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Compound probiotic fermentation promotes feed utilization in weaned piglets via biosynthesis and metabolism of amino acid

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Research Article

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Abstract

Probiotics represent a promising alternative to antibiotics in livestock production. This study investigated the effects of compound probiotic fermentation (FAM, comprising *Lactobacillus acidophilus* and *Bacillus subtilis*) on nitrogen utilization and nutrient digestibility in weaned piglets. A total of 180 piglets (28 days old; initial weight: 8.21 ± 0.67 kg) were allocated to three groups: control (basal diet), FAM (basal diet + 0.1% FAM), and antibiotic (basal diet + 55 mg/kg kitasamycin + 75 mg/kg chlortetracycline). After a 30-day trial, FAM supplementation significantly increased apparent nutrient digestibility of crude protein and ether extract, enhanced duodenal and jejunal digestive enzyme activities, and reduced fecal nitrogen excretion and serum urea nitrogen levels ($p < 0.05$). Serum metabolomics revealed that FAM upregulated metabolites linked to energy metabolism (e.g., creatine, L-carnosine), which are metabolites of amino acid metabolism, and enriched pathways such as amino acid biosynthesis and protein digestion. These findings demonstrate that FAM improves nitrogen utilization efficiency and gut health in piglets via biosynthesis and metabolism of amino acids, offering a viable alternative to antibiotics.

Introduction

Weaning-associated challenges, including diarrhea, growth retardation, and elevated mortality rates, pose significant economic and welfare concerns in swine production (Singer et al. 2003). Historically, antibiotics have been widely employed to mitigate these issues by enhancing growth performance and disease resistance. However, their prolonged use has raised global alarms due to unintended consequences such as antimicrobial resistance, drug residues, and gut microbiota dysbiosis (Nwobodo et al. 2022; Uddin et al. 2021). In response, over 40 countries have implemented regulatory restrictions on antibiotic use in livestock, driving the urgent need for safe and sustainable alternatives (Bjrnsson 2017).

Probiotics have emerged as a promising solution, demonstrating the capacity to modulate immune responses, strengthen intestinal barrier function, and competitively exclude pathogens (Sanders et al. 2019; Slizewska et al. 2021). Among these, *Lactobacillus acidophilus* and *Bacillus subtilis* – approved by the Ministry of Agriculture and Rural Affairs as feed additives – exhibit synergistic benefits. *Lactobacillus acidophilus* enhances nutrient absorption and growth performance in piglets through the production of bioactive metabolites such as organic acids and antimicrobial peptides (Lee et al. 2016; Sanchez et al. 2019). Concurrently, *B. subtilis* secretes extracellular enzymes (e.g., proteases, amylases) that improve feed digestibility, while also fostering a favorable gut environment by reducing oxidative stress and suppressing *Escherichia coli* colonization (Ding et al. 2021; He et al. 2023). Critically, *B. subtilis* enhances the survival of *Lactobacillus* strains via oxygen scavenging and co-metabolite production, underscoring the superiority of multi-strain probiotics over single-strain applications (Li et al. 2021).

Building on prior evidence that compound probiotic fermentation (FAM®, a co-culture of *L. acidophilus* and *B. subtilis*) improved feed intake and growth performance while reduced diarrhea incidence in weaned piglets (Xie et al. 2022), as detailed in Supplementary Table S1, this study investigates its mechanistic effects on nitrogen utilization and nutrient metabolism. Using a comparative approach with antibiotic-treated and control groups, we evaluated FAM's impact on (1) apparent nutrient digestibility (AND), (2) fecal nitrogen excretion, (3) digestive enzyme activity, and (4) serum metabolomic profiles. Our findings provide novel insights into how FAM enhances nitrogen retention and metabolic efficiency, offering a viable strategy to optimize swine productivity while aligning with global antibiotic-reduction initiatives.

Table 1. Ingredients and nutrient composition of the base diets

Items	g/kg
Ingredients	
Corn	585
Soybean meal	130
Extruded soybeans	150
Whey powder	50
Fish meal	30
Salt	3
$CaHCO_3$	10
Limestone	12
Bran	20
Premix[a]	10
Total	1,000
Nutrient levels[b]	
Lysine	12.1
Methionine	3.30
Cystine	3.60
Digestible energy ($MJ \cdot kg^{-1}$)	13.3
Crude protein	193.5
Ether extract	51.7
Crude ash	35.8
Crude fiber	25.4
Calcium	9.20
Phosphorus	7.30

[a]The premix provided per kg dry matter of diet: vitamin A 5,000 IU, vitamin B_1 3.0 mg, vitamin B_1 6.5 mg, vitamin B_6 2.4 mg, vitamin D 2,000 IU, vitamin E 20 IU, vitamin K 1.0 mg, biotin 0.4 mg, folic acid 1.45 mg, pantothenic acid 23.0 mg, niacin 1 mg, Fe ($FeSO_4$) 200 mg, Cu ($CuSO_4$) 6 mg, Mn ($MnSO_4$) 30 mg, Zn ($ZnSO_4$) 80 mg, I (KI) 0.2 mg, Se (Na_2SeO_3) 0.3 mg.
[b]Except for the calculated digestible energy, the rest are measured values.

Materials and methods

Animals, diets, and experimental design

The experimental protocol followed the standards of the Animal Care and Use Committee of Zhejiang University (SYXK 2012-0178). One hundred eighty piglets (Duroc × Landrace × Yorkshire hybrid) weaned at 28 days old (average weight of 8.21 ± 0.67 kg) were randomly allocated to three groups with three pens per group, 20 piglets per pen. The three groups consisted of (1) the control group (C), which was fed with basal diet; (2) the FAM group (F), which was fed with basal diet supplemented with 0.1% FAM; and (3) the antibiotic group (A), which was fed with basal diet supplemented with 55 mg/kg kitasamycin and 75 mg/kg chlortetracycline. The basal diet was formulated to meet the nutrient needs for weaned piglets as recommended by the National Research Council (NRC, 1998), with the composition details provided in Table 1. FAM® was provided by Zhejiang Kangwan Dechuan Technology Co., Ltd. (Shaoxing, China), which is a co-fermentation product containing *L. acidophilus* ($\geq 1 \times 10^6$ CFU/g) and *B. subtilis* ($\geq 1 \times 10^6$ CFU/g).

All piglets were housed in a single barn but allocated to three distinct pens within separate spatial regions, ensuring standardized environmental conditions across groups. Husbandry practices conformed to commercial-scale swine farm protocols, including consistent implementation of cleaning, disinfection, and biosecurity measures to maintain optimal hygiene, ventilation, and temperature control. Piglets were kept in pens with unrestricted access to food and water. The experiment period lasts 30 days following a 10-day adaptation period. The intake of feed was recorded during the experiment.

Sample collection and preparation

From 27 to 29 days, for each replicate, 200 g of feces was collected daily, mixed with 10% HCl at a volume ratio of 5:1, and stored at −20°C for further analysis. At the beginning and end of the experiment, each piglet was weighed and recorded, with a 12-hour fasting period before weighing but free access to water. After the feeding trial, two piglets with an average weight were selected from each pen. A total of 18 piglets were slaughtered following 12 h fast. First, 5 g of liver inner lobe was cut in a 2 mL freezing tube. Blood samples were collected from carotid artery and centrifuged at 3000 × g at 4°C for 15 min to obtain the serum. The entire gastrointestinal tract was then excised. The digesta samples from the duodenum were collected and placed into 50 mL falcon tubes. The mucosal samples were scraped from the center of the jejunum using a blade and placed into 2 mL tubes. The samples obtained above were snap frozen in liquid nitrogen and then stored at −80°C until further analysis.

Apparent nutrient digestibility

Fecal samples were dried at 65°C and pulverized to pass a 1.0-mm screen. The ash, ether extract (EE), crude protein (CP), calcium (Ca), and phosphorus (P) contents of the diets and feces were determined in accordance with the standard procedures established by the Association of Official Agricultural Chemists (AOAC (Association of Official Analytical Chemists) 2000). We determined AND using the acid-insoluble ash method, which is characterized by its simplicity, cost-effectiveness, and reliability. The calculation formula is presented below (Van Keulen and Young 1977):

$$\text{AND } (\%) = [1 - (\text{IF} \times \text{nf}) / (\text{if} \times \text{NF})] \times 100$$

where AND is the apparent nutrient digestibility (%), IF is the acid-insoluble ash content (%) in the feed, nf is the nutrient content (%) in the feces, if is the acid-insoluble ash content (%) in the feces, and NF is the nutrient content (%) in the feed.

Fecal nitrogen excretion

The nitrogen content in feces was determined using the Kjeldahl method. The Nesslerization method was used for detecting the ammonium-N. 1 g of feces was soaked in 2.0 mol/L KCl to extract ammonium-N. Then, 10 mL of the filtrate was transferred to a colorimetric tube, and 1 mL of Nessler's reagent was added. The absorbance was determined at a wavelength of 410 nm, and the concentration of ammonium-N was calculated by standard curve.

Digestive enzymatic activity

0.1 g of digesta sample and 0.1 g of mucosal sample were weighed and homogenized with saline (1:9; wt/v) and three steel balls for

20 min (4000 × g, 4°C). The supernatant was used for the determination of the activities of trypsin, lipase, amylase, sucrase, lactase, and maltase following the instructions provided in the commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Antioxidant capacity

The antioxidant capacity of total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) were assessed using ELISA kits according to the instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing city, China).

Serum biochemistry parameters

Blood urea nitrogen (BUN), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in serum were measured according to the instructions provided with kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Serum metabolomic analysis

Serum metabolome analysis was conducted by liquid chromatography-tandem mass spectrometry (LC–MS/MS). LC–MS/MS analysis was conducted on the UHPLC system (Agilent 1290, USA) together with AB Sciex TripleTOF 6600 system (Q-TOF, Concord, ON, Canada).

The file format of the serum metabolome analysis was converted to common data format using MSConvert. Data from LC–MS was pretreated using XCMS 1.41.0. Further multivariate statistical analysis was performed on the SIMCA (Version 14.1, MKS Data Analytics Solutions, Concord, ON, Canada). Orthogonal projections to latent structures discriminant analyses (OPLS-DA) were used for detecting responses of dependent variables to independent variables in all groups. The Q^2 predictive ability parameter and R^2Y goodness-of-fit parameter were calculated to assess model quality using seven-fold cross-validation. Metabolite set enrichment analysis and pathway analysis were performed separately for each identified metabolite and biomarker pathway using the web-based tool MetaboAnalyst, yielding-related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Statistical analysis

Statistical analysis of the data was performed using SPSS software 26.0. All data are presented as mean. Significance was set at $p < 0.05$, and $0.05 \leq p < 0.10$ was viewed as a tendency toward significance. The Kolmogorov–Smirnov test was used to assess the normality of the data distribution. Comparisons between two groups were made using the unpaired Student's *t*-test with Welch's correction or the Mann–Whitney *U* test. For comparisons involving more than two groups, one-way ANOVA was employed to determine significant differences among groups, followed by Bonferroni or Dunnett's T3 post-hoc multiple comparison tests.

Result

AND and fecal nitrogen excretion

As shown in Table 2, in piglets supplemented with FAM or antibiotics, the AND of CP and EE was increased significantly ($p < 0.05$). Additionally, FAM or antibiotics decreased fecal N ($p < 0.05$) and NH_3-N ($p < 0.01$), and the antibiotic group showed higher fecal NH_3-N levels ($p < 0.05$) compared to the FAM group (Table 3).

Digestive enzymatic activity

Based on the positive effect of FAM on the apparent digestibility of piglets, we then measured changes in the activity of intestinal digestive enzymes in piglets. Digestive enzymes are one of the key factors affecting digestibility. In the duodenum (Table 4), FAM or antibiotics supplementation enhanced ($p < 0.05$) the activities of trypsin, lipase, and amylase compared to the control group. Similarly, in the jejunal mucosa (Fig. 2B), the supplementation of FAM or antibiotics enhanced the activities of sucrase, lactase, and maltase ($p < 0.05$).

Serum biochemistry parameters

To evaluate the impact of FAM on the nutritional and health status of piglets, we conducted tests on serum biochemical parameters. As shown in Table 5, the ALT levels were higher in the antibiotic group compared to control and FAM groups ($p < 0.05$), and the BUN levels were lower in FAM and antibiotic groups ($p < 0.05$). No significant differences were observed in the ALP, AST, or TP levels between groups.

Serum metabolomics analysis

We further conducted serum metabolomics analysis to assess the metabolic status and explored the mechanisms underlying the effects on digestion efficiency and nitrogen utilization. The untargeted LC–MS/MS approach was used to analyze the serum metabolite profiles. The total ion chromatogram (TIC) curves of quality control (QC) samples showed that the response intensity and retention time of each chromatographic peak largely overlapped, indicating that there was minimal variation due to instrument error throughout the experimental process (Fig. 1A). The QC samples were closely clustered in the principal component analysis (PCA), reflecting good repeatability (Fig. 1B). The R^2X for PCA was 0.548, suggesting the reliability of the PCA model (Supplementary Table S2). The orthogonal partial least squares discriminant analysis (OPLS-DA) score plot for the serum samples of control, FAM, and antibiotic groups showed clear clustering in Fig. 1C, indicating that FAM or antibiotics significantly influenced the serum metabolome. The Q^2 values of the OPLS-DA, which exceeded 0.5, indicate that model remained stable and reliable (Supplementary Table S2). Furthermore, the regression line in the response permutation testing displayed an upward trend, suggesting that the models did not overfit (Fig. 1D).

Serum differential metabolomics

The volcano plots further illustrated the differences between groups (Fig. 2A). For FAM and control groups, there were 34 different metabolites. For FAM and antibiotic groups, there were 22 different metabolites. For antibiotic and control groups, there were 18 different metabolites. In addition, the differential metabolites are shown in Fig. 2B. The results revealed that, compared to the control group, 24 metabolites, including decanoyl-L-carnitine, L-carnosine, and creatine, were significantly upregulated in the FAM group. In comparison, 10 metabolites, such as L-carnitine,

Table 2. Effect of FAM on apparent nutrient digestibility in weaned piglets

	Treatments				
Items	C	F	A	SEM	*p*-value
DM (%)	74.83[b]	79.91[a]	77.50[a]	0.320	0.003
CP (%)	66.62[b]	73.64[a]	71.22[a]	2.06	<0.001
EE (%)	63.24[b]	68.73[a]	70.81[a]	3.03	0.004
CA (%)	50.54	49.83	51.62	0.521	0.157
Ca (%)	52.75	56.83	53.34	1.27	0.213
P (%)	42.21	43.65	40.35	0.955	0.131

C: control group; F, FAM group; A, antibiotic group. CP, crude protein; EE, ether extract; CA, crude ash. Values are presented as the means, $n = 6$.
[a-b] The mean values within rows with different letters differ significantly ($p < 0.05$).

Table 3. Effect of FAM on fecal nitrogen excretion in weaned piglets

	Treatments				
Items	C	F	A	SEM	*p*-value
N (mg · kg^{-1})	4.61[b]	4.23[a]	4.25[a]	0.123	0.003
NH_3-N (mg · kg^{-1})	4.81[b]	2.85[ab]	3.01[a]	0.628	<0.001

C: control group; F, FAM group; A, antibiotic group. N, nitrogen; NH_3-N, ammonia nitrogen. Values are presented as the means, $n = 6$.
[a-b] The mean values within rows with different letters differ significantly ($p < 0.05$).

Table 4. Effect of FAM on digestive enzymatic activity in weaned piglets

	Treatments				
Items	C	F	A	SEM	*p*-value
Duodenum(U · mg protein^{-1})					
Trypsin	244.32[b]	312.65[a]	324.40[a]	24.9	<0.001
Amylase	28.87[b]	36.37[a]	35.23[a]	2.33	0.006
Lipase	30.25[b]	37.45[a]	38.92[a]	2.68	<0.001
Jejunum mucosa(U · mg protein^{-1})					
Sucrase	15.05[b]	20.82[a]	21.18[a]	1.98	0.017
Lactase	5.77[b]	7.57[a]	7.77[a]	0.636	0.024
Maltase	15.40[b]	19.57[a]	18.30[a]	1.23	0.003

C: control group; F, FAM group; A, antibiotic group. N, nitrogen; NH_3-N, ammonia nitrogen. Values are presented as the means, $n = 6$.
[a-b] The mean values within rows with different letters differ significantly ($p < 0.05$).

Table 5. Effect of FAM on serum biochemistry parameters in weaned piglets

	Treatments				
Items	C	F	A	SEM	*p*-value
BUN (mmol · L^{-1})	5.24[B]	2.47[A]	2.51[A]	0.92	<0.001
TP (g · L^{-1})	60.76	57.74	56.97	1.17	0.416
AST (U · L^{-1})	87.35	88.55	96.57	2.91	0.449
ALP (U · L^{-1})	378.91	423.12	392.31	13.088	0.121
ALT (U · L^{-1})	102.56[b]	110.21[b]	147.86[a]	14.001	<0.001

C: control group; F, FAM group; A, antibiotic group. BUN, blood urea nitrogen; TP, total protein; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase. Values are presented as the means, $n = 6$.
[A-B] The mean values within rows with different capital letters differ extremely significantly ($p < 0.01$).
[a-b] The mean values within rows with different lowercase letters differ significantly ($p < 0.05$).

S-methyl-5'-thioadenosine, and hypoxanthine, were significantly downregulated. Compared to the antibiotic group, 13 metabolites, including acetylcarnitine, L-carnosine, creatine, and creatinine, were significantly upregulated in the FAM group, while 9 metabolites, such as L-pipecolic acid and D-(+)-melibiose, were significantly downregulated. Compared to the control group, 15

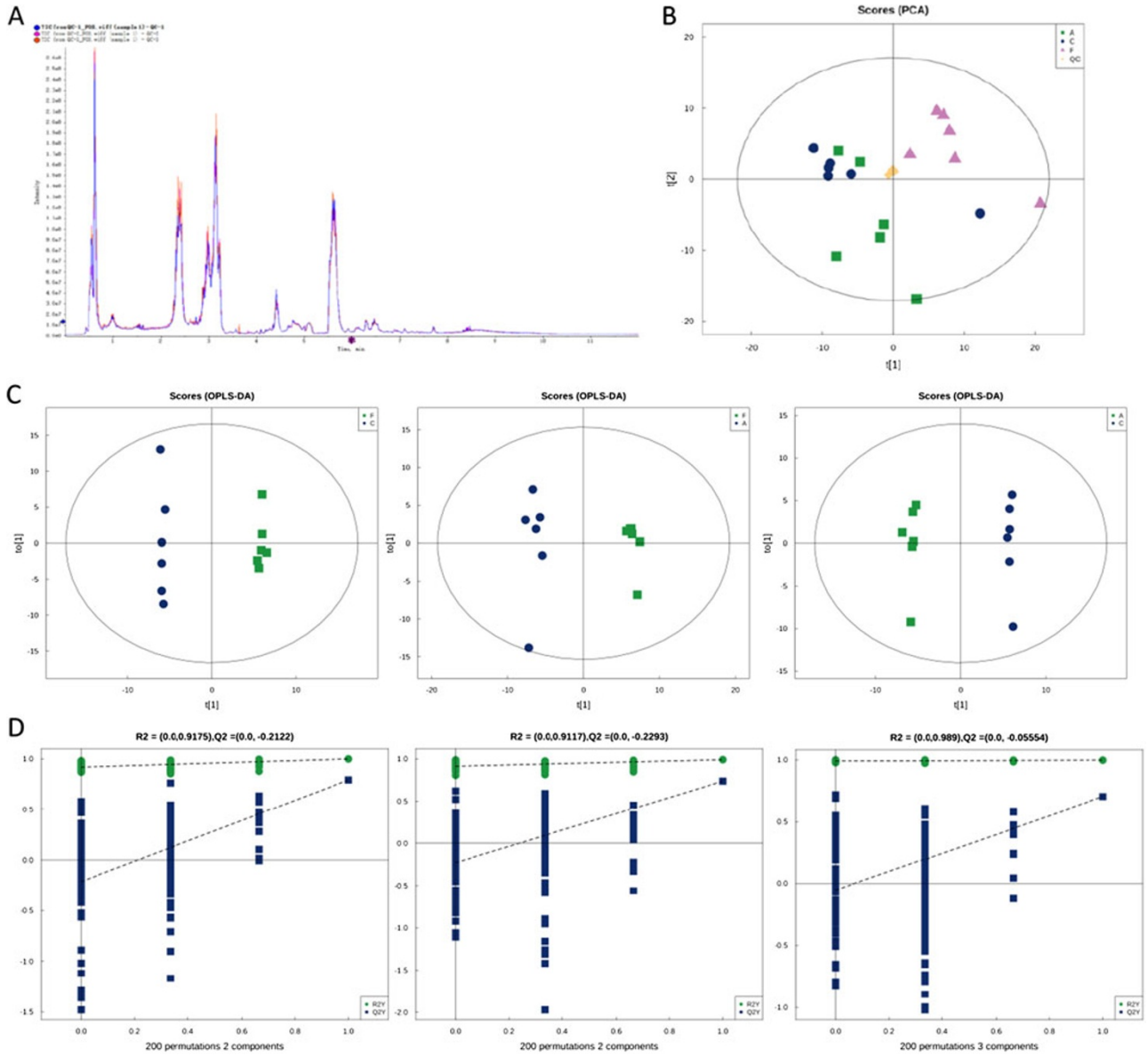

Figure 1. (A) The total ion chromatogram plot of quality control samples. (B) Principal component analysis score plot of QC and serum samples with 95% confidence interval. (C, D) Orthogonal partial least squares discriminant analysis (C) and response permutation testing of serum samples (D). The sequence of each group of images from left to right is as follows: F vs C, F vs A, and A vs C. The following pictures are in the same order. C: control group; F, FAM group; A, antibiotic group.

metabolites, including trimethoprim, arginine-glutamate, glycerophosphocholine, and indole-3-pyruvate acid, were significantly upregulated in the antibiotic group, while acetylcarnitine, 1-aminocyclohexanecarboxylic acid, and hydrocortisone were significantly downregulated. These findings suggest that both FAM and antibiotics can cause significant alterations in serum metabolites of piglets.

Metabolic pathway enrichment analysis of differential metabolites

To identify the significantly different metabolic pathways between groups, we further conducted metabolite enrichment analysis. The metabolic pathway enrichment analysis is shown in Fig. 3. For FAM and control groups, there were significant differences in biosynthesis of amino acids, protein digestion and absorption, ABC transporters, and other pathways. For FAM and antibiotic groups, significant differences emerged in N metabolism, the biosynthesis of amino acids and unsaturated fatty acids, ABC transporters, and other pathways. For antibiotic and control groups, there were significant differences in ABC transporters, galactose metabolism, pyrimidine metabolism, and other pathways. Moreover, compared to the control and the antibiotic groups, the FAM group exhibited enhanced amino acid metabolic activity, including biosynthesis of arginine, valine, leucine and isoleucine, and metabolism of alanine, aspartate, glutamate, histidine, arginine, and proline.

Serum and liver antioxidant parameters

The results for the piglet serum and liver antioxidant ability are presented in Table 6. No significant differences were observed in serum T-AOC and T-SOD across the three groups. Compared

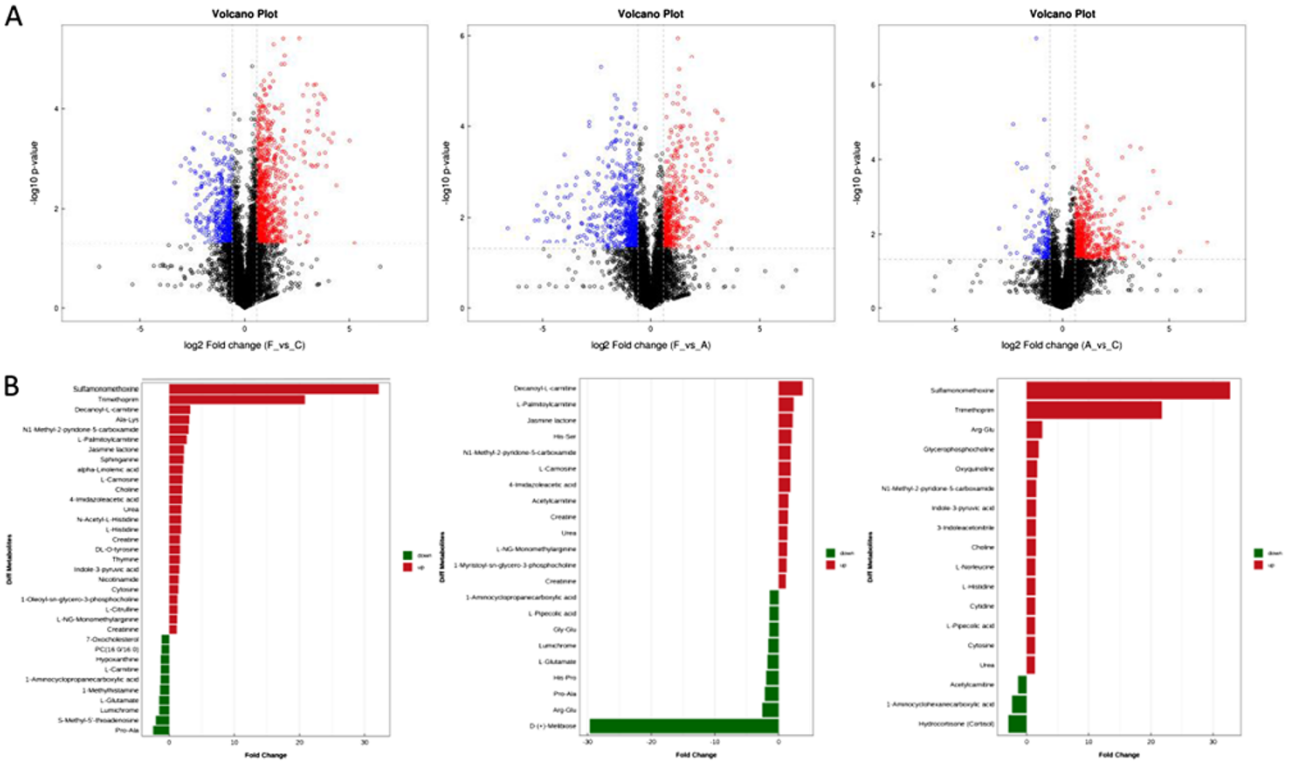

Figure 2. (A) Volcano plots showed the relationship between log2 (fold change) and −log10(*p*-value). (B) LEfSe of serum differential metabolite.

to the control group, serum GSH-Px was significantly increased and serum MDA was significantly decreased ($p < 0.05$) in both FAM and antibiotic groups. Compared to the control and antibiotic groups, T-AOC in liver was significantly increased in the FAM group ($p < 0.05$), while T-SOD showed no significant differences between three groups. Compared to control group, GSH-Px in liver was increased ($p < 0.05$) and MDA in liver was decreased ($p < 0.05$) in both FAM and antibiotic groups, with no significant differences between FAM and antibiotic groups ($p > 0.05$).

Discussion

This study confirms that compound probiotic fermentation (FAM) enhances nutrient utilization in weaned piglets through multifaceted mechanisms. Consistent with prior findings (Xie et al. 2022) demonstrating that the FAM group showed superior average daily gain (ADG), feed conversion ratio (FCR), and diarrhea incidence compared to the control group while performing comparably to the antibiotic (A) group, FAM supplementation significantly elevated the apparent digestibility of CP and EE, respectively, paralleling improvements in duodenal trypsin, amylase, and lipase activities. Concurrently, a reduction in fecal N and NH_3-N concentrations was observed, further explaining FAM's role in promoting lean muscle deposition and nitrogen recycling. Moreover, FAM caused a rise in the concentrations of L-carnosine, creatine, and acetylcarnitine in serum and had a positive effect on metabolic pathways such as amino acids biosynthesis, unsaturated fatty acids biosynthesis, and protein digestion and absorption. Notably, FAM outperformed antibiotics in safety metrics, as evidenced by elevated serum ALT levels in the antibiotic group, a marker of potential hepatotoxicity (Yin et al. 2023).

The synergistic utilization of multiple microbial strains holds the potential to amplify probiotic efficacies through intricate inter-strain interactions. Unlike single-strain formulations, the combined use of *L. acidophilus* and *B. subtilis* in FAM leverages complementary functional roles: *Lactobacillus* acidifies the intestinal environment through lactic acid production, inhibiting pathogens and stimulating endogenous enzyme activity (Gao et al. 2022), while *Bacillus* secretes extracellular hydrolases that break down complex nutrients (Sella et al. 2015). Such interactions can stimulate the biosynthetic potential of the constituent strains, thereby fostering host growth and augmenting the overall salutary impact on host health (Selegato and Castro-Gamboa 2023). This aligns with studies showing multi-strain probiotics outperform single-strain formulations in viability and metabolic output (Moussavi et al. 2023), even rivaling fecal microbiota transplantation in restoring gut health (Kurt et al. 2023). Hence, the observed improvement in digestion and absorption capabilities can plausibly be ascribed to the co-fermentation of diverse microbial strains. This connection bridges the gap between the observed benefits of FAM and the broader context of multi-strain probiotic strategies, highlighting the importance of strain combinations in achieving optimal results.

The digestibility of nutrients is a crucial factor influencing the growth response in weaned piglets (Jones and Patience 2014). In light of the recent implementation of antibiotic bans, there has been a notable increase in the use of probiotics and their fermentation products as feed additives. Lan et al. (2016) reported that *L. acidophilus* increased the digestibility of dry matter, nitrogen, crude fiber, and gross energy in weaned piglets. Yuan et al. (2017) found that soybean meal fermented by *B. subtilis, Hansenula anomala*, and *Lactobacillus casei* increased the digestibility of CP, EE, Ca, and P of piglet. Similarly, Dowarah et al. (2018) found that

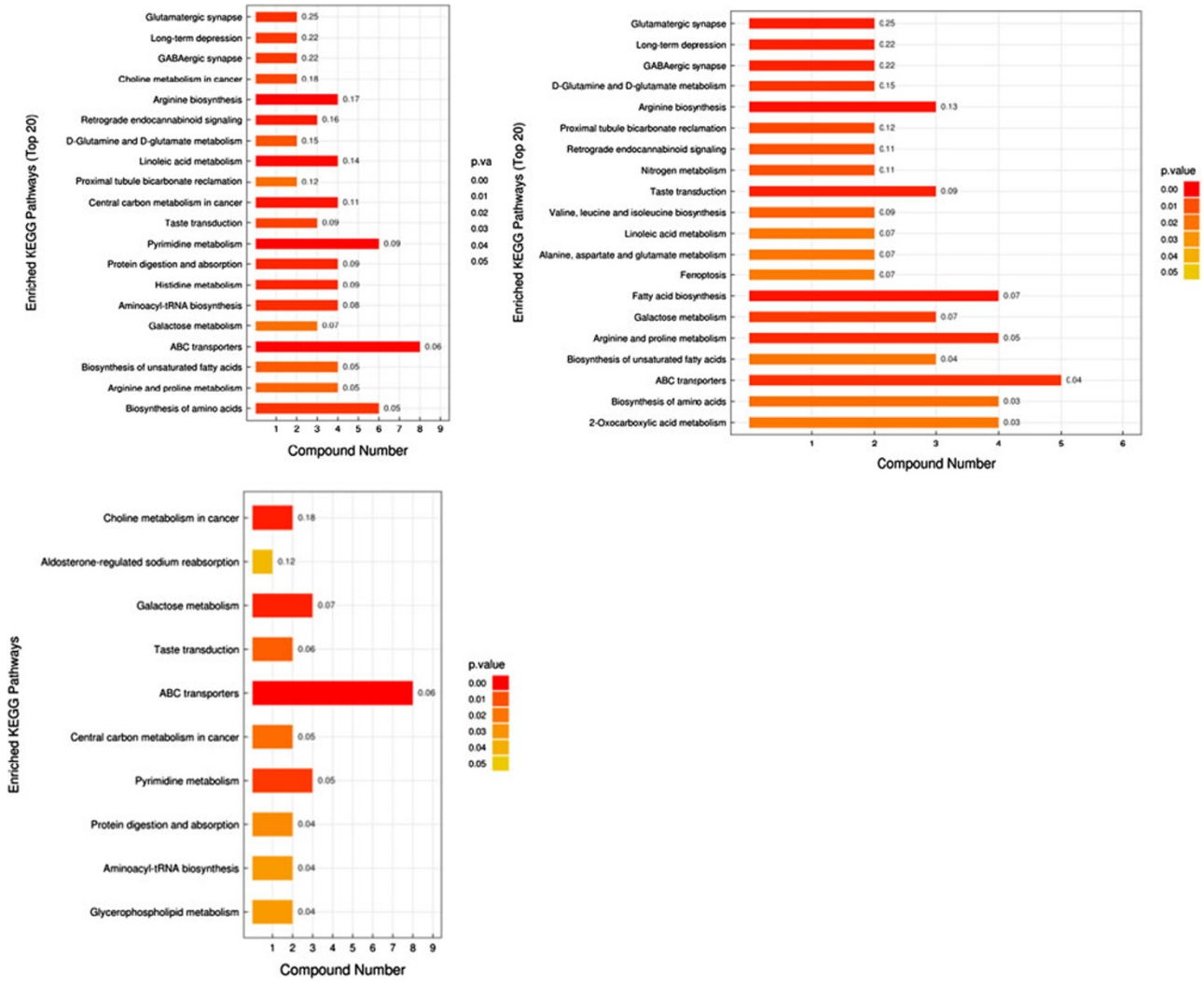

Figure 3. The KEGG pathway enrichment analysis of serum different metabolites.

Table 6. Effect of FAM on antioxidant capacity of serum and liver in weaned piglets

	Treatments				
Items	C	F	A	SEM	*p*-value
Serum					
T-AOC (mmol · L^{-1})	0.80	0.85	0.94	0.411	0.134
T-SOD (U · mL^{-1})	27.67	30.03	30.18	0.812	0.115
GSH-Px (U · mL^{-1})	632.14[b]	739.29[a]	712.5[a]	32.2	0.032
MDA (nmol · mL^{-1})	6.99[a]	4.92[b]	4.67[b]	0.735	<0.001
Liver					
T-AOC (mmol · g^{-1})	0.41[b]	0.51[a]	0.46[b]	0.029	0.003
T-SOD (U · mg^{-1})	108.66	113.19	112.72	1.44	0.078
GSH-Px (U · mg^{-1})	725.22[b]	805.16[a]	789.73[a]	24.5	<0.001
MDA (nmol · mg^{-1})	7.25[a]	6.21[b]	6.76[b]	0.300	0.011

C: control group; F, FAM group; A, antibiotic group. MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase. Values are presented as the means, $n = 6$.
[a-b] The mean values within rows with different letters differ significantly ($p < 0.05$).

the digestibility of CP and EE was better in pigs fed *Pediococcus acidilactici* FT28. This study demonstrated that FAM significantly enhances the ATD of CP and EE. Furthermore, the increase in serum ALT levels in the antibiotic group suggests liver damage in piglets, possibly due to hepatocellular necrosis or increased membrane permeability (Shaban et al. 2022; Sookoian and Pirola 2015). These findings cumulatively suggest that FAM represents a viable, efficacious, and safer substitute for antibiotics.

Nutrient digestion is closely linked to the activity of digestive enzymes, which directly affects the efficiency and performance of the digestive system. Research indicates that digestive enzyme activity drops to about one-third of pre-weaning levels one week after weaning, typically taking up to two weeks for these activities to return to or exceed pre-weaning levels (Shi et al. 2022). In animal experiments, *B. subtilis* was shown to increase protease and amylase activities (Liu et al. 2017), while *Lactobacillus lactis* enhanced the activities of protease, amylase, and lipase (Kong et al. 2021). Similarly, the current study demonstrates that the activities of duodenal digestive enzymes and jejunal mucosal disaccharidases of piglets in the FAM group were elevated, corresponding to improved digestibility. The positive effects of FAM on digestive enzyme activity are likely due to reduced intestinal pH (Shi et al. 2022). *Lactobacillus acidophilus* can produce lactic acid, which lowers intestinal pH and inhibits pathogenic bacteria (Deng et al. 2022). Meanwhile, *B. subtilis* forms spores with strong survival capabilities in the gastrointestinal tract, exhibiting acid resistance and thermal stability while promoting the growth of *Lactobacillus* (Cutting 2011). Therefore, the synergistic action of these two strains in FAM likely contributed to improving the intestinal environment, thereby enhancing digestive enzyme activity and AND.

Increased nitrogen utilization efficiency is one of the manifestations of improved nutrient digestibility. We observed that FAM reduced fecal N and NH_3-N content, as well as serum BUN levels, of which the reduction is inversely correlated with the feed's biological value (Bassily et al. 1982). These results indicate an improvement in nitrogen utilization efficiency, which partly explains the enhanced digestibility of piglets. Previous studies have shown that probiotics exhibit such effect. For instance, adding a mixture containing active dry yeast to the diet of dairy cows resulted in a reduction of fecal NH_3-N content over a period of 7 to 35 days (Vasil and Evgeni 2023). In another study conducted by Zhao and Kim, it was observed that feed mixed with *Lactobacillus reuteri* and *Lactobacillus plantarum* reduced fecal NH_3-N in weaned piglets (Zhao and Kim 2015). A possible explanation for these findings may be the enhancement of intestinal flora, which aids in converting small non-protein waste in the blood into microbial proteins that can be digested and absorbed (Zheng et al. 2020).

Serum metabolites reflect changes in metabolic activity, providing insights into underlying biochemical processes. In this study, FAM promoted amino acid metabolism in piglets, increasing the levels of metabolites such as L-carnosine, creatine, and acetylcarnitine. Additionally, FAM enhanced differential metabolic pathways including amino acid biosynthesis and protein digestion and absorption. Carnosine, a dipeptide constituted by β-alanine and L-histidine, is endowed with the capacity to scavenge reactive oxygen species and neutralize α,β-unsaturated aldehydes that are formed during lipid peroxidation under oxidative stress. Increased carnosine concentration through blood meal or β-alanine reduces drip loss in pork, increases lean meat yield, decreases backfat thickness, and enhances muscle antioxidant capacity (Park et al. 2014; Wang et al. 2022). Creatine, along with phosphocreatine and creatine kinase, functions as an intracellular energy transfer system. Creatine metabolism in fats can induce thermogenesis and combat obesity (Greenhill 2017). Studies have shown that creatine supplementation promotes muscle energy metabolism, enhances protein synthesis, and improves growth and feed efficiency, while preventing rapid pH decline post-slaughter (Ying et al. 2013; Young et al. 2005, 2007).

The up-regulation of metabolites, amino acid biosynthesis and protein digestion and absorption aligns with the positive changes in AND, BUN, and nitrogen excretion. Furthermore, the experimental results indicate that FAM supplementation significantly enhanced the metabolic pathways of arginine, branched-chain amino acids (valine, leucine, and isoleucine), aspartate, alanine, proline, and histidine. These amino acids perform critical biological functions including, promoting urea cycle and nitric oxide production (Gao K et al., Posset R et al. 2024), regulating muscle metabolism (Zhang L et al. 2022), participating in protein and nucleotide biosynthesis (Bröer S and Bröer A 2017, Holeček M 2023), facilitating nitrogen transport (Zhang X et al. 2007), promoting collagen synthesis (Li and Wu 2017), and contributing to carnosine formation (Wang et al. 2022). Consequently, these metabolic modifications provide evidence for FAM's capacity to enhance whole-body nitrogen utilization efficiency.

Weaning stress generates excess free radicals, disrupting redox balance and causing oxidative damage to lipids, proteins, and DNA. SOD and GSH-Px scavenge free radicals, while MDA, a product of lipid peroxidation, serves as a marker for oxidative damage. These indicators are critical for assessing antioxidant status in animals. Tang et al. (2024) reported that combined use of *Lactobacillus plantarum, Lactobacillus rhamnosus*, and *Bifidobacterium longum* reduced serum MDA levels in weaned piglets and pregnant sows, while increasing GSH-Px and SOD levels. Similarly, Li et al. (2019) found that *Lactobacillus delbrueckii* reduced serum and liver MDA levels within 4 weeks while enhancing liver GSH-Px activity. In this study, FAM significantly enhanced liver T-AOC and increased GSH-Px levels, while reducing MDA levels in both serum and liver; in line with the improvement of metabolism, it is suggested that FAM can enhance the metabolic functions, promote protein deposition, and thereby improve the antioxidant capacity.

Conclusion

In conclusion, 0.1% FAM enhances weaned piglet performance through a tripartite mechanism: (1) enzymatic nutrient hydrolysis via strain synergy, (2) nitrogen conservation through biosynthesis and metabolism of amino acids, and (3) antioxidant mitigation of weaning stress. By elevating digestibility indices (CP, EE) and reducing nitrogen waste, FAM not only improves ADG, FCR, and diarrhea incidence, but also addresses environmental concerns linked to intensive swine production. Its safety profile, contrasted with antibiotic-associated hepatotoxicity, positions FAM as a sustainable alternative in the era of antimicrobial stewardship. Future research should explore FAM's long-term impacts on gut microbiota resilience and carcass quality to fully realize its translational potential.

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M.H. and L.-z.X. contributed equally to this study.

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