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Abstract

The periparturient period is associated with metabolic and immunological challenges in sows that may influence lactational performance and piglet growth. Astragalus polysaccharides (APS) are botanical bioactive ingredients with immunomodulatory and metabolic regulatory properties, but their effects on maternal physiological adaptation and mammary responses remain incompletely understood. This study evaluated dietary APS supplementation in 24 multiparous sows receiving either a basal diet or 400 mg/kg APS from 20 days before farrowing until weaning. APS supplementation increased sow feed intake (p = 0.0461) and tended to increase estimated milk yield (p = 0.0808) and piglet average daily gain (p = 0.0864). Mammary alveolar area was greater (p < 0.0001), while serum IgG, IgM, and IL-10 concentrations were higher after BH correction (adjusted p < 0.05); circulating pro-inflammatory cytokines showed no significant between-group differences. Exploratory serum metabolomics suggested increases in 2,5-diaminopentanoic acid, 5-aminovaleric acid, creatinine, and phosphatidylethanolamine species, together with decreases in chenodeoxycholic acid glycine conjugate and hypotaurine. Exploratory mammary transcriptomic analysis highlighted immune-related KEGG pathways, particularly IL-17, TNF, and NF-κB signaling, together with representative genes involved in cytokine and chemokine signaling, epithelial adaptation, and tissue regulation. Pooled exploratory metabolomic-transcriptomic analysis further identified distinct association patterns. Amino acid-related metabolites were mainly associated with immune and chemokine genes, bile acid-related metabolites with immune and xenobiotic-response genes, and phosphatidylethanolamine species with genes involved in mammary cellular activity. Collectively, APS supplementation was associated with enhanced maternal humoral immune status and mammary alveolar development, accompanied by serum metabolic and mammary transcriptional responses that may contribute to maternal physiological adaptation during lactation.

Keywords: Astragalus polysaccharides, Periparturient sows, Mammary transcriptomics, Serum metabolomics, Immune regulation.

Introduction

The periparturient period is characterized by profound physiological, metabolic, and immunological adaptations in sows. During this period, maternal nutrients are extensively repartitioned to support fetal development, mammary gland activation, and milk synthesis, resulting in increased metabolic burden and immune challenges [1, 2]. At the same time, inadequate metabolic adaptation or immune dysregulation during the periparturient period may negatively affect lactational performance, maternal health, and piglet growth [1]. Therefore, nutritional strategies

capable of supporting maternal metabolic homeostasis and immune adaptation during late gestation and lactation are of considerable importance in modern swine production.

Among the physiological systems involved in maternal adaptation, the mammary gland plays a central role in nutrient utilization, milk synthesis, and lactational function. As a highly dynamic secretory tissue, the mammary gland undergoes extensive structural and functional remodeling during the transition from gestation to lactation [3]. This remodeling is characterized by epithelial proliferation, alveolar expansion, and secretory activation, which together determine the capacity for milk production and support the nutritional demands of suckling piglets [3, 4]. In addition to its secretory function, the mammary gland also requires appropriate local immune regulation to maintain tissue integrity and physiological function during lactation [5]. Therefore, nutritional interventions that support mammary structural development and immune homeostasis may contribute to improved lactational performance and maternal health.

During late gestation and lactation, various nutritional supplements are commonly incorporated into sow diets to improve reproductive performance, milk production, immune function, and the growth and health of piglets. Among these, Astragalus polysaccharides (APS), the major bioactive polysaccharides isolated from *Astragalus membranaceus*, have been widely studied because of their immunomodulatory, antioxidant, anti-inflammatory, and metabolic regulatory properties [6]. A previous study in weaned piglets has shown that APS supplementation can improve immune function, antioxidant status, nutrient utilization, and growth performance [7]. In sows, APS supplementation has been reported to enhance colostrum immune components and improve lactation-related traits [8, 9]. In addition, APS has been associated with the regulation of cholesterol metabolism and amino acid availability in pigs [10, 11].

Despite these findings, the mechanisms by which APS influences maternal physiological adaptation during lactation remain incompletely understood. Most previous studies have primarily focused on growth performance, antioxidant capacity, or immune-related parameters [8], whereas relatively limited attention has been paid to maternal metabolic alterations and mammary functional responses under APS supplementation. Moreover, whether APS-associated systemic metabolic changes are coordinated with mammary tissue responses during lactation remains unclear. Integrated multi-omics approaches may therefore provide additional insight into the biological mechanisms underlying APS-mediated maternal adaptation. Therefore, this study investigated the effects of dietary APS supplementation on reproductive and lactational performance, mammary morphology, serum immune indices, serum metabolomic profiles, and mammary transcriptomic responses in periparturient sows. Integrated metabolomic

and transcriptomic analyses were also performed to explore the potential relationships between systemic metabolic alterations and mammary transcriptional responses under APS supplementation. We hypothesized that dietary APS supplementation would support maternal metabolic and immune adaptation and mammary function during the periparturient period.

Materials and Methods

Animal ethics

The experimental protocol was approved by the Animal Ethics Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Ethics Approval Code: IAS2022-112).

Animals and experimental treatments

A total of 24 second-parity Landrace × Yorkshire sows with comparable reproductive status and body condition were included in this study. Backfat thickness was assessed at the P2 site 20 days before the expected farrowing date and ranged from 19 to 22 mm. During late gestation, sows were restrictively fed approximately 2.5 kg of diet per day, whereas feed and water were offered *ad libitum* after farrowing. Piglets remained with their dams and were naturally suckled until weaning. Temperature and humidity in the farrowing room were automatically controlled using ventilation and cooling equipment (Henan Hengyin Automation Technology Co., Ltd., Henan, China).

The 24 sows were randomly allocated to the CON and APS groups (n = 12 per group) using a random-number sequence. Treatment assignments were retained by one investigator and concealed from personnel responsible for animal weighing, sample collection, histological measurements, and laboratory analyses; treatment codes were disclosed for statistical analyses. The APS group received a basal diet supplemented with 400 mg/kg commercial APS powder, whereas the CON group received the basal diet. The APS inclusion level was selected based on our preliminary evaluation in periparturient sows, with reference to previous dietary APS studies in pigs supporting an effective supplementation range that includes 400 mg/kg [12, 13]. Commercial APS powder was supplied by Beijing Centre Biology Co., Ltd. (Beijing, China). According to the manufacturer's certificate of analysis, the product contained 705 mg/g polysaccharides (expressed as glucose equivalents) and met the quality requirements specified in Agricultural and Rural Affairs Announcement No. 2272 [14]. The dietary intervention was initiated 20 days before farrowing and maintained throughout lactation until weaning. Diet composition, expressed on an as-fed basis, is provided in Table S1. Nutrient levels were calculated based on standard feed composition tables [15].

Production performance measurements

Piglets were weaned at 21 days of age. From farrowing to weaning, total litter size, numbers of liveborn, stillborn, and weak piglets, number of piglets at weaning, sow feed intake, and individual piglet body weights were recorded. Weak piglets were defined as liveborn piglets weighing < 1.0 kg at birth [16]. When necessary, cross-fostering was restricted to sows within the same dietary treatment. A total of four piglets in the CON group and eight piglets in the APS group were cross-fostered during lactation. Piglets that died during lactation were recorded and weighed at death. Total litter size was the sum of liveborn and stillborn piglets. Mean birth weight and birth litter weight were based on all liveborn piglets delivered by each sow. Mean weaning weight was calculated only from piglets that remained with their biological dams throughout lactation and survived to weaning, excluding piglets fostered into or out of the litter. Piglets remained assigned to their biological dams when calculating the number of piglets at weaning, survival rate at weaning, and weaning litter weight.

Live birth rate and stillbirth rate were calculated as the numbers of liveborn and stillborn piglets divided by total litter size, respectively. Weak piglet rate was calculated as the number of weak piglets divided by the number of liveborn piglets. Survival rate at weaning was calculated as the number of piglets at weaning divided by the number of piglets born alive. Piglet average daily gain was calculated using the same piglets included in the mean weaning weight calculation, as the difference between mean weaning weight and the corresponding mean birth weight of the same piglets, divided by lactation days. Sow average daily feed intake was calculated as total lactation feed intake divided by lactation days. Estimated milk yield was derived from total weight gain of the actual nursing litter, including weight gain before death, assuming that 4 g of milk were required for 1 g of piglet weight gain [17]. Feed conversion ratio of milk was calculated as total sow feed intake divided by estimated milk yield.

Serum and mammary tissue sampling

At weaning, blood samples were collected from sows via anterior vena cava puncture. After clotting at room temperature, samples were centrifuged at 2000 × g for 10 min at 4°C, and the serum was aliquoted and stored at -80°C for subsequent ELISA and metabolomic analyses.

Mammary tissue was collected from all 12 sows per treatment at weaning using a Bard® MAGNUM® reusable core biopsy instrument (MG1522; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) fitted with a disposable core biopsy needle (MN1413, 14 G × 13 cm) with a 22 mm throw depth. Biopsy sites were located approximately 5 cm dorsal to the nipple areola of the second and third mammary glands on the left side [18]. Three biopsy cores were obtained from each gland. Tissue samples intended for histological evaluation were fixed in 4%

paraformaldehyde, whereas samples used for molecular analyses were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Mammary histological evaluation

Mammary tissue samples fixed in 4% paraformaldehyde were dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned at a thickness of 5 μm. Tissue sections were subsequently deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) for histological examination. Histological evaluation was performed according to previously described procedures [19].

Histological images were captured at 80× magnification using a Leica DM3000 LED optical microscope (Leica Microsystems, Wetzlar, Germany). Mammary gland morphology, including alveolar structure, epithelial organization, and luminal characteristics, was compared between experimental groups. For quantitative analysis, one representative tissue section from each of eight randomly selected sows per group was evaluated, and the areas of ten morphologically intact alveoli from each section were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The ten alveolar measurements were averaged to obtain one value for each sow, and sow-level means were used for statistical comparison (n = 8 biological replicates per group).

Serum immune index determination

Serum concentrations of immunoglobulins (IgA, IgG, and IgM) and cytokines (IL-1β, IL-6, IL-8, and IL-10) were determined in all serum samples (n = 12 per group) using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' protocols. Immunoglobulin ELISA kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), whereas cytokine ELISA kits were purchased from Invitrogen (Thermo Fisher Scientific, USA). Briefly, serum samples and standards were incubated in antibody-coated microplates, followed by binding of the enzyme conjugate and chromogenic detection. Absorbance was measured at 450 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and sample concentrations were calculated based on the corresponding standard curves.

Serum metabolome analysis

Metabolite extraction and LC-MS/MS-GC-MS detection

Metabolomic analysis was performed according to the manufacturer's protocol (Majorbio Bio-Pharm Technology Co., Ltd.). Untargeted serum metabolomic profiling was performed in six sows randomly selected from the 12 sows in each treatment using integrated LC-MS/MS and GC-MS platforms. The same individuals were

subsequently used for mammary transcriptomic analysis. Serum samples were divided into two aliquots for parallel metabolite extraction and instrumental detection.

For LC-MS/MS analysis, serum metabolites were extracted using methanol/water solution containing L-2-chlorophenylalanine as the internal standard. After vortexing, ultrasonic extraction, low-temperature incubation, and centrifugation according to the standard operating procedures of Majorbio Bio-Pharm Technology Co., Ltd., the supernatant was transferred to injection vials for LC-MS/MS analysis.

For GC-MS analysis, serum samples were extracted using methanol/water solution followed by chloroform-assisted extraction. After centrifugation, the supernatant was dried under nitrogen gas and derivatized with methoxyamine hydrochloride in pyridine, followed by BSTFA containing 1% TMCS before GC-MS detection.

LC-MS/MS analysis was performed using a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, USA). GC-MS analysis was conducted using an Agilent 8890B gas chromatograph coupled with an Agilent 5977B mass selective detector fitted with a DB-5MS capillary column (40 m × 0.25 mm × 0.25 μm). All metabolomic analyses were performed on the Majorbio Cloud platform (https://cloud.majorbio.com).

Metabolomic data processing

Raw LC-MS/MS files were processed using Progenesis QI software for peak extraction, retention time correction, peak alignment, and normalization. GC-MS raw data were analyzed using MassHunter Workstation Quantitative Analysis software (version 10.0.707.0). Metabolite annotation was performed by matching spectral information against public and in-house databases, including Human Metabolome Database (HMDB), METLIN, NIST, Fiehn, MS-DIAL, and the Majorbio database.

Pooled quality control samples were prepared by mixing equal aliquots from all serum samples and analyzed throughout the instrumental sequence to monitor analytical stability. The processed metabolomic matrices were subsequently uploaded to the Majorbio Cloud platform (https://cloud.majorbio.com) for downstream analyses.

Mammary transcriptome analysis

RNA extraction and sequencing

Total RNA was isolated from mammary tissue samples obtained from the same six randomly selected sows in each group using TRIzol Reagent (Thermo Fisher Scientific, USA). RNA quantity and purity were evaluated using a NanoDrop spectrophotometer, and RNA integrity was assessed before library construction using an Agilent 5300 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Following quality assessment, all RNA samples

fulfilled the required criteria and were subsequently processed for library construction.

Messenger RNA was enriched using oligo (dT)-attached magnetic beads and fragmented. cDNA libraries were then constructed through reverse transcription, end repair, adapter ligation, and PCR amplification. Library quality was examined using an Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on the Illumina NovaSeq 6000 platform using a paired-end 150 bp strategy.

Transcriptomic data processing

Raw sequencing reads were subjected to quality control to remove adapter sequences and low-quality reads. Clean reads were mapped to the *Sus scrofa* reference genome using HISAT2. Gene expression levels were quantified using HTSeq based on mapped reads. The processed expression matrix was used for differential expression and functional enrichment analyses described in the Statistical analysis section. The raw data were deposited in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA1468988.

Correlation analysis

To further investigate the coordinated responses between serum metabolism and mammary transcriptional regulation under APS supplementation, integrated multi-omics correlation analysis was performed. Metabolites and genes meeting the nominal screening criteria and represented in the exploratory KEGG pathways were selected for subsequent analysis. Pairwise Spearman correlations were calculated in R software (version 4.3.1) using matched serum metabolomic and mammary transcriptomic data from all 12 sows pooled across the CON and APS groups (n = 6 per treatment). Correlation results were visualized using heatmaps and interaction networks.

qRT-PCR validation of transcriptomic data

To examine the expression patterns obtained from RNA sequencing, 11 representative candidate genes identified by the nominal screening criteria were selected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. qRT-PCR validation was performed using mammary tissue samples from all 12 sows per treatment (n = 12/group). Total RNA was isolated from mammary tissue samples using TRIzol reagent (Thermo Fisher Scientific, USA), and RNA quality was evaluated prior to downstream analysis. First-strand cDNA was synthesized from total RNA using a commercial reverse transcription kit following the manufacturer's protocol.

Gene amplification was conducted using SYBR Green-based qPCR chemistry on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, USA). GAPDH served as the housekeeping gene for data normalization. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are provided in Table S2. Primers were commercially synthesized by Sangon Biotech (Shanghai, China).

Statistical analysis

Statistical analyses for reproductive performance, serum immune indices, mammary histology, and qRT-PCR validation were performed using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). Data normality was assessed using the Shapiro-Wilk test. Depending on data distribution, differences between the CON and APS groups were analyzed using either Student's t-test or the Mann-Whitney U test. For the serum immune panel (seven analytes) and targeted qRT-PCR panel (11 genes), *p* values from the group comparisons were adjusted within each panel using the Benjamini-Hochberg procedure, and adjusted $p < 0.05$ was considered statistically significant. For the other individual phenotypic outcomes, nominal $p < 0.05$ was considered statistically significant. No multiple-testing correction was applied to the exploratory metabolite-gene correlation analysis; the reported correlation *p* values are unadjusted.

For mammary transcriptomic analysis, gene expression levels were quantified using HTSeq (version 0.9.1). Differentially expressed genes (DEGs) between the CON and APS groups were identified using DESeq2 in R software (version 4.3.1). Genes with $|\log2FoldChange| > 1$ and nominal $p < 0.05$ were considered DEGs for exploratory analysis. Principal component analysis (PCA) was performed using the Factoextra package (version 1.0.7). KEGG pathway enrichment analysis of DEGs was conducted using KOBAS 2.0; pathways with nominal $p < 0.05$ were considered enriched. In addition, Benjamini-Hochberg correction was applied to gene-level *p* values, and the corresponding results are summarized in Table S3. GSEA was also performed using all expressed genes ranked by the signal-to-noise metric with gene-set permutations; gene sets with adjusted $p < 0.05$ were considered significantly enriched (Table S4).

For serum metabolomic analysis, principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using the ropls package in R software (version 4.3.1). OPLS-DA was performed on the full metabolomic dataset using Pareto scaling, and model performance was assessed using R^2Y, Q^2, and 200 permutation tests. Differential metabolites were screened based on OPLS-DA-derived VIP > 1 and nominal $p < 0.05$ for exploratory analysis. Benjamini-Hochberg correction was also evaluated across the tested metabolites, with results summarized in Table S3. KEGG pathway enrichment analysis was subsequently performed using the nominally selected metabolites.

For integrated multi-omics analysis, Spearman's correlation coefficients between the selected metabolites and DEGs were calculated using matched samples pooled across treatments. Correlations with $|r| > 0.5$ and nominal $p < 0.05$ were retained for exploratory interpretation; these *p* values were not adjusted for multiple testing.

Results

Effects of APS supplementation on maternal and lactation-related phenotypes

Table 1 presents the reproductive performance, lactation traits, and growth performance of sows and their piglets in the CON and APS groups. Compared with the control group, APS supplementation of sow diets tended to increase piglet average daily gain ($p = 0.0864$) and sow estimated milk yield ($p = 0.0808$). Moreover, APS supplementation significantly increased sow daily feed intake ($p = 0.0461$), whereas other parameters did not differ significantly ($p > 0.05$).

APS supplementation increased mammary alveolar development in sows

Representative hematoxylin and eosin (H&E)-stained sections of mammary gland tissue are shown in Figure 1. Compared with the control group, mammary glands from the APS group exhibited enlarged alveolar lumens, expanded alveolar structures, and a more compact and uniform alveolar arrangement. Quantitative morphometric analysis further demonstrated that the mean alveolar area was significantly greater in the APS group than in the control group ($p < 0.0001$), indicating enhanced mammary alveolar development in response to APS supplementation.

Effects of APS supplementation on serum immunoglobulins and cytokines in sows

Serum ELISA results are shown in Figure 2. In Figure 2A, IgG and IgM concentrations were higher in the APS group after BH correction (adjusted $p < 0.05$), whereas IgA showed an increasing trend (adjusted $p = 0.0535$). In Figure 2B, IL-1β, IL-6, and IL-8 did not differ significantly between groups (adjusted $p > 0.05$), while serum IL-10 was higher in the APS group (adjusted $p < 0.05$).

Serum metabolomic alterations in sows receiving APS supplementation

Serum metabolomic profiling was performed to evaluate the systemic metabolic response to APS supplementation (Figure 3). As shown in the volcano plot (Figure 3A), 63 differential metabolites were identified based on the exploratory screening criteria (VIP > 1 and nominal $p < 0.05$), including 30 increased and 33 decreased metabolites in the APS group, whereas 1454 metabolites did not meet these combined criteria. However, none of the metabolites remained significant after Benjamini-Hochberg correction (adjusted $p < 0.05$; Table S3). As shown in the PCA score plot (Figure 3B), APS and CON samples partially overlapped, although a tendency toward separation was observed along PC1 (PC1 = 22.80%, PC2 = 16.20%). This pattern indicated a moderate shift in the overall serum metabolic profile following APS supplementation. Exploratory OPLS-DA showed separation between APS and CON samples (Figure 3C). The model yielded $R^2X = 0.559$, $R^2Y = 0.992$, and $Q^2 = 0.657$, with 200-permutation

validation results provided in Figure S1. Exploratory Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the nominally selected metabolites is shown in Figure 3D. The enriched pathways included autophagy-related pathways (Autophagy other and Autophagy animal). Other enriched pathways involved lipid metabolism and signaling (Cholesterol metabolism, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, and Retrograde endocannabinoid signaling) and amino acid-related metabolism (Arginine biosynthesis, Arginine and proline metabolism, Taurine and hypotaurine metabolism, and D-amino acid metabolism). In addition, several pathways related to disease and host response were enriched, including Chemical carcinogenesis receptor activation and Kaposi sarcoma-associated herpesvirus infection. Key differential metabolites contributing to these pathways are summarized in Figure 3E (fold change, APS vs CON). Among them, 2,5-diaminopentanoic acid showed the most pronounced increase in the APS group, while hypotaurine, phenobarbital, and chenodeoxycholic acid glycine conjugate were decreased. Several lipid-related metabolites, including PE (16:0/20:2(11Z,14Z)) and PE (18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), were also increased, together with 5-aminovaleric acid and creatinine. These changes suggest coordinated alterations in amino acid metabolism, bile acid-related components, and phospholipid profiles in sow serum.

Mammary transcriptomic alterations in sows receiving APS supplementation

RNA-seq and KEGG enrichment analyses were used to characterize mammary transcriptional responses to APS supplementation (Figure 4). The volcano plot (Figure 4A) shows the global distribution of differentially expressed genes (DEGs) between APS and CON groups. A total of 162 genes were up-regulated and 93 were down-regulated in the APS group under the nominal differential-expression criteria. However, only two genes remained significant after Benjamini-Hochberg correction (adjusted $p < 0.05$; Table S3). Principal component analysis (PCA) showed partial overlap between APS and CON samples, with a tendency toward separation (Figure 4B). This pattern suggests that APS altered the overall gene expression profile despite moderate intergroup variability. The top 20 KEGG pathways identified by the exploratory enrichment analysis (nominal $p < 0.05$) are shown in Figure 4C, most of which are associated with immune and inflammatory signaling processes. These included the IL-17, TNF, NF-κB, Toll-like receptor, and C-type lectin receptor signaling pathways, as well as cytokine-cytokine receptor interaction. Other enriched pathways included Lipid and atherosclerosis and the AGE-RAGE signaling pathway in diabetic complications. As a complementary analysis, GSEA of the full ranked transcriptomic dataset further supported the predominantly immune-related enrichment pattern observed in the exploratory KEGG analysis. Multiple immune-related gene sets, including IL-17, TNF, NF-κB signaling and other immune-associated pathways, reached

significance after false discovery rate (FDR) correction (Table S4). To further illustrate transcriptional changes within the enriched pathways, 18 representative DEGs from the top 20 KEGG pathways (excluding unannotated genes) are shown in Figure 4D. Among these, several cytokines and chemokines were up-regulated in the APS group, including *IL-6*, *IL-1A*, *TNF*, *CXCL8*, *CXCL2*, *CCL2*, *CCL4*, and *CCL8*. Other immune-associated or regulatory genes, including *OSM*, *IL-5*, *IL-11*, *PTGS2*, *EGR3*, *PLK3*, and *ATP6V1C2*, were also up-regulated. In contrast, *AGER*, *CYP1A1*, and *KIT* were down-regulated. These exploratory findings suggest that APS supplementation modulated the expression of key genes involved in immune communication and inflammatory signaling pathways in mammary tissue.

Associations between serum metabolites and mammary gene expression

Correlation analysis was used to examine relationships between serum differential metabolites and mammary DEGs (Figure 5). As shown in Figure 5A, several nominally significant pooled correlations were observed between selected serum metabolites and mammary DEGs from the exploratory KEGG pathways. Among all metabolites, creatinine showed the most consistent associations and was positively correlated with multiple inflammatory and chemokine genes, including *CXCL8* ($p < 0.01$), *PLK3* ($p < 0.01$), *IL-1A*, *CXCL2*, *TNF*, *OSM*, *CCL4*, and *PTGS2* ($p < 0.05$). In addition, 5-aminovaleric acid was positively correlated with *IL-6*, *CCL2*, *CXCL2*, *CCL4*, and *IL-11* ($p < 0.05$). Similarly, 2,5-diaminopentanoic acid showed positive correlations with *TNF*, *PLK3*, *OSM*, *CCL4*, and *PTGS2* ($p < 0.05$), suggesting a close association with key inflammatory regulators. Among metabolites related to bile acids and exogenous compound, chenodeoxycholic acid glycine conjugate was negatively correlated with *IL-5* and *TNF* ($p < 0.05$) and positively correlated with *CYP1A1* ($p < 0.05$). Phenobarbital was negatively correlated with *IL-6*, *CCL2*, and *IL-11* ($p < 0.05$). Regarding lipid species, PE (16:0/20:2(11Z,14Z)) was positively correlated with *CXCL2* ($p < 0.05$), and PE (18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) was positively correlated with *ATP6V1C2* ($p < 0.05$). Hypotaurine showed a significant negative correlation with *IL-5* ($p < 0.05$). qRT-PCR validation using mammary samples from all 12 sows per treatment showed expression patterns generally consistent with the RNA-seq results, with all 11 targeted comparisons meeting the BH-adjusted $p < 0.05$ threshold (Figure 5B). Overall, these exploratory pooled correlations described patterns of co-variation between serum metabolites and mammary gene expression across the CON and APS groups.

Discussion

In the present study, dietary APS supplementation significantly increased sow feed intake during lactation and tended to increase piglet average daily gain (ADG). Estimated milk yield, derived from the total body weight gain of

the actual nursing litter, also tended to increase. Because this value was indirectly estimated from nursing-litter weight gain rather than directly measured, it should not be interpreted as direct evidence of increased milk production. Previous studies have shown that APS possesses immunomodulatory and antioxidant activities and can improve immune and antioxidant status in pigs and sows [7]. Maternal metabolic homeostasis and physiological status during late gestation and lactation are important for maintaining sow performance and may influence milk quality and subsequent piglet performance [1]. Therefore, the biological effects of APS may partly contribute to the phenotypic responses observed in the present study. The tendency toward increased piglet ADG may be related to the reported effects of APS on immune regulation, oxidative status, nutrient utilization, and gut health [7]. These systemic effects may improve maternal physiological status during lactation, potentially influencing the nutrient and bioactive composition of milk and thereby supporting piglet growth. Previous studies have also shown that APS supplementation improves nutrient digestibility and growth performance in piglets and growing pigs [20], which is consistent with the growth trend observed in the present study. The increased feed intake in APS-supplemented sows may reflect improved adaptation to the nutritional demands of lactation. Greater feed intake may also increase nutrient availability for maternal maintenance and lactation and could therefore partly contribute to the observed mammary and litter responses. This finding agrees with previous reports of improved feed intake and lactation-related performance in sows receiving APS supplementation [8]. Together, these findings suggest that maternal APS supplementation may support sow adaptation during lactation and contribute to the growth potential of suckling piglets.

The observed lactation-related phenotypic responses were accompanied by clear structural changes in mammary tissue, including enlarged alveolar lumens, expanded alveolar structures, and a more compact alveolar arrangement. The mammary alveolus is the basic functional unit for milk synthesis and secretion, and its size is closely associated with secretory activity [3]. The increased alveolar area in the APS group therefore suggests enhanced mammary structural development during the periparturient period. Given the reported immunomodulatory and metabolic regulatory properties of APS, it is plausible that APS supplementation may have contributed to a more favorable systemic and mammary microenvironment, thereby supporting alveolar expansion and functional readiness for milk production [6]. Because milk composition was not measured, the present study also cannot determine whether APS altered milk nutrient or bioactive composition. Nevertheless, the histological findings provide structural evidence of mammary remodeling consistent with the observed lactation-related phenotypic

responses. Previous studies have also shown that APS can affect lactation-related traits in sows [8] and regulate mammary epithelial cell function [21], which is consistent with the mammary changes observed here.

APS supplementation also modulated maternal humoral immune status. Serum ELISA showed significantly increased IgG and IgM concentrations, with IgA showing an increasing trend. Because immunoglobulins are central mediators of humoral immunity [22], these results suggest improved systemic humoral immune status at weaning, within the broader periparturient period characterized by pronounced physiological and immunological transition [2]. APS has been widely recognized as an immunomodulatory polysaccharide [7]. In sows, maternal circulating immunoglobulins contribute to the immune components present in colostrum and milk [23]. Therefore, the increased serum immunoglobulins observed in the APS group may be associated with enhanced maternal humoral immune status at weaning. Previous studies similarly reported that APS supplementation enhanced immunoglobulin levels in sow colostrum and milk, including IgG and IgM [9], supporting the immunomodulatory role of APS during the periparturient period. In addition, APS significantly increased IL-10 concentration. Because IL-10 is an important anti-inflammatory cytokine involved in limiting excessive inflammatory responses and maintaining immune homeostasis [24], the elevated IL-10 level suggests that APS may contribute to a more regulated immune environment during lactation. Similar regulation of IL-10 expression by APS has been reported in porcine immune cells [25]. Together, the increased immunoglobulins and elevated IL-10 suggest that APS may support maternal immune adaptation while helping maintain immune homeostasis during the periparturient period.

Exploratory serum metabolomics suggested changes in selected metabolic processes. Although PCA revealed partial overlap between groups, 63 metabolites meeting the nominal screening criteria and their KEGG enrichment patterns suggested possible changes in amino acid, lipid, and related metabolic pathways. Because the periparturient period is already characterized by major shifts in energy use and nutrient partitioning [2], the metabolomic profile in the APS group may reflect targeted metabolic modulation superimposed on normal physiological remodeling. KEGG analysis indicated that APS may affect pathways related to autophagy, lipid metabolism, and amino acid metabolism. This suggests coordinated regulation of nutrient recycling, lipid handling, and nitrogen-associated metabolism during the transition from gestation to lactation. Autophagy is a core mechanism of stress adaptation and nutrient recycling that also interacts with immune regulation [26], and APS has been reported to influence autophagy-related signaling in experimental models [27]. Lipid pathway enrichment, especially cholesterol metabolism, is biologically meaningful because lipid transport and membrane remodeling are highly active during lactation and are closely related to mammary secretory function [28]. APS has also been shown to influence

cholesterol and bile acid metabolism in vivo [10]. Among amino acid pathways, arginine-related metabolism is notable because it is linked to both immune regulation and nitrogen utilization [29]. APS has been reported to improve amino acid digestibility and serum amino acid availability, including arginine, in pigs [11]. Enrichment of taurine and hypotaurine metabolism also suggests regulation of antioxidant-related processes, which may help maintain cellular stability during the metabolic challenge of early lactation [30]. In addition, although some enriched KEGG pathways were labeled as disease-related, they are more likely to represent shared host response or receptor signaling modules rather than actual pathological processes. For example, the enrichment associated with phenobarbital may reflect regulation of xenobiotic-metabolizing enzymes and detoxification pathways rather than disease occurrence [31]. Overall, these exploratory results suggest that APS may modulate systemic metabolic adaptation during the periparturient period.

At the metabolite level, increased 2,5-diaminopentanoic acid, 5-aminovaleric acid, and creatinine suggest altered amino acid-related metabolism during lactation. 5-aminovaleric acid is a lysine degradation product [32], whereas 2,5-diaminopentanoic acid corresponds to ornithine. Creatinine reflects creatine turnover and whole-body energy buffering, and creatine metabolism is closely linked to amino acid utilization through glycine, arginine, and methionine metabolism [33]. These changes are consistent with the increased demands for amino acid substrates and energy during lactation [2]. Consistently, dietary APS has been reported to improve amino acid digestibility and increase circulating amino acid concentrations in swine [11]. The increase in phosphatidylethanolamine (PE) species suggests enhanced membrane phospholipid turnover related to membrane curvature, vesicle formation, and secretory activity [34], and APS has previously been linked to glycerophospholipid metabolism [35]. The decrease in chenodeoxycholic acid glycine conjugate suggests altered bile acid composition, which may reflect reorganization of lipid handling during early lactation rather than impaired absorption [36]. Reduced hypotaurine may indicate greater utilization of antioxidant metabolites under lactational oxidative demand. Hypotaurine can neutralize reactive oxygen species [37], consistent with previous evidence that APS enhances antioxidant capacity [7]. Together, these metabolite changes suggest that APS may modulate nutrient and energy metabolism, membrane remodeling, bile acid homeostasis, and antioxidant responses during early lactation.

RNA-seq analysis suggested that APS supplementation may alter immune-related mammary transcriptional responses. The exploratory KEGG analysis highlighted IL-17, TNF, NF-κB, Toll-like receptor, and C-type lectin receptor signaling pathways, as well as cytokine-cytokine receptor interaction. These findings suggest that APS may influence mammary immune-related gene programs during the periparturient period. This predominantly immune-

related enrichment pattern was further supported by GSEA of the full ranked transcriptomic dataset, which identified FDR-significant enrichment of multiple immune-associated pathways, particularly IL-17, TNF, and NF-κB signaling. Such enrichment is biologically plausible because the lactating mammary gland requires active local immune defense to maintain tissue integrity and milk safety [38]. Previous studies similarly showed that APS modulates immune signaling and cytokine expression in porcine immune cells, including pathways related to TLR, NF-κB, and TNF signaling [39], supporting the transcriptomic patterns observed here.

At the gene level, APS supplementation was associated with coordinated upregulation of several cytokines and chemokines in mammary tissue, including *IL-6*, *IL-1A*, *TNF*, *CXCL8*, *CXCL2*, *CCL2*, *CCL4*, and *CCL8*. *IL-6*, *IL-1A*, and *TNF* participate in the coordination of early immune responses [5], whereas *CXCL8* and *CXCL2* are mainly involved in neutrophil recruitment [40]. *CCL2*, *CCL4*, and *CCL8* contribute to the recruitment and activation of monocytes, macrophages, and other leukocyte populations [41]. Together, the coordinated increase in these genes suggests that APS may enhance local immune readiness in mammary tissue during the periparturient period. This interpretation is consistent with previous reports that APS regulated *IL-6* and *TNF-α* expression through NF-κB and MAPK signaling pathways [42]. Importantly, these transcriptional changes occurred in mammary tissue without corresponding increases in circulating pro-inflammatory cytokines, while serum IL-10 was increased. This pattern highlights distinct immune response profiles at the systemic and mammary levels under APS supplementation. In this context, increased expression of cytokine- and chemokine-related genes may reflect enhanced local immune preparedness or immune surveillance [43]. This is consistent with the need for the lactating mammary gland to maintain tissue integrity and defense capacity without triggering excessive inflammation [44]. In addition, *OSM*, *IL-5*, and *IL-11* were also up-regulated. *OSM* is associated with epithelial differentiation and tissue remodeling [45], *IL-5* supports immune cell development and antibody-related responses [46], and *IL-11* is involved in epithelial survival and tissue repair [47]. Their increased expression therefore suggests that APS may contribute to epithelial adaptation together with local immune regulation in mammary tissue. This interpretation is consistent with previous studies showing that APS can regulate cytokine expression, increase *IL-11*, and enhance antibody-related immune responses in vivo [48]. Moreover, several non-cytokine genes were also altered. *PTGS2* and *PLK3* were up-regulated, suggesting potential involvement of prostaglandin-related inflammatory regulation and stress-responsive cellular adaptation [49, 50]. Other differentially expressed genes, including *ATP6V1C2*, *EGR3*, *AGER*, *CYP1A1*, and *KIT*, were associated with intracellular transport, early transcriptional responses, oxidative metabolism, inflammatory signal amplification, and tissue homeostasis [51-55]. These changes suggest that APS not only

influenced mammary immune signaling but also affected epithelial adaptation, intracellular transport, and tissue homeostasis. Similar regulation of inflammatory and oxidative pathways by APS has been reported in other models [7]. Collectively, these transcriptional changes suggest that APS supported a coordinated mammary adaptation involving immune responsiveness, epithelial regulation, and tissue remodeling during the periparturient period.

Exploratory pooled metabolomic and transcriptomic correlations suggested patterns of co-variation between selected circulating metabolites and mammary gene expression across the two treatment groups. Among the metabolites, creatinine showed the broadest positive correlations with genes involved in immune signaling and tissue adaptation, including *CXCL8*, *IL-1A*, *TNF*, *CCL4*, *OSM*, *PLK3*, and *PTGS2*. Because creatinine is an end product of creatine metabolism [33], it is unlikely to directly regulate these genes, and its altered abundance may instead reflect metabolic changes accompanying mammary transcriptional responses related to immune and tissue processes under APS supplementation. Similar metabolite-transcript relationships involving creatinine have also been reported in integrated omics analyses under immune challenge, in which creatinine was identified as a metabolite associated with immune-related transcriptional responses [56]. Meanwhile, 5-aminovaleric acid was positively correlated with *IL-6*, *CCL2*, *CXCL2*, *CCL4*, and *IL-11*, whereas 2,5-diaminopentanoic acid showed positive correlations with *TNF*, *PLK3*, *OSM*, *CCL4*, and *PTGS2*. These associations suggest coordinated variation between amino acid-derived metabolites and mammary genes related to cytokine signaling, chemokine recruitment, and tissue responses. Previous studies have also linked 5-aminovaleric acid and ornithine-related metabolism to inflammatory states [57, 58]. In contrast, chenodeoxycholic acid glycine conjugate was negatively associated with *IL-5* and *TNF* but positively associated with *CYP1A1*. Bile acids are signaling metabolites that can regulate cytokine production through receptors such as Farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) [36, 59]. Interactions between bile acid signaling and xenobiotic metabolism pathways such as *CYP1A1* regulation have also been reported [60]. Therefore, bile acid-related metabolic variation may accompany mammary transcriptional responses related to immune and xenobiotic processes. In addition, phosphatidylethanolamine species were positively correlated with genes related to immune signaling and cellular responses, including *CXCL2* and *ATP6V1C2*. Because phosphatidylethanolamine is an important membrane phospholipid involved in membrane structure and vesicle formation [34], these associations may reflect coordinated changes between phospholipid metabolism and mammary cellular activity under APS supplementation. Overall, these pooled associations suggest that serum metabolic variation may accompany mammary transcriptional responses related to mammary immune and functional adaptation during the periparturient period.

Several limitations should be noted. First, the correlations between serum metabolites and mammary gene expression were associative and did not establish causality. Because the correlation analysis pooled animals from both treatment groups, some associations may partly reflect treatment-related differences rather than independent within-group relationships. Second, transcriptomic analysis was conducted at the whole-tissue level and could not distinguish contributions from different mammary cell populations. Third, metabolomic analysis was limited to serum because milk collection from periparturient sows was difficult. Serum metabolomics may therefore not fully capture mammary secretory metabolism. Fourth, metabolomic and transcriptomic analyses were conducted in six sows per treatment. This limited sample size, together with the multiple-testing burden, may have reduced the statistical power to identify individual features after FDR correction. As most nominally identified genes and all nominally identified metabolites did not remain significant after FDR correction, the corresponding individual-feature findings and downstream analyses based on these candidates should be considered exploratory. The OPLS-DA model may also be susceptible to overfitting, while the integrated correlations may be sensitive to individual variation; both should therefore be interpreted cautiously. Fifth, only one APS inclusion level was evaluated; therefore, a dose-response relationship and the optimal supplementation level could not be determined in the present study. Future studies incorporating milk metabolomics, cell-type-specific transcriptomics, and functional validation may help clarify the mechanisms linking systemic metabolic changes with mammary immune and secretory responses under APS supplementation. Nevertheless, this study has important strengths. APS supplementation was evaluated across multiple biological levels, including reproductive and lactational performance, mammary morphology, serum immune indices, serum metabolomics, and mammary transcriptomics. This integrative design links phenotypic outcomes with molecular responses and provides evidence that APS may support mammary immune responsiveness, tissue adaptation, and lactation-related function in periparturient sows.

Conclusions

In conclusion, dietary supplementation with APS increased sow feed intake and showed tendencies toward higher estimated milk yield and piglet average daily gain during lactation. APS supplementation was also associated with enlarged mammary alveolar area and improved maternal humoral immune status at weaning, as reflected by increased serum IgG and IgM concentrations and elevated IL-10. Exploratory multi-omics analyses further suggested changes in systemic metabolic profiles and mammary transcriptional responses. Specifically, serum metabolomic analysis suggested increases in selected amino acid-related metabolites and phosphatidylethanolamine species, together with alterations in bile acid-related and antioxidant-associated metabolites, potentially involving

nutrient utilization, membrane remodeling, and metabolic homeostasis. At the transcriptional level, exploratory analysis highlighted changes in genes related to cytokine signaling, chemokine activity, epithelial function, and tissue regulatory processes in mammary tissue. These transcriptional changes suggested potential involvement in immune regulation, epithelial adaptation, and tissue remodeling, with complementary GSEA providing further support for the immune-related pathway enrichment. Pooled exploratory correlation analysis further identified associations between circulating metabolites and mammary transcriptional responses, particularly those related to immune activity and tissue adaptation. Together, these findings suggest that APS supplementation may influence maternal metabolic adaptation, humoral immune responses, and mammary transcriptional programs during the periparturient period. These results support the further evaluation of standardized APS preparations as botanical functional ingredients for maternal nutrition during physiologically demanding periods.

Ethics approval and consent to participate

The experimental protocol was approved by the Animal Ethics Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Ethics Approval Code: IAS2022-112).

Consent for publication

Not applicable.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

The datasets generated and analyzed during the current study are included in this published article and its

supplementary information files. The RNA sequencing data have been deposited in the NCBI Sequence Read Archive under accession number PRJNA1468988. The serum metabolomics raw data have been deposited in the MetaboLights repository under accession number MTBLS16072. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Jiaheng Li: Conceptualization; data curation; formal analysis; writing-original draft, writing-review & editing. Li Zhang: Conceptualization; data curation; formal analysis, writing-review & editing. Chengzeng Luo: Software; writing-review & editing. Jiujun Duan: Formal analysis; methodology, writing-review & editing. Zeou Wei and Huixin Wang: Software; resources; supervision; writing-review & editing. Ruqing Zhong and Liang Chen: Software; resources, writing-review & editing. Lianhua Zhang: Conceptualization; funding acquisition; project administration; resources; supervision; writing-review & editing. Martine Schroyen and Hongfu Zhang: Funding acquisition; methodology; project administration; resources, writing-review & editing.

Declaration of generative AI

No AI tools were used in this article.

Notes

Five piglet growth variables-birth weight, birth litter weight, weaning weight, weaning litter weight, and piglet average daily gain-presented in this study were also included in a companion manuscript originating from the same sow-piglet experiment. In that manuscript, these variables were included primarily to characterize offspring growth performance, and only piglet average daily gain was further discussed in relation to maternal APS supplementation. The two studies were designed to address different scientific objectives. The companion manuscript focuses on offspring intestinal morphology, microbiota, colonic metabolomics, and colonic transcriptomic responses, whereas the present study focuses on maternal physiological and mammary responses. No maternal serum metabolomic, mammary morphological, serum immune, or mammary transcriptomic data presented here are included in the companion manuscript.

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Tables and Figures

Table 1 Reproductive performance, lactation traits, and growth performance of sows and their piglets in two groups

Parameters	CON	APS	Pooled SD	*p* value
Litter size, head	11.83 ± 2.92	13.58 ± 3.03	2.97	0.1636
Number of piglets born alive, head	11 ± 2.95	12.58 ± 2.91	2.93	0.1993
Live birth rate, %	100 (88.1, 100)	93.49 (88.98, 100)	NA	0.6165
Stillbirth rate, %	0 (0 ,9.6)	0 (0, 7.14)	NA	0.9667
Number of weak piglets, head	0 (0, 0)	0 (0, 1)	NA	0.2125
Weak piglet rate, %	0 (0, 0)	0 (0, 0.08)	NA	0.1262
Birth weight, g	1508.25 ± 161.15	1425.25 ± 198.64	180.87	0.2731
Birth litter weight, g	16117.15 ± 4122.16	17498.5 ± 3546	3844.89	0.3884
Number of piglets at weaning, head	9.83 ± 2.76	11.17 ± 2.89	2.82	0.2597
Survival rate at weaning, %	95.83 (86.11, 100)	93.3 (87.56, 100)	NA	0.8724
Weaned weight, g	6051.75 ± 463.15	6371.79 ± 766.98	633.55	0.2290
Weaned litter weight, g	60080.53 ± 18563.02	69501.88 ± 13044.44	16042.8	0.1644
Piglet average daily gain, g/day	216.36 ± 17.86	235.55 ± 32.44	26.19	0.0864
Sow daily feed intake, g/day	5913.32 ± 752.84	6507.57 ± 617.34	688.43	0.0461
Estimated milk yield, g	194474.54 ± 36271.99	218323.65 ± 26881.51	31923.92	0.0808
Feed conversion ratio of milk	0.65 ± 0.15	0.63 ± 0.1	0.12	0.7345

Data are presented as mean ± SD and analyzed using Student's t-test for normally distributed variables, or as median (lower quartile, upper quartile) and analyzed using the Mann-Whitney U test for non-normally distributed variables. Pooled SD was calculated as sqrt [(SD CON² + SD APS²)/2], as the sample size was equal between the CON and APS groups. NA indicates that pooled SD was not applicable to variables analyzed using the Mann-Whitney U test. CON, control group; APS, Astragalus polysaccharide group; NA, not applicable; n = 12 per group.

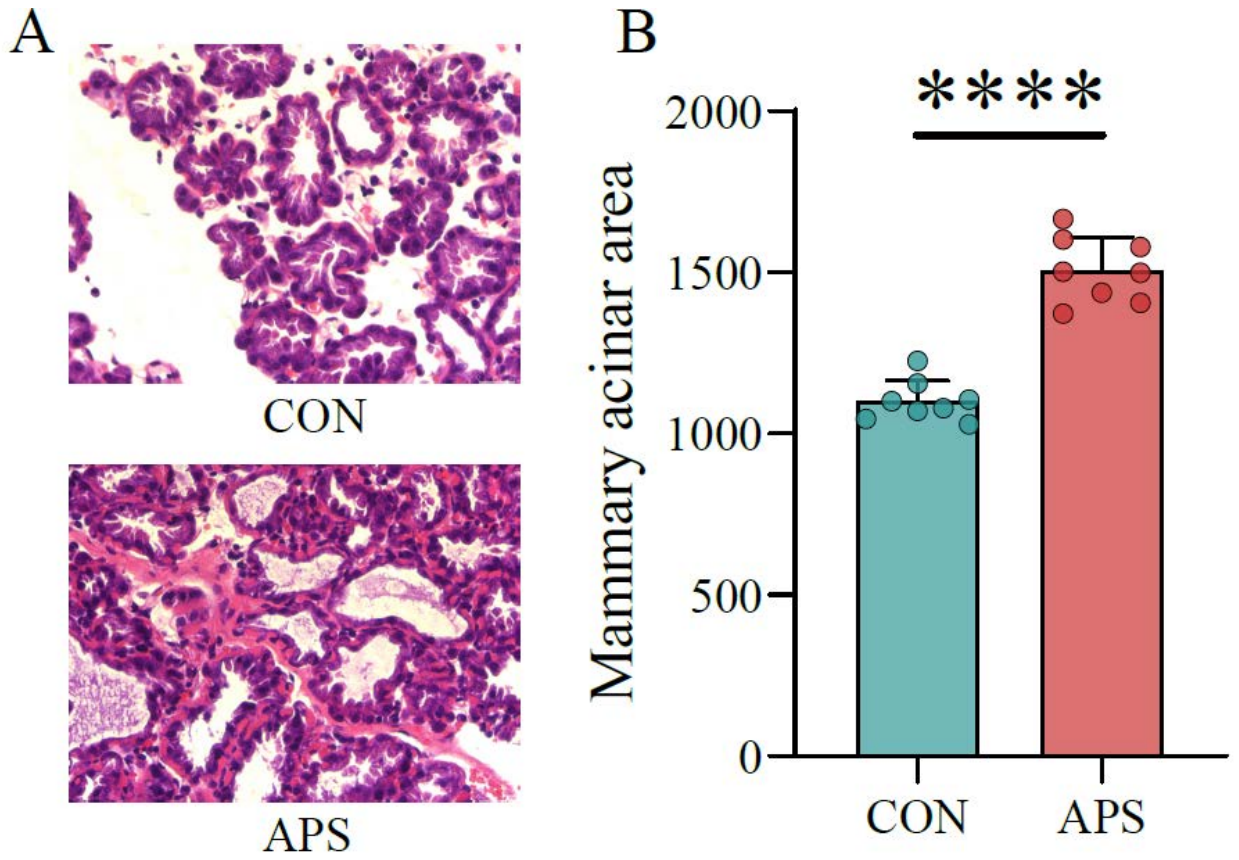


Figure 1. Effects of APS supplementation on mammary morphology in periparturient sows.

(A) Representative H&E-stained mammary tissue sections from the CON and APS groups. (B) Mammary alveolar area. CON = control group; APS = Astragalus polysaccharide group; H&E = hematoxylin and eosin staining; Data are presented as the mean ± SD, including all individual data points; Each data point represents the mean area of ten alveoli measured from one sow; Normality of data was determined by the Shapiro-Wilk test; Statistical significance was determined by Student's t-test; n = 8; **** $p < 0.0001$.

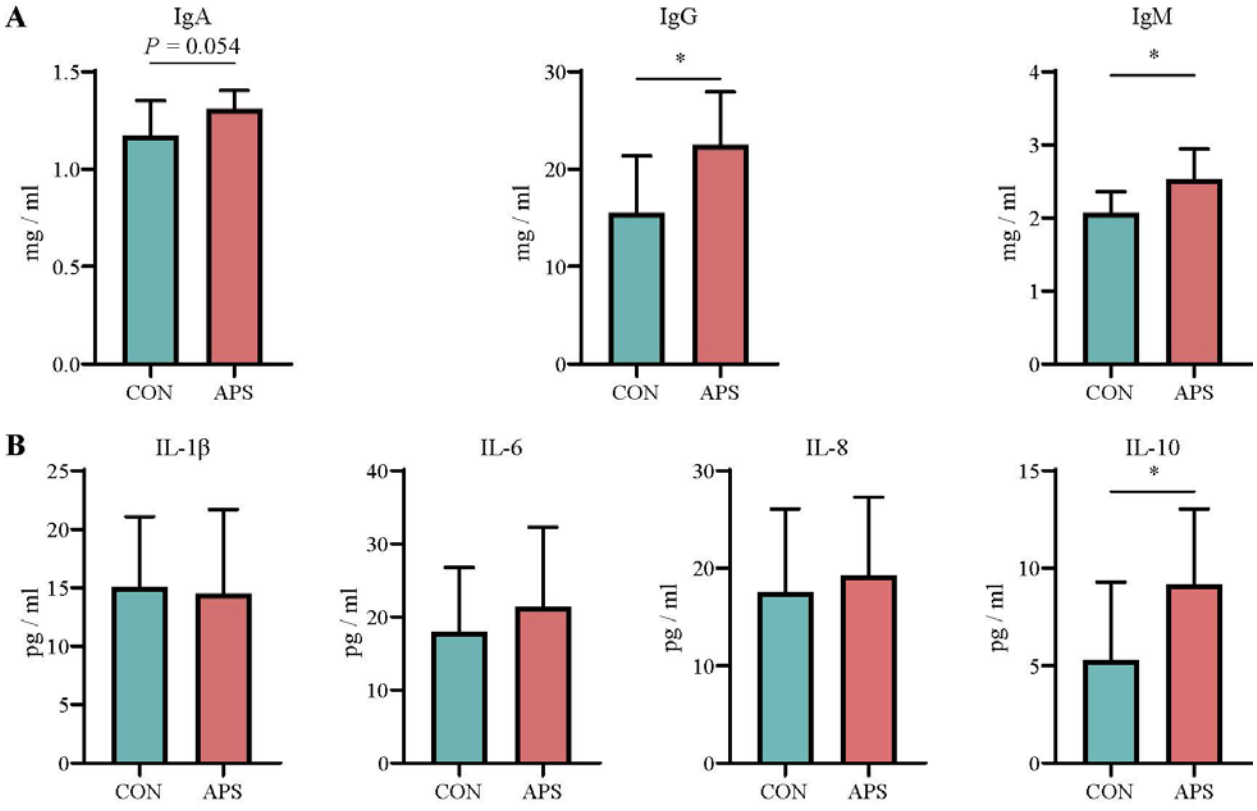


Figure 2. Effects of APS supplementation on serum immunoglobulin concentrations and cytokine levels in periparturient sows.

(A) Serum immunoglobulin concentrations, including IgA, IgG, and IgM. (B) Serum inflammatory cytokine concentrations, including IL-1β, IL-6, IL-8, and IL-10. CON = control group; APS = Astragalus polysaccharide group; Data are presented as the mean ± SD. Normality of data was determined by the Shapiro-Wilk test; Statistical significance was determined by Student's t-test; *p* values were adjusted across the seven serum immune indices using the Benjamini-Hochberg procedure; n = 12; * adjusted $p < 0.05$.

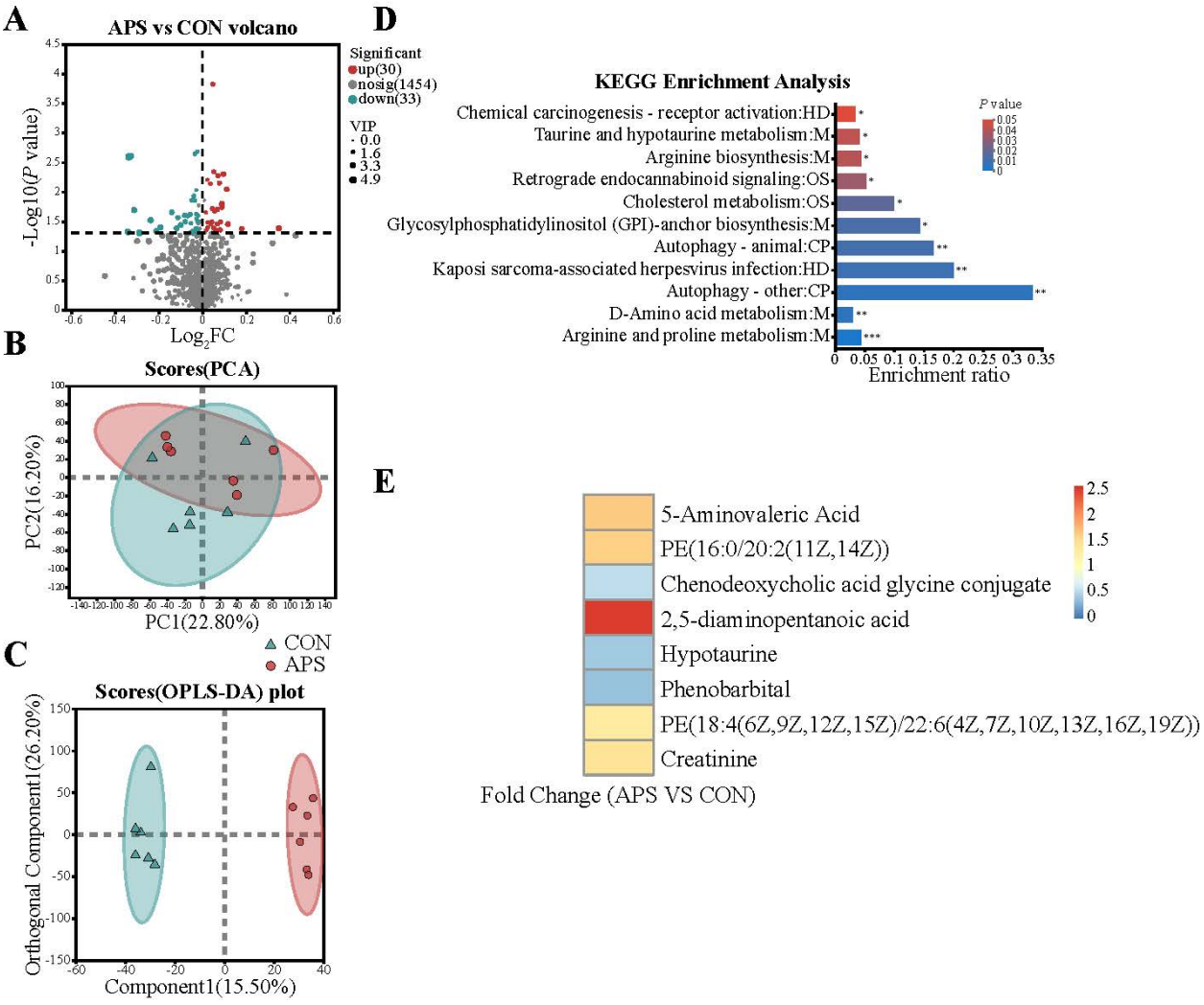


Figure 3. Serum metabolomic alterations in periparturient sows following APS supplementation. (A) Volcano plot of differential serum metabolites (APS vs CON) (VIP > 1, nominal $p < 0.05$). (B) PCA of serum metabolites. (C) OPLS-DA of serum metabolites. (D) KEGG enrichment analysis of differential serum metabolites. (E) Heatmap of fold changes for eight differential serum metabolites involved in enriched KEGG pathways. CON = control group; APS = Astragalus polysaccharide group; PCA = principal component analysis; OPLS-DA = orthogonal partial least squares-discriminant analysis; VIP = variable importance in projection; n = 6.

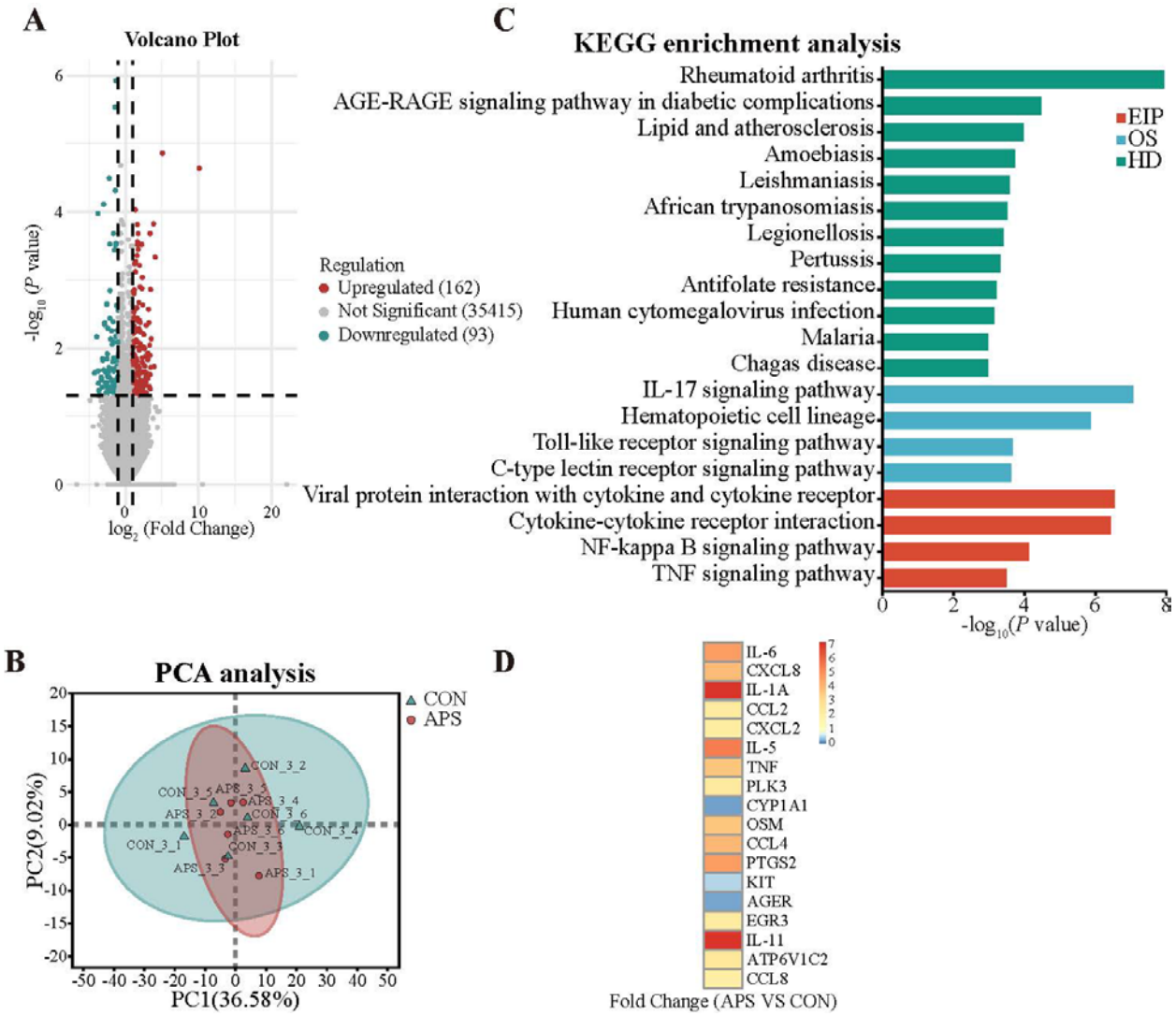


Figure 4. Mammary transcriptomic responses to APS supplementation in periparturient sows.

(A) Volcano plot of differentially expressed genes (DEGs) in mammary tissue between the APS and CON groups. (|log2FoldChange| > 1, nominal $p < 0.05$). (B) PCA of mammary transcriptomic profiles. (C) KEGG enrichment analysis of mammary DEGs. (D) Fold change heatmap of annotated DEGs from the top 20 enriched KEGG pathways. CON = control group; APS = Astragalus polysaccharide group; DEG = differentially expressed gene; PCA = principal component analysis; n = 6.

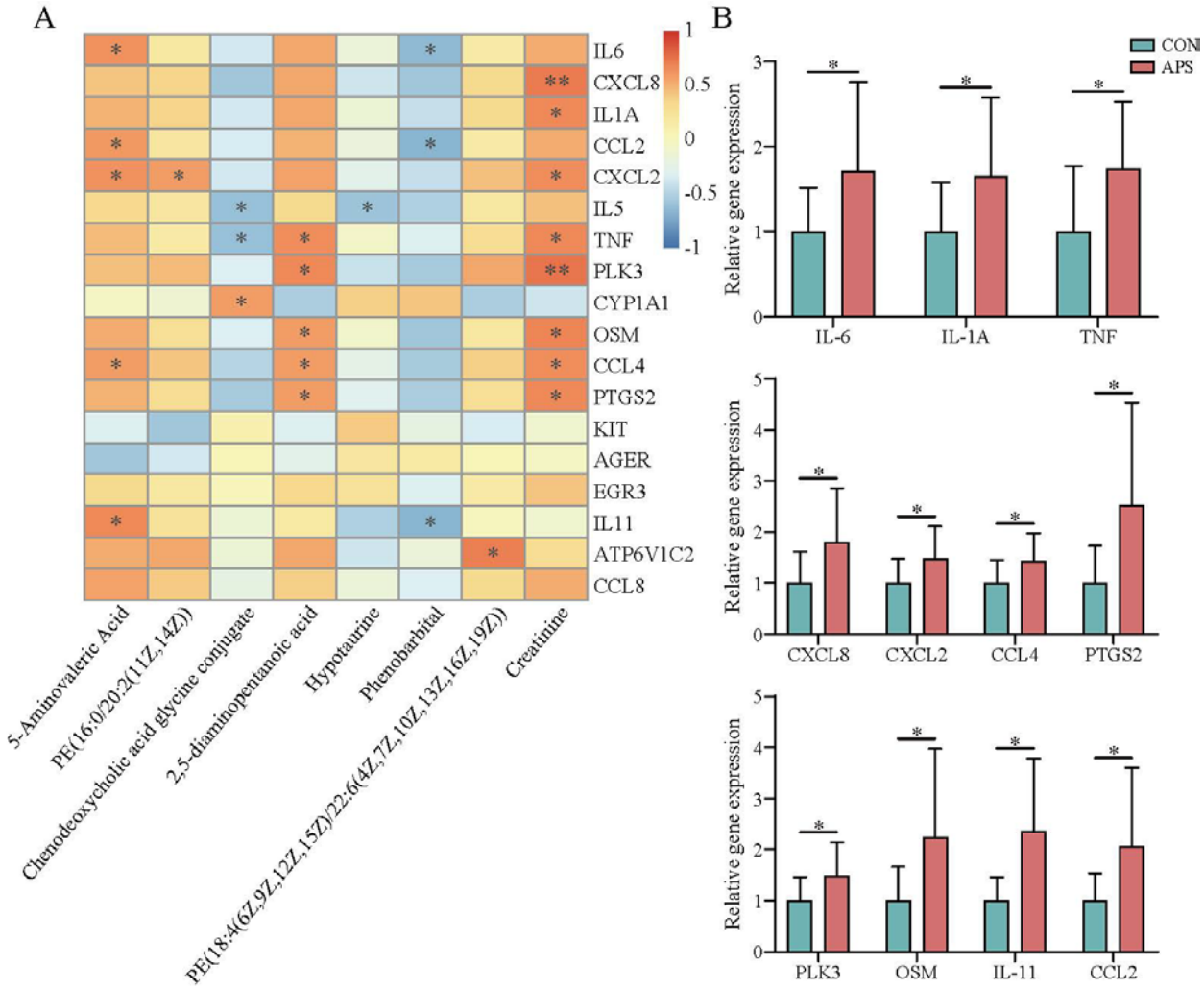


Figure 5. Associations between serum metabolites and mammary gene expression, with qRT-PCR validation of representative genes.

(A) Correlation heatmap between differential serum metabolites and mammary differentially expressed genes (DEGs). (B) qRT-PCR validation of representative genes related to immune signaling, epithelial function, and tissue regulatory processes. qRT-PCR = quantitative real-time polymerase chain reaction; DEGs = differentially expressed genes; Correlations in panel A were calculated using Spearman's method with paired data pooled from both treatment groups (n = 12; six sows per group). Nominal *p* values were reported without multiple-testing adjustment (* $p < 0.05$, ** $p < 0.01$). For qRT-PCR in panel B, data are presented as mean ± SD (n = 12 sows per group). Statistical significance was assessed using Student's t-test followed by Benjamini-Hochberg correction across the 11 genes (* adjusted $p < 0.05$).