCP versus CON groups

<table>
<thead>
<tr>
<th>Metabolic pathways</th>
<th>KEGG ID</th>
<th>Hits</th>
<th>P-value</th>
<th>Upregulated</th>
<th>Downregulated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate digestion and absorption</td>
<td>map04973</td>
<td>1</td>
<td>0.092</td>
<td></td>
<td></td>
<td>Sucrose</td>
</tr>
<tr>
<td>Tyrosine metabolism</td>
<td>map00350</td>
<td>1</td>
<td>0.245</td>
<td>L-Tyrosine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolactin signaling pathway</td>
<td>map04917</td>
<td>1</td>
<td>0.039</td>
<td>L-Tyrosine</td>
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<td>Glycerophospholipid metabolism</td>
<td>map00564</td>
<td>4</td>
<td>0.016</td>
<td></td>
<td>LysotPC(18:0), LysotPC(18:1(9Z))</td>
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</tr>
<tr>
<td>PPAR signaling pathway</td>
<td>map03230</td>
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<td>0.018</td>
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<td></td>
<td>9(S)-HODE</td>
</tr>
<tr>
<td>Pantothenate and CoA biosynthesis</td>
<td>16</td>
<td>1</td>
<td>0.102</td>
<td>Pantothenic Acid</td>
<td></td>
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</tr>
<tr>
<td>Taurine and hypotaurine metabolism</td>
<td>16</td>
<td>1</td>
<td>0.082</td>
<td>Taurine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP).
2Hits represents the number of KEGG compound IDs annotated to pathways in this metabolism.
3The P-value is an uncorrected P-value, and P-values less than 0.05 are considered enrichment terms.
Figure 4. Spearman’s correlation analysis between rectal feces microbiota from genus level by LefSE analysis and (A) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters, (B) SCFAs. (C) milk performance. (D) differential metabolites.

Correlation analysis between differential metabolites and (E) SCFAs. (F) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (G) milk performance. A mechanism map for linking the microbiota and the metabolites involved in the metabolic pathway of enrichment (H). CON = control (basal diet); AOC = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOC). The correlation or difference between groups with significance was represented as ***p < 0.001, **p < 0.05.
Figure 4 (Continued). Spearman’s correlation analysis between rectal feces microbiota from genus level by LefSE analysis and (A) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (B) SCFAs. (C) milk performance. (D) differential metabolites. Correlation analysis between differential metabolites and (E) SCFAs. (F) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (G) milk performance. A mechanism map for linking the microbiota and the metabolites involved in the metabolic pathway of enrichment (H). CON = control (basal diet); AOCPC = * Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCPC). The correlation or difference between groups with significance was represented as ***P < 0.001, **P < 0.05.
Li et al.: Rectal microbiomes and serum…

Figure 4 (Continued). Spearman’s correlation analysis between rectal feces microbiota from genus level by LefSE analysis and (A) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (B) SCFAs. (C) milk performance. (D) differential metabolites. Correlation analysis between differential metabolites and (E) SCFAs. (F) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (G) milk performance. A mechanism map for linking the microbiota and the metabolites involved in the metabolic pathway of enrichment (H). CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP). The correlation or difference between groups with significance was represented as ***P < 0.001, **P < 0.05.
It was found that increased milk production is related to the body’s ability to resist oxidative stress (Banakar et al., 2021). In lactating animals, ROS and reactive nitrogen species (RNS) are metabolites generated during metabolism. Redox imbalance occurs when the body’s antioxidant defenses fail to counteract increased ROS and RNS (Zarkovic et al., 2020). The increased activity, such as SOD, CAT, and GSH-Px, and the decreased the content of MDA exhibited better antioxidants in animals (Zheng et al., 2021). Some cytokines, such as IL-1 and TNF-α, are markers of the inflammatory response of animals: the increase of cytokines can activate the production of iNOS and NO in the inflammatory process, which regulates inflammatory responses (Florescu et al., 2023). The current study showed that AOCP supplementation improved lactating donkeys’ immune systems and antioxidant capacity, which was also one of the reasons why AOCP increased lactation performance, as observed by Yu et al. (2021) in dairy cows.

Alterations in antioxidant and immune status were closely related to the composition of the rectal microbiota and the concentration of their metabolites, SCFAs (Li et al., 2023), which may be attributed to the prebiotic properties of polysaccharides (Li et al., 2020). To evaluate the balance or imbalance of the hindgut microbiota in donkeys, digestive contents are typically collected in the cecum and the colon (Liu et al., 2019). Having no direct access to these gut regions prevents access to their contents, whereas feces are readily accessible. Samples of feces and the bacterial analysis of the feces were used to assess the macrobiotic ecosystem of the hindgut of the equine, as well as metabolites produced by the rectal microbiota which are associated with the antioxidant and immune functions of the host (Grimm et al., 2017; Deledda et al., 2021). Therefore, the feces samples in this study were selected to evaluate the effect of AOCP on the gut microbiota.

As a result of our study, supplementation with AOCP improved antioxidant and immune function in lactating donkeys, which may be partly attributed to changes in the composition of the intestinal microbiota, increasing the abundance of beneficial bacteria (Ruminococcus, Lactobacillus, Unclassified_f_Prevotellaceae, and Fibrobacter) and its metabolites (propionate, butyrate, and isovalerate) concentrations and reducing pathogenic bacteria (Clostridium_sensu_stricto_1) abundance. Similar results have been reported by Shi et al. (2022) in broilers and Yu et al. (2021) in dairy cows. With the increasing relative abundance of Clostridium_sensu_stricto_1, inflammatory factors were secreted (Gu et al., 2021).

Figure 4 (Continued). Spearman’s correlation analysis between rectal feces microbiota from genus level by LefSE analysis and (A) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (B) SCFAs. (C) milk performance. (D) differential metabolites. Correlation analysis between differential metabolites and (E) SCFAs. (F) serum oxidative, inflammatory status, nutrient digestibility, and biochemical parameters. (G) milk performance. A mechanism map for linking the microbiota and the metabolites involved in the metabolic pathway of enrichment (H). CON = control (basal diet); AOCP = Artemisia ordosica crude polysaccharide (basal diet with 1.0 g/kg DM AOCP). The correlation or difference between groups with significance was represented as ***P < 0.001, **P < 0.05.
et al., 2022). The current research also indicated that the *Clostridium_sensu_stricto_1* genus was negatively correlated with propionate, butyrate, and TVFA contents and positively correlated with MDA, NO, and ROS concentrations. These results are consistent with Wei’s study on dairy cows (Wei et al., 2023). Therefore, the effect of AOCP on donkey’s health status and lactation performance through the fecal microbiota and its metabolite SCFAs.

Researchers have identified positive and negative correlations between gut bacteria and metabolite, and gut microbiota affected multiple metabolic pathways (Zhang et al., 2022). Using Pearson correlation analysis, our results found that diet-induced changes in rectal microflora abundance led to changes in the metabolome. Glycerophospholipids are the major lipid component of cell membranes and have a wide range of biological functions in cell differentiation, proliferation, and apoptosis (Mukhopadhyay et al., 2023). LysoPC, a product of fatty acid oxidation, is produced when phosphatidylcholines (PC) are partially hydrolyzed by removing a fatty acid moiety (Sun et al., 2022) as well as inhibiting proinflammatory cell secretion (Li et al., 2020). As Chen et al. (2023) demonstrated, oxidative stress may impair lipid metabolism and prevent the biosynthesis of cell membranes. Additionally, choline is one of the significant components of phospholipids and a major methyl donor in the organism. It is directly involved in polyamine synthesis, promoting the growth of cells and the synthesis of proteins (Zhong et al., 2021). Choline levels may be elevated because of oxidative modifications to phospholipid membranes, disrupting membrane structure, and accumulating membrane constituents. Choline levels may decrease due to the body’s efforts to repair damaged membrane structures, resulting in the depletion of lysoPCs and choline-mediated glycerophospholipid metabolism in our study. A significant decrease in choline content was observed in the AOCP group. There was also a negative correlation between serum metabolite choline and the abundance of *Lactobacillus*, *Ruminococcus*, and the *unclassified_f_Prevotellaceae* genus in rectal flora, as well as the concentrations of TVFA, propionate, and butyrate in rectal contents. Conversely, choline content was positively correlated with NO and ROS concentrations and negatively correlated with IgG content, CP, ALB in serum, and DM digestibility. Thus, AOCP probably reduces oxidative stress by alleviating fatty acid oxidation and promoting cell membrane synthesis. Recently, Sun et al. (2022) found that Fuzhuan brick tea crude polysaccharides were similar to ours in mice. In addition, it was found that choline (Tsukahara et al., 2017) may be involved in the hypoglycemic process as a potential agonist of Peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is a potential target for anti-inflammatory therapies (Reddy et al., 2018). According to recent research, 9(S)-HODEs produced by lipid metabolism contribute to the development and formation of cancer (Jiang et al., 2018). Our research found that 9(S)-HODEs were reduced in the highly enriched PPARγ signaling pathway, with a negative correlation with TVFA and propionate concentrations and a positive correlation with NO levels. As shown above, AOCP supplementation activates the PPARγ signaling pathway and reduces ROS, NOS, and cytokine production. Similar results were reported by Richard et al. (2014) in mice. Carbohydrates are essential for bacterial growth, proliferation, and colonization (Cerqueira et al., 2020). Sucrose, the main product of dietary starch, is finally digested into glucose, which affects the host’s glycolysis (Dai et al., 2022). Decreased glucose levels in glycolytic products lead to insufficient energy being provided by carbohydrates. In response, fatty acid oxidation is increased to meet energy requirements. The accumulation of late glycosylation end products caused by high glucose levels leads to chronic glycosylation of proteins and tissue damage. Interestingly, the sucrose content of AOCP was significantly reduced, suggesting that AOCP has the potential to reduce carbohydrate and glucose production. Various studies have shown that plant polysaccharides have inhibitory effects on starch digestion and have recently received much attention for their antihyperglycemic effects (Yu et al., 2019; Xiao et al., 2019). Tyrosine metabolism was altered by AOCP treatment in the current research. Tyrosine is an essential substrate for milk protein synthesis (Zhang et al., 2022). Our study revealed L-tyrosine enrichment in tyrosine metabolism and the prolactin signaling pathway. Prolactin is necessary to maintain metabolism in mammary cells (Hannan et al., 2021). Tyrosine, through its role in the regulation of prolactin, is a critical factor in the maintenance of lactation as well as the synthesis and secretion of milk fats and proteins (Hannan et al., 2021). It is consistent with donkey lactation performance in our study. Tyrosine metabolism can also result in succinic acid generated in the TCA cycle, affecting oxidative balance and energy metabolism homeostasis (Teodorak et al., 2017). We found that L-Tyrosine levels were negatively associated with the abundance of the *Clostridium_sensu_stricto_1* genus, NO, and ROS concentrations. In contrast, they were positively associated with TVFA, propionate, butyrate concentrations, CP, TP, and milk protein levels. Accordingly, AOCP increased dairy donkeys’ antioxidant capacity and milk protein content. Based on the above analysis, Figure 4H provides a mechanism for linking the microbiota and the metabolites involved in the metabolic pathway of enrichment.
Similarly, Mayengbam et al. (2019) demonstrated that microbiome abundance and composition changes affect host metabolism. However, the causal relationship and mechanisms between the microbiome and metabolome still need further study and exploration.

In summary, dietary supplementation with AOPC increased the diversity of donkey rectal microflora, and their microbial abundance was strongly correlated with metabolite content, antioxidants, immunity, nutrient digestibility, and lactation performance. Based on this finding, AOPC and the gut microbiota were able to reshape the exclusive structure of the microbiome and alter metabolites and metabolic pathways, which promote antioxidant, immune, and nutrient digestibility in donkeys. Consequently, donkey lactation performance is improved. Additionally, this study provides new insights into the potential mechanisms by which AOPC interferes with the “microbiome-metabolome” axis to influence donkey lactation performance. As a result of the findings, specific associated microorganisms and metabolites may be potential targets for modifying animal lactation performance through specific dietary intake.

CONCLUSIONS

Supplementing lactating donkey diets with AOPC (1.0 g/kg DM) promotes antioxidant and anti-inflammatory status, nutrient digestion, and lactation performance. Changes in the composition and metabolites of the gut microflora may be causing this condition. Supplementation of AOPC promotes the colonization of beneficial bacteria while inhibiting pathogenic bacteria and up-regulating and downregulating a range of metabolites in lactating donkeys. Furthermore, Pearson correlation analysis revealed a high correlation between differential bacterial genera and metabolite changes, suggesting that their combined effects may be responsible for increased lactation performance. A new perspective is provided as a result of this study on developing dietary strategies to improve the productivity of donkeys in intensive feeding systems.

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