

Dietary supplementation with fermented *Broussonetia papyrifera* enhances tissue mineral deposition and intestinal microbiome in finishing pigs

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ABSTRACT. The objective of this study was to investigate the dietary effects of fermented *Broussonetia papyrifera* (FBP) on serum biochemistry, mineral deposition, and intestinal microbiota in finishing pigs. Thirty-six grower pigs (18 males and 18 females, with an initial body weight of 12.25 ± 0.15 kg) were selected and randomly assigned to two dietary treatment groups: CON group fed a basal diet, and treatment group fed an isonitrogenous and isocaloric FBP diet (20% FBP). Each treatment included 6 replicates with 3 pigs in each replicate, and the feeding trial lasted 6 months. The results showed that dietary FBP significantly reduced ammonia, alanine aminotransferase, aspartate aminotransferase and urea nitrogen in the serum of finishing pigs. In addition, the FBP diet significantly reduced the concentration of iron, calcium, magnesium, zinc, and manganese in serum, as well as magnesium and manganese levels in faeces, while significantly increasing selenium, copper, and iron concentrations in the liver and iron and selenium in muscles. Dietary FBP raised the abundance of *Terrisporobacter*, *Clostridium butyricum*, *Akkermansia*, and *Turicibacter*, while decreasing the abundance of *Lactobacillus* in the colon. The abundance of *Unclassified_k_norank_d_Bacteria* was closely related to iron and magnesium concentrations. In conclusion, these results suggest that the dietary effect of FBP on absorption, metabolism and deposition of minerals could be attributed to an altered microbial community in the colon. We recommend further studies to unravel the underlying mechanisms by which gut microbes modulate mineral deposition.

Introduction

The hybrid *Broussonetia papyrifera* (BP) has been widely planted and utilised as an unconventional feed source in recent years (An et al., 2021; Zhou et al., 2022). It is rich in amino acids, fatty acids, vitamins, and a variety of biologically active compounds such as flavonoids, polysaccharides, alkaloids, terpenes, and diphenylpropane (Chao et al., 2006; Hanting et al., 2011). BP has mainly

been used in ruminant nutrition to improve growth performance of beef cattle (Xiong et al., 2021; Wen et al., 2022), carcass traits of sheep (Sheng et al., 2021), antioxidant capacity of dairy goats (Zhang et al., 2022), and immune response of dairy cows (Si et al., 2018; Hao et al., 2020). Polyphenols and flavonoids present in BP are compounds primarily responsible for the increased antioxidant activity and immunity of host animals. In addition, BP can affect the ruminant microflora and be utilised

by rumen microbes to produce different metabolites, e.g., butyric acid and acetic acid (Han et al., 2022). To date, very few studies have explored the nutritional and medicinal effects of BP in monogastric animals. Dietary supplementation of fermented BP has been shown to improve serum superoxide dismutase (SOD), feed intake, average egg weight, and *Lactobacillus* abundance in the caecum of laying hens (Niu et al., 2022). The inclusion of BP leaf extract has been reported to improve pork meat quality by increasing intramuscular fat and amino acid contents in muscles (Yang et al., 2014; Zhang et al., 2019; Song et al., 2020). The inclusion of BP in pig feed was found to increase the relative abundance of Bacteroidetes and decrease the relative abundance of Firmicutes in the colon (Duan et al., 2022).

Plant feed sources may impact the absorption, deposition and transport of trace elements in animals. The addition of phytase-treated plant feedstuff was demonstrated to increase the absorption of dietary minerals by common carp (Nwanna et al., 2007). The macroalga *Enteromorpha* sp. was able to enhance the deposition of Cr, Mn, Fe, Cu, and Zn in meat of finishing pigs and reduce Cu excretion in faeces (Michalak et al., 2015). At present, the effects of dietary fermented BP (FBP) on mineral deposition and intestinal microbiota in finishing pigs remain unexplored. Therefore, the purpose of this study was to investigate the influence of FBP supplementation in the diet on mineral contents in serum, muscle, liver, and faeces, as well as changes in the intestinal microbial community in finishing pigs.

Material and methods

Animal ethics

All animal care procedures and experimental protocols applied in this study were approved by the Animal Care and Use Committee of the Tianjin Institute of Industrial Biotechnology of the Chinese Academy of Sciences (Approval No. 20200708).

Animal management and experiment design

A single factor unpaired group design with two types of diets: a control (CON) diet and a fermented *Broussonetia papyrifera* (FBP) diet, was used in the animal experiment. A total of 36 piglets (Duroc × Landrace × Large Yorkshire, half males and half females) with an average initial body weight of 12.2 ± 0.15 kg were randomly allocated to two treatment groups: the CON group fed with

Table 1. Ingredients and composition of the experimental diets for finishing pigs (as-fed basis, %)

Items	Groups	
	CON	FBP
Ingredients, %		
maize	63	43
soybean meal	10	3
oil bran	15	20
rice bran	8	10
fermented <i>Broussonetia papyrifera</i>	0	20
CaHPO ₄	0	0
limestone	0	0
salt	0	0
premix ¹	4	4
total	100	100
Chemical composition		
digestible energy, MJ/kg	12.28	12.38
lysine	0.6	0.6
methionine	0.2	0.2
crude protein, %	14.47	14.44
crude fibre, %	8	10.82
total calcium, %	0.4–1.1	0.47–1.19
total phosphorus, %	0.66	0.68

CON – control group, FBP – basal diet + 20% fermented *Broussonetia papyrifera*; ¹ finisher period: supplied per kg of diet: IU: vit. A 10800, vit. D₃ 4000, vit. E 40; mg: vit. K₃ 4, vit. B₆ 6, vit. B₂ 12, vit. B₁₂ 0.05, biotin 0.2, folic acid 2, niacin 50, D-calcium pantothenate 25, Cu (as copper sulphate) 25, Fe (as ferrous sulphate) 100, Mn (as manganese oxide) 40, Zn (as zinc oxide) 80, I (as potassium iodide) 0.5, Se (as sodium selenite) 0.3; values of digestible energy, methionine and lysine were calculated, while the others were measured

a basal diet and the FBP group fed with a basal diet supplemented with 20% FBP (energy and nitrogen equivalently formulated). Each group was housed in 6 pens (2.0 × 1.20 × 1.00 m, length × width × height), 3 pigs each, and each pen was considered a replicate. All experimental diets met the nutritional requirements for finishing pigs. The ingredients and nutritional composition are shown in Table 1, while FBP mineral composition is provided in Table 2. The experimental animals were fed twice daily (at 9:00 and 18:00), and water was available *ad libitum*.

Table 2. Mineral content in fermented *Broussonetia papyrifera*, mg/g

Items	Fermented <i>Broussonetia papyrifera</i>
Ca	37.377
Mg	12.086
P	4.682
Cu	0.022
Fe	1.588
Mn	0.269
Se	0.117
Zn	0.187

all values in Table 2 were measured

After one week of acclimation, the feeding trial lasted for 6 months and the pig rearing environment, including temperature, humidity and light, met standard requirements.

Sample collection

At end of the experiment, one pig was selected from each pen and fasted overnight. The blood was collected from the jugular vein and centrifuged at 8000 g for 10 min at 4 °C. After blood collection, the pigs were slaughtered and liver (20 g) and *longissimus dorsi* muscle (20 g) were collected, stored in liquid nitrogen, and transferred to -80 °C prior to analysis. Colon contents were collected from each finishing pig and immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

Serum biochemical analysis

Serum biochemical indices were determined using a BC-6800 automatic analyzer (Mindray, Shanghai, China), including levels of serum total protein (TP), albumin (ALB), blood urea nitrogen (BUN), and blood ammonia (NH₃L), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities, as well as glucose (GLU), and ceruloplasmin (CER) levels, and unsaturated iron binding capacity (UIBCI). Serum immunoglobulin G (IgG) and immunoglobulin M (IgM) were assayed using OUSAID Bio kits (OUSAID Bio, Shanghai, China) following standard instructions.

Mineral content determination

Inductively coupled plasma optical emission spectrometry (ICP-OES-5110, Agilent, Changsha, China) was used to determine the elemental concentrations of Ca, P, Fe, Zn, Cu, and Mn in finishing pig samples. Freeze-dried samples of muscles, liver, faeces (2.00 ± 0.05 g), and serum samples (0.3 ml) were prepared in triplicate. Concentrated nitric acid (8 ml) and hydrogen peroxide (1 ml) were added to each sample, which was then placed in a digestion furnace until digestion was completed. The acid rush program was set at 180 °C for 90 min. If digestion was not complete within this time, it was extended appropriately until one drop of liquid remained in the tube, and then 1% nitric acid was added. The samples with added nitric acid were poured into 10 ml test tubes. The digestion jars were rinsed three times with 1% nitric acid, and the cleaning solution was also poured into the 10 ml test tubes. Finally, the solution in each test tube was adjusted to a final volume of 10 ml.

Colonic microbiota analysis

Total DNA extraction and high-throughput sequencing analysis of the collected colonic content samples were performed by a commercial service of Shanghai Meiji Biotechnology Co., Ltd (Shanghai, China). To obtain species classification information corresponding to each operational taxonomic unit (OTU), the Bayesian algorithm of the ribosomal database project (RDP) classifier was employed for taxonomic analysis of the representative OTU sequences with a 97% similarity level. The bacterial composition of each sample was determined at all classification levels, including phylum, class, order, family, genus, and species. Alpha-diversity was assessed using richness parameters (Chao 1 index and Ace index) and diversity parameters (Shannon diversity index and Simpson index). Significant differences in α -diversity between the two treatment groups were determined using Student's t test. A Venn diagram was used to present the distribution of common and unique species in the two treatments. Beta-diversity was determined using principal coordinate analysis (PCoA). Significant differences in bacterial species between the two experimental groups were determined using the Mann-Whitney U-test. Potential functional profiles of bacterial communities, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) were generated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2, <https://github.com/picrust/picrust2>) and annotated using the MetaCyc and ENZYME databases. Student's t test was used to compare significant differences in KEGG level 1 and 3 pathways between the two experimental groups. Spearman's analysis was used to analyse potential associations between intestinal microbiota and mineral elements based on the relative abundance at the phylum and genus levels using a numerical matrix of correlation coefficients calculated using the R package.

Statistical analysis

The raw data were preliminarily sorted using Excel 2019. Independent sample t-test was performed using IBM SPSS Statistics 20.0 (IBM Corporation, Summers, NY, USA), and graphs were produced using GraphPad 8.0.2 (GraphPad Software, San Diego, CA, USA). Data are presented as means and the standard error of the mean (SEM). Statistical values were interpreted as follows: $0.05 < P < 0.1$ indicated a significant trend, $P < 0.05$ indicated a significant difference, and $P < 0.01$ indicated an extremely significant difference.

Results

Effects of dietary FBP on serum biochemical parameters

The impact of the FBP diet on serum parameters in finishing pigs was determined (Table 3). Compared to the CON group, feeding FBP significantly reduced the values of NH_3L , ALT, AST ($P < 0.05$), and BUN ($P < 0.01$). On the other hand, there were no significant changes observed in TP, ALB, GLU, UIBCI, and CER nor in immune parameters, including serum IgG and IgM titres ($P > 0.05$).

Table 3. Effect of dietary fermented *Broussonetia papyrifera* on serum biochemical parameters of finishing pigs

Items	Groups		P-value
	CON	FBP	
TP, g/l	83.883 ± 4.218	79 ± 2.688	0.345
ALB, g/l	59.567 ± 2.049	52.417 ± 2.540	0.649
BUN, mmol/l	5.95 ± 0.612 ^a	3.783 ± 0.147 ^c	0.007
NH_3L , $\mu\text{mol/l}$	634.8 ± 195.8 ^a	350.4 ± 49.84 ^b	0.034
ALT, U/l	74.35 ± 10.266 ^a	71.083 ± 2.728 ^b	0.011
AST, U/l	97.5 ± 24.142 ^a	73.5 ± 8.617 ^b	0.041
ALP, U/l	146 ± 8.012	138.167 ± 14.918	0.199
GLU, mmol/l	6.4 ± 0.841	5.65 ± 0.765	0.841
UIBCI, $\mu\text{mol/l}$	90.18 ± 5.116	72.82 ± 1.939	0.053
CER, g/l	0.092 ± 0.009	0.088 ± 0.012	0.556
IgG, g/l	3.43 ± 0.388	3.935 ± 0.304	0.605
IgM, g/l	1.533 ± 0.117	1.646 ± 0.120	0.932

CON – control group, FBP – fermented *Broussonetia papyrifera* diet, TP – total protein, ALB – albumin; BUN – urea nitrogen, NH_3L – blood ammonia, ALT – alanine aminotransferase, AST – aspartate aminotransferase, ALP – alkaline phosphatase, GLU – glucose, UIBCI – unsaturated iron binding capacity, CER – ceruloplasmin, IgG – immunoglobulin G, IgM – immunoglobulin M; the data are presented as means ± standard error of the mean ($n = 6$), ^{ab} – means within the rows with different superscripts are significantly different at $P < 0.05$, ^{ac} – means within the rows with different superscripts are significantly different at $P < 0.01$

Dietary effects of FBP on mineral deposition in finishing pigs

Mineral concentrations in serum, liver, muscle, and faeces of finishing pigs were determined and are presented in Table 4. In relation to the CON treatment, dietary FBP significantly decreased the concentration of Fe, Ca, Mg, Zn, and Mn ($P < 0.05$), without affecting serum Cu, Se, and P levels ($P > 0.05$). In the liver, dietary FBP significantly increased the concentrations of Se, Cu, and Fe ($P < 0.05$), without causing significant changes in Ca, Mg, Mn, and Zn levels ($P > 0.05$). In muscle tissue, FBP supplementation significantly increased the concentrations of Fe and Se ($P < 0.05$), while Ca, Mg, P, and Zn levels remained

Table 4. Effect of dietary fermented *Broussonetia papyrifera* on the mineral content in finishing pigs

Items	Groups		<i>P</i> -value
	CON	FBP	
Serum			
Ca, mg/ml	0.749 ± 0.072 ^a	0.379 ± 0.034 ^c	<0.001
Mg, mg/ml	0.152 ± 0.016 ^a	0.034 ± 0.007 ^c	<0.001
P, mg/ml	0.210 ± 0.007	0.194 ± 0.004	0.087
Cu, µg/ml	2.951 ± 0.102	2.797 ± 0.165	0.446
Fe, µg/ml	18.15 ± 0.859 ^a	13.39 ± 1.336 ^b	0.013
Mn, µg/ml	1.360 ± 0.278 ^a	0.230 ± 0.012 ^c	0.003
Se, µg/ml	0.307 ± 0.019	0.321 ± 0.002	0.240
Zn, µg/ml	20.69 ± 1.667 ^a	12.26 ± 0.875 ^c	0.001
Liver			
Ca, mg/g	0.862 ± 0.029	0.902 ± 0.039	0.425
Mg, mg/g	0.604 ± 0.031	0.631 ± 0.016	0.468
P, mg/g	10.92 ± 0.496	11.87 ± 0.144	0.065
Cu, mg/g	0.074 ± 0.007 ^c	0.17 ± 0.012 ^a	<0.001
Fe, mg/g	0.763 ± 0.067 ^c	1.102 ± 0.024 ^a	0.002
Mn, µg/g	6.722 ± 0.407	5.853 ± 0.277	0.108
Se, µg/g	0.392 ± 0.042 ^b	0.577 ± 0.045 ^a	0.022
Zn, mg/g	0.198 ± 0.021	0.168 ± 0.004	0.589
Muscle			
Ca, mg/g	0.788 ± 0.038	0.845 ± 0.022	0.226
Mg, mg/g	0.996 ± 0.021	1.072 ± 0.046	0.164
P, mg/g	8.608 ± 0.213	9 ± 0.281	0.292
Cu, µg/g	4.416 ± 0.607	7.867 ± 1.793	0.065
Fe, µg/g	59.64 ± 2.768 ^c	82.25 ± 3.359 ^a	<0.001
Mn, µg/g	2.937 ± 0.212	3.548 ± 0.162	0.093
Se, µg/g	0.324 ± 0.034 ^c	0.517 ± 0.029 ^a	0.002
Zn, µg/g	90.6 ± 3.216	97.55 ± 5.789	0.319
Faeces			
Ca, mg/g	22.78 ± 1.016	24.74 ± 1.936	0.389
Mg, mg/g	5.986 ± 0.259 ^a	4.575 ± 0.225 ^c	0.002
P, mg/g	10.32 ± 0.629	8.916 ± 0.636	0.148
Cu, mg/g	0.399 ± 0.010	0.368 ± 0.020	0.193
Fe, mg/g	1.858 ± 0.136	1.79 ± 0.114	0.711
Mn, mg/g	0.390 ± 0.010 ^a	0.320 ± 0.023 ^b	0.018
Se, µg/g	0.171 ± 0.016 ^c	0.282 ± 0.014 ^a	<0.001
Zn, mg/g	0.474 ± 0.017	0.406 ± 0.026	0.055

CON – control group, FBP – fermented *Broussonetia papyrifera* diet; the data are presented as means ± standard error of the mean ($n = 6$); ^{ab} – means within the rows with different superscripts are significantly different at $P < 0.05$; ^{ac} – means within the rows with different superscripts are significantly different at $P < 0.01$

unchanged ($P > 0.05$). In faecal samples, dietary FBP significantly increased the concentration of selenium ($P < 0.05$) and decreased the concentrations of Mn and Mg ($P < 0.05$). Ca, P, Cu, and Fe levels in faeces showed no significant changes ($P > 0.05$).

Correlation analysis of mineral deposition in different organs of animals from the CON and FBP groups is shown in Figure 1. Both hierarchical cluster analysis (HCA) and principal component

analysis (PCA) revealed different organ dependent clustering between the two experimental groups for eight minerals: Ca, Mg, P, Cu, Fe, Mg, Se, and Zn (Figure 1A and 1B). Ca and Zn concentrations were significantly decreased only in serum, while Cu was solely significantly enriched in the liver. Reduced levels of Mg and Mn were detected both in serum and faeces. The concentration of Fe showed a decline in serum and an increase in the liver and muscle. Se deposition was higher in the liver, muscle, and faeces (Figure 1C). Spearman correlation analysis indicated several associations: serum Ca positively correlated with serum Mg and Zn, and negatively correlated with liver Fe, and muscle Fe and Se. Serum Fe showed negative correlations with liver and faecal Se levels. On the other hand, serum Mg was positively correlated with serum Zn and

faecal Mg, while negatively correlated with liver Fe and muscle Fe and Se levels. Mn in serum was positively correlated with Mn in the liver. Serum levels of Zn correlated positively with faecal Se, while negatively with muscle Fe and Se, as well as faecal Se concentrations (Figure 1D).

Effects of dietary FBP on colonic microbiota

The influence of FBP supplementation on colonic microbiota of finishing pigs is presented in Figure 2. The inclusion of FBP did not significantly change Shannon- and Simpson-based α diversity indices, but tended to increase α -diversity of the colonic microbiota based on ACE and Chao1 (Figure 2A–D). A total of 733 species were identified, including 513 shared species between the CON and FBP groups, as well as 54 and 166 unique

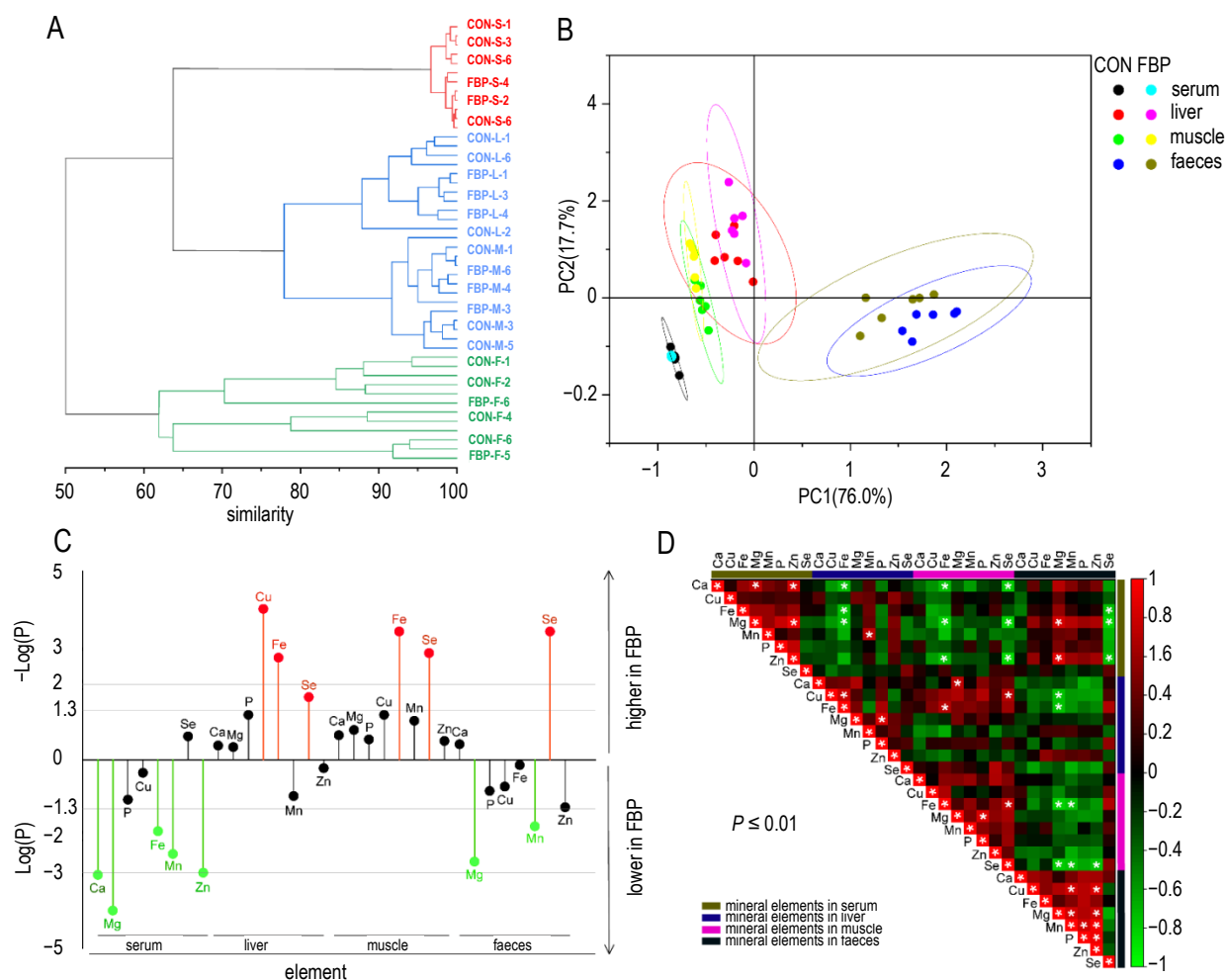


Figure 1. Effect of dietary fermented *Broussonetia papyrifera* on mineral deposition in different organs of pigs: (A) Cluster analysis for CON and FBP. Numbers denote individual samples; (B) Principal component analysis (PCA); (C) The P -value of FBP and CON were compared for significance. The horizontal and vertical axes represent the 8 elements studied, and the comparison of the concentrations of each element in CON and FBP, respectively. A red dot indicates that an element has a higher concentration in the FBP than in the CON group; a green dot indicates that an element has a lower concentration in FBP than in CON; a black dot indicates that the difference in element concentrations in COM and FBP was not significant; (D) Correlation analysis of mineral elements; red and green in the upper triangular squares represent positive and negative correlations, respectively; * denotes significant difference at $P < 0.01$, # denotes trend at $P < 0.001$ level

CON – control group, FBP – fermented *Broussonetia papyrifera* diet

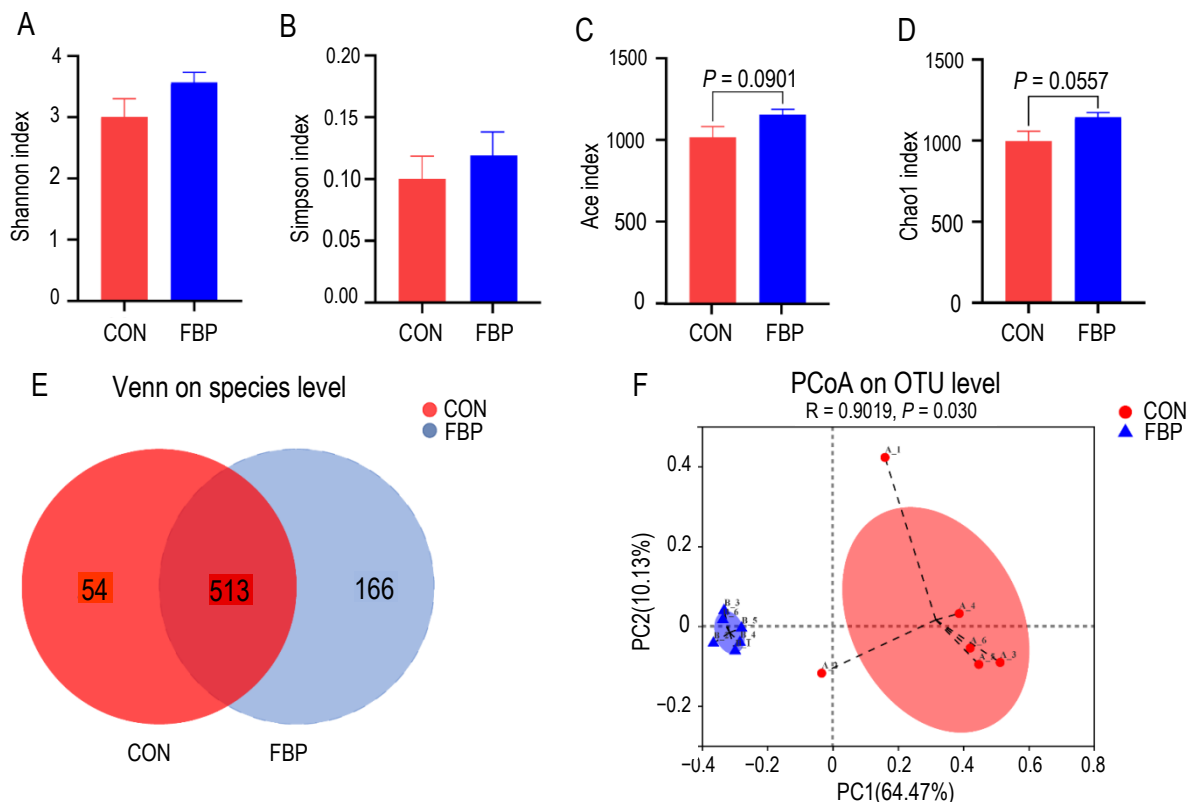


Figure 2. Effect of dietary fermented *Broussonetia papyrifera* on gut microbial diversity of pigs. (A) Shannon index; (B) Simpson index; (C) ACE index; (D) Chao1 index; (E) Venn Diagram; (F) Principal coordinate analysis (PCoA) of samples based on unweighted UniFrac distances. CON – control group, FBP – fermented *Broussonetia papyrifera* diet

species in the CON and FBP groups, respectively (Figure 2E). Unweighted UniFrac distance-based PCA showed differentially clustered colonic microbiota between the two experimental groups (Figure 2F). At the phylum level, Firmicutes, Verrucomicrobia, Actinobacteriota, Spirochaetes, Bacteroidetes, and Proteobacteria were predominant. The abundance of Verrucomicrobia and Actinobacteriota was significantly increased by the FBP diet ($P < 0.05$) (Figure 3A). At the genus level, the dominant taxa were *Lactobacillus*, *Streptococcus*, *Clostridium*, and *Treponema*. Further, the abundance of *Terrisporobacter*, *Clostridium butyricum*, *Akkermansia*, and *Turicibacter* sp. H121 was significantly increased, while the abundance of *Lactobacillus* was significantly decreased as a result of FBP inclusion (Figure 3B–C). Functional prediction analysis using PICRUSt showed that dietary FBP significantly enhanced pathways associated with organic systems, genetic information processing, cellular processes, metabolism, and environmental information processing at KEGG level 1 (Figure 4A). At KEGG level 3, analysis revealed 50 pathways that exhibited differential changes between the two treatment groups. Among these, FBP addition significantly upregulated methane metabolism, biosynthesis and

biodegradation of secondary metabolites, bacterial secretion system, energy metabolism, minerals absorption and nitrogen metabolism (Figure 4B). Notably, FBP dietary treatment significantly enriched the bacterial mineral absorption pathway.

Correlation between mineral deposition and colonic microbiota

Spearman's correlation analysis was conducted to analyse the association between the concentrations of 8 minerals and the differentially altered genera in the colon (Figure 5). Serum concentrations of Ca, Mg, Mn, or Zn were negatively correlated with the abundance of *Unclassified_k_norank_d_Bacteria*, *Clostridium_sensu_stricto_6*, *Terrisporobacter*, *Turicibacter*, *Cellulosilyticum*, *Unclassified_c_Clostridia*, *Turicibacter*, *Clostridium_sensu_stricto_1*, while positively correlated with the abundance of *Lactobacillus* and *Peptococcus*. Liver Fe and Cu levels were positively correlated with *Unclassified_k_norank_d_Bacteria*, *Clostridium_sensu_stricto_6*, *Terrisporobacter*, *Turicibacter*, *Cellulosilyticum*, *Unclassified_c_Clostridia*, *Turicibacter*, *Clostridium_sensu_stricto_1*, UCG-005, *norank_f_Erysipelotrichaceae*, and negatively correlated with *Lactobacillus* and *Peptococcus*.

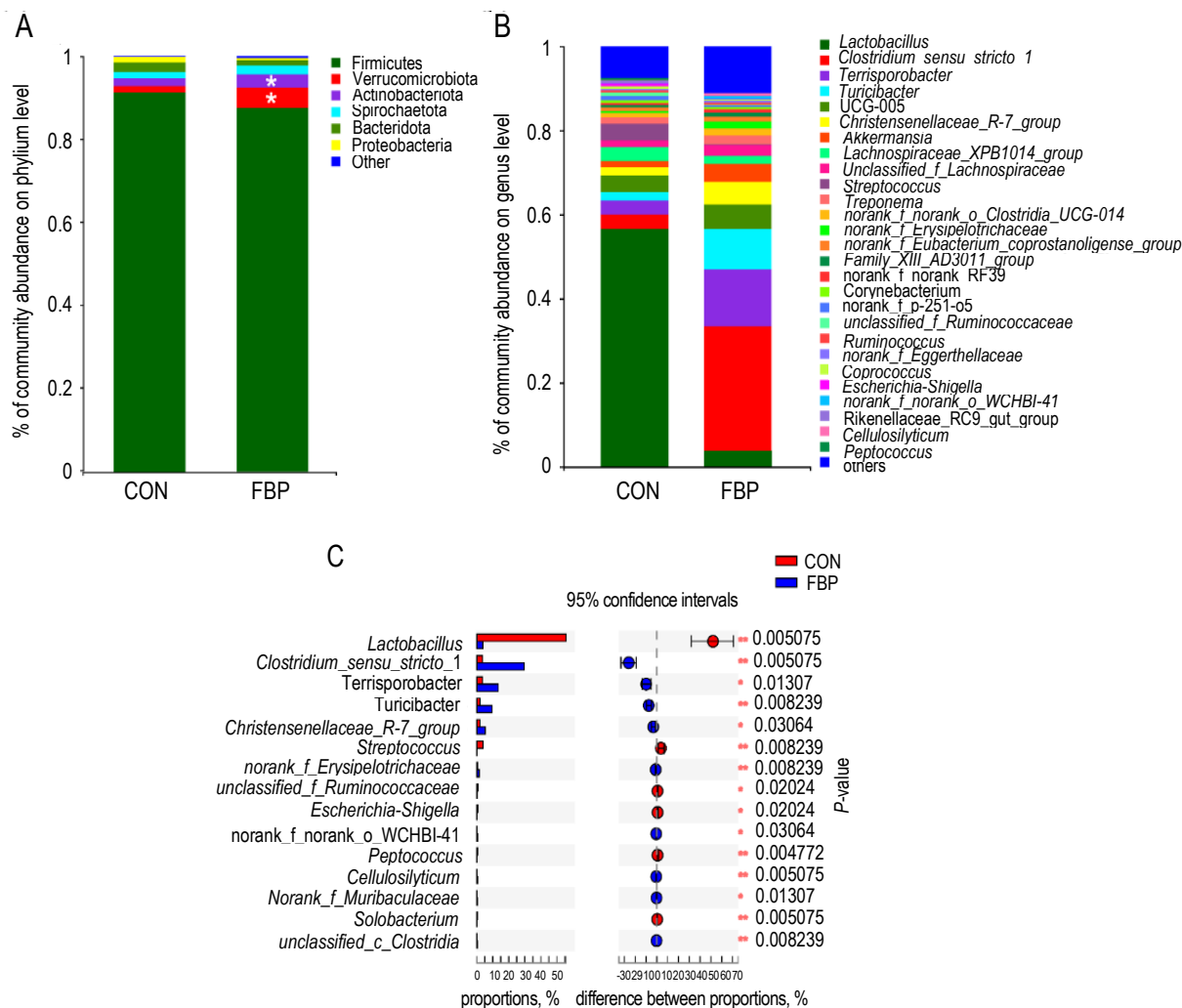


Figure 3. Effect of dietary FBP on gut microbial composition of pigs. (A) Phylum level, * $P < 0.05$; (B) Genus level. Data are means of six replicates; (C) Genus-level data on gut microbial composition of the control group and the fermented *Broussonetia papyrifera* diet group were imported into the R statistical analysis package (version 3.3.1) and the scipy package for Python software for statistical analysis. Data presented are derived from 6 animals per group ($n = 6$). Statistical differences were assessed using the Mann-Whitney U-test and were considered significant at $P < 0.05$

CON – control group, FBP – fermented *Broussonetia papyrifera* diet

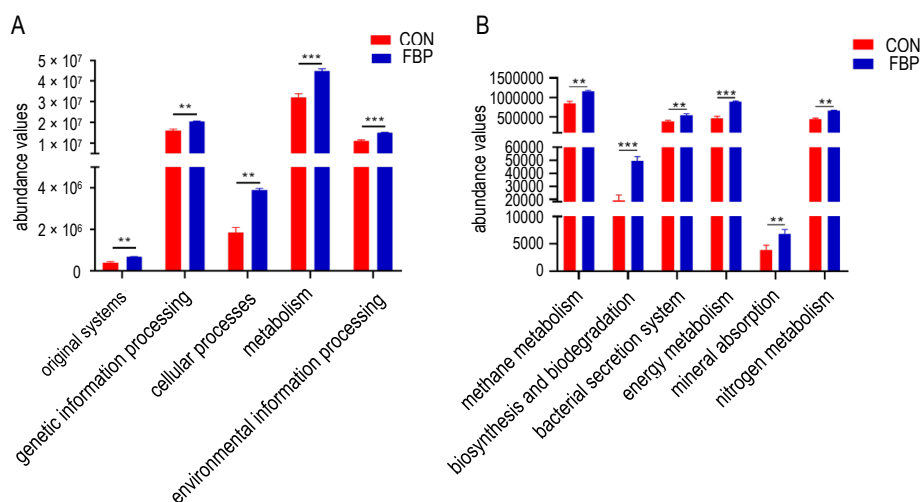


Figure 4. Effect of dietary fermented *Broussonetia papyrifera* on gut microbiota functionality based on KEGG analysis. (A) KEGG pathway at level 1; (B) KEGG pathway at level 3. ** denotes significant difference at $P < 0.01$, *** denotes trend at $P < 0.001$

CON – control group, FBP – fermented *Broussonetia papyrifera* diet

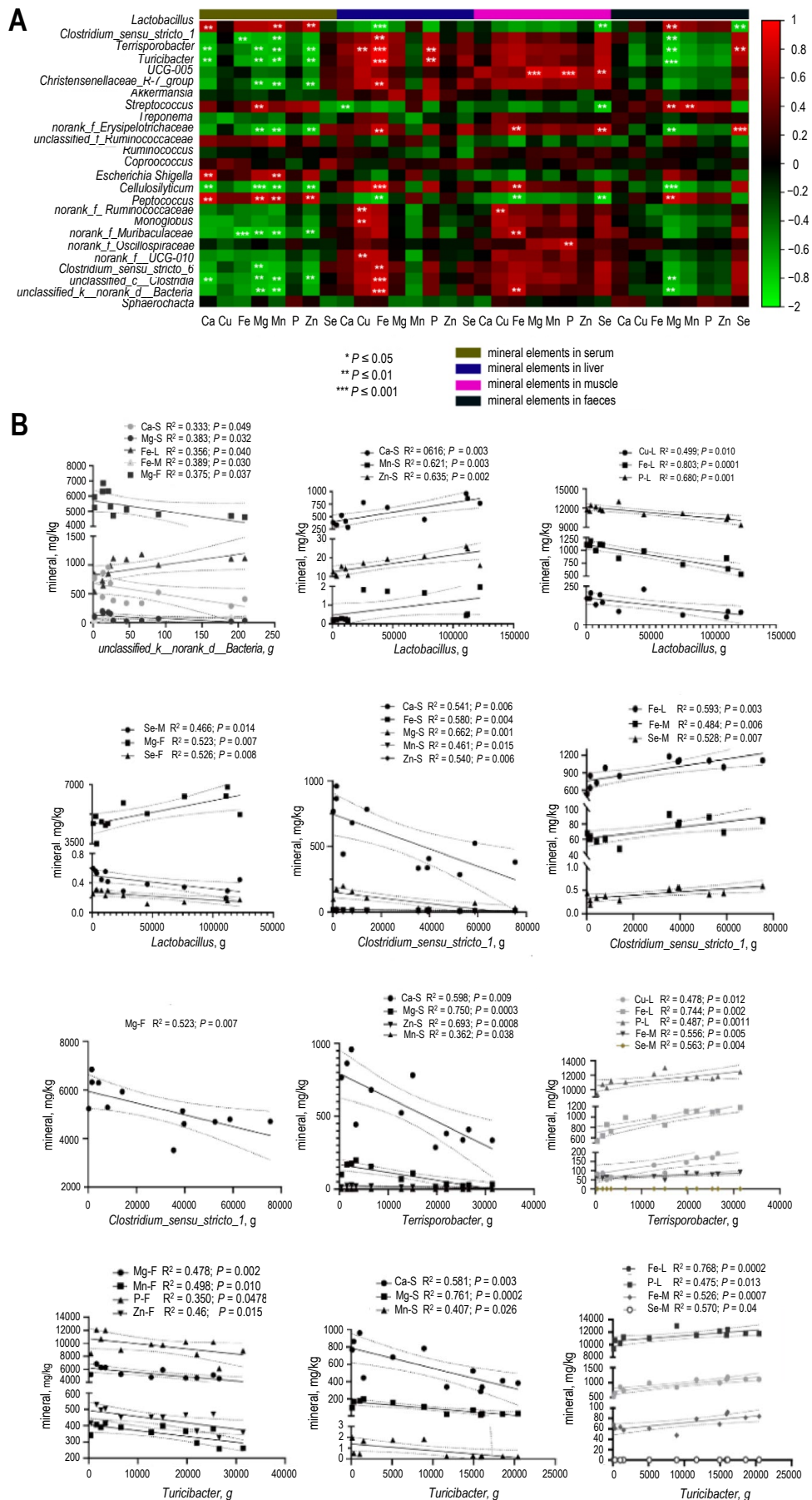


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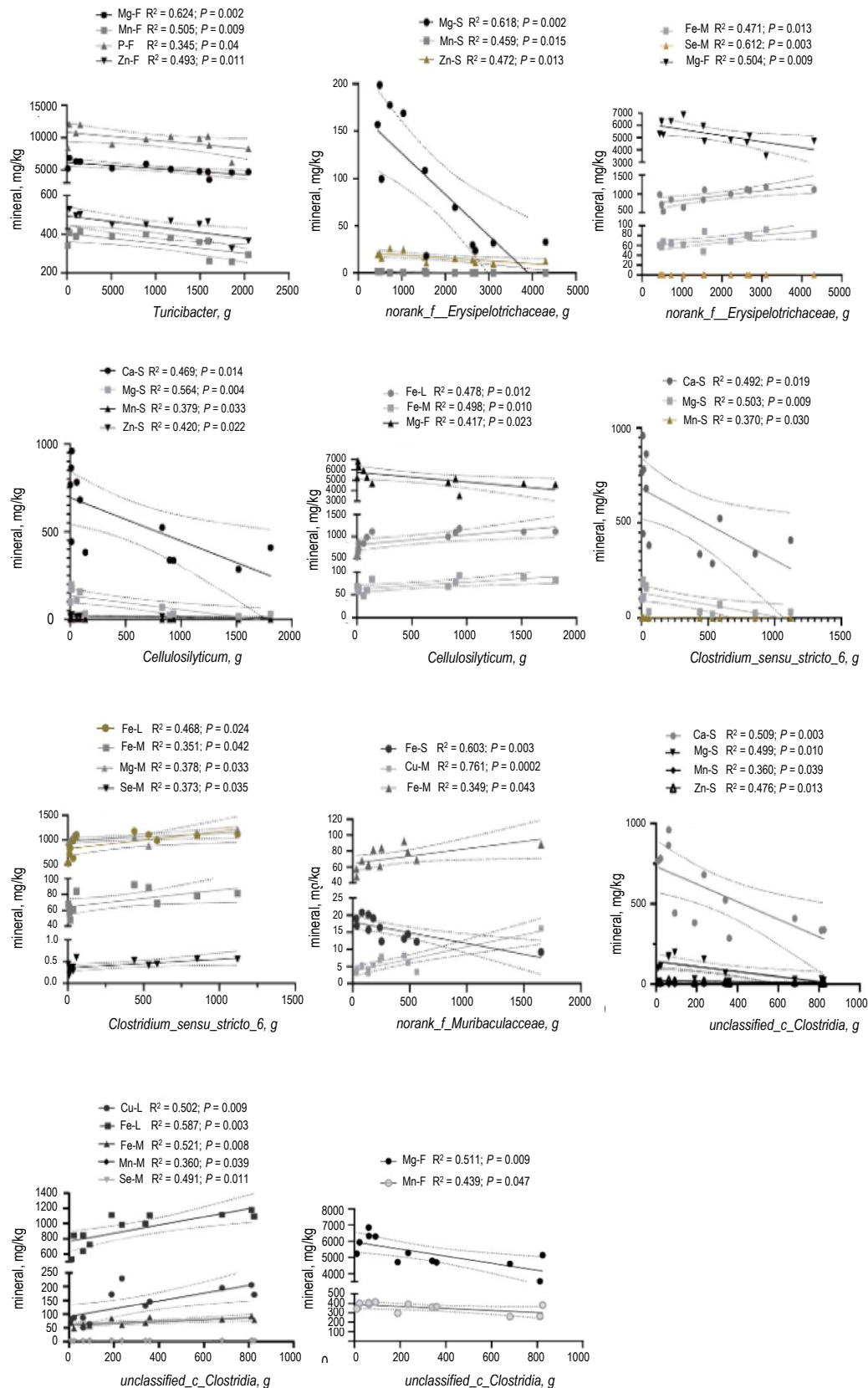


Figure 5. Correlation analysis between mineral elements and differentially altered genera. (A) Heat map of Spearman's correlation analysis between microbes and minerals. Red and green in the rectangle chart represent positive and negative correlations, respectively. Significance and correlation coefficient was analysed using Spearman's correlation analysis in graphs. * denotes a significant difference at $P < 0.05$, ** denotes a significant difference at $P < 0.01$, *** denotes a trend at $P < 0.001$; (B) Linear correlation analysis between microbes and minerals. B. Fitted lines from linear models with 95% confidence intervals

CON – control group, FBP – fermented *Broussonetia papyrifera* diet; S – serum, L – liver, M – muscle, F – faeces

Fe content in the muscle was significantly positively correlated with *Unclassified_k_norank_d_Bacteria*, *Unclassified_c_Clostridia*, *Clostridium_sensu_stricto_6*, *norank_f_Muribaculaceae*, *Cellulosilyticum*, *norank_f_Erysipelotrichaceae*, UCG-005, *Terrisporobacter*, *Turicibacter*, and *Clostridium_sensu_stricto_1*, while negatively correlated with *Peptococcus*. Muscle Se content was positively correlated with *Unclassified_c_Clostridia*, *Clostridium_sensu_stricto_6*, *norank_f_Erysipelotrichaceae*, UCG-005, *Terrisporobacter*, *Turicibacter*, *Clostridium_sensu_stricto_1* and negatively correlated with *Lactobacillus*, *Streptococcaceae* and *Peptococcus*. Faecal Mn or Mg concentrations were negatively correlated with *Unclassified_k_norank_d_Bacteria*, *Unclassified_c_Clostridia*, *Cellulosilyticum*, *norank_f_Erysipelotrichaceae*, *Terrisporobacter*, *Turicibacter*, *Clostridium_sensu_stricto_1*, and positively correlated with *Peptococcus*, *Streptococcus*, and *Lactobacillus*.

Discussion

This study investigated the effects of supplementation with FBP on the mineral status and intestinal microbiota of finishing pigs. Serum biochemical parameters can be employed to gather basic information on the health of finishing pigs. We found that the analysed biochemical indices in both experimental groups were within normal ranges, indicating that the pigs were healthy, and the FBP diet was safe. Mineral content in blood, on the other hand, can reflect bioavailability and deposition of these elements *in vivo* (Olukosi et al., 2018). A higher content of minerals in the diet can lead to their higher accumulation in liver and muscle (Cabrera and Saadoun, 2014; Wang et al., 2019). This study found that serum levels of Ca, Mg, Zn, Mn, and Fe decreased in the FBP diet group. The decrease in Fe levels corresponded with a decrease in UIBCI in serum, while the Cu levels were consistent with CER results. The concentration of Se, Cu, and Fe significantly increased in the liver, and Cu, Mg, Fe, and Se concentrations were elevated in the muscle (Figure 1D). Mineral correlation analysis from all sampling sites (Figure 1C) also showed that most minerals in serum and faeces were negatively correlated with those in the liver and muscle. Burrow et al. (2020) has argued that mineral distribution is highly organ- and diet- dependent, with mutual interactions occurring between the elements. The FBP diet increased the content of some minerals in the liver and muscle, which could explain the observed decrease in serum mineral levels. Mineral elements typically have low bioavailability *in vivo*,

and excessive accumulation in faeces can cause environmental pollution (Tan and Yin, 2017). For example, metal elements, such as Cd, Cu, Mg, or Mn in faeces can be deposited in the environment, stressing vegetation and crops, which can result in plant death and ecological imbalance (Dominguez et al., 2010). Yin et al. (2022) pointed out that the concentration of metal ions in faeces could be reduced by adjusting the form of minerals in the diet. The present results showed that FBP significantly reduced Mg and Mn concentrations in faeces, suggesting a reduced environmental burden from excessive mineral accumulation. Additionally, the study also demonstrated higher bioavailabilities of these trace minerals as a result of FBP supplementation.

The intestinal microbiota can be shaped by dietary supplementation, and its impaired biodiversity has been associated with various health issues and diseases (Krga and Glibetic, 2022; Krupa-Kozak and Drabińska, 2022). Previous researcher has found that minerals, such as Ca (Weaver, 2015) and Fe (Tang et al., 2017), could affect intestinal microbial diversity. In this study, the FBP diet elevated intestinal microbial diversity, which might be attributed to the rich mineral and cellulose contents of FBP. The FBP diet also altered the gut microbial composition of finishing pigs, increasing the abundance of *Christensenellaceae*, *Clostridium*, *Clostridium_sensu_stricto_1*, *Clostridium_butyricum*, *Verrucomicrobia*, *Terrisporobacter*, *Akkermansia* and *Turicibacter*. Among these, *Christensenellaceae*, a health-related genus within the phylum Firmicutes, has been commonly reported in the distal colon of humans and animals (Waters and Ley, 2019). The FBP diet is closely associated with *Christensenellaceae*, which is involved in shifting intestinal metabolism from carbohydrate fermentation to protein catabolism. This metabolic shift results in lower levels of protein-derived metabolites in serum and elevated levels of potentially harmful protein-derived metabolites in urine (Roager et al., 2016; Beaumont et al., 2017). This could explain the significant reduction in serum BUN and NH_3L levels observed in this study. *Clostridium_butyricum* is a beneficial bacteria that produces butyric acid, which is important for intestinal mucosal regeneration and repair, as well as growth promotion of other beneficial gut bacteria (Cassir et al., 2016). A previous study has found that *Clostridium_sensu_stricto_1*, a *Clostridium* genus, can be regulated by a diet rich in flavonoids, which has a beneficial effect on the proliferation of *Clodistrium_burietyum* (Wu et al., 2017).

In this study, an increased abundance of *Clostridium* was found in the FBP group, which could be attributed to the high flavonoid content of FBP (Niu et al., 2022). *Akkermansia muciniphila* (Akk), a representative species of the phylum Verrucomicrobia, has attracted much attention as a next-generation probiotic. *Akkermansia* is a mucinolytic bacterial genus commonly found in the human intestinal microbial community. The abundance of *Akkermansia* is known to reduce inflammatory status and can be restored when intestinal inflammation is attenuated (Nakashima et al., 2022). In the current study, the higher abundance of *Akkermansia* and *Verrucomicrobia* in the FBP group implied that FBP could promote the colonisation of *Akkermansia*, potentially helping to inhibit intestinal inflammation. *Terrisporobacter*, a spore-forming genus linked to carbohydrate metabolism (Wang et al., 2022), was also enriched in the FBP-supplemented animals. This suggested an increase in the production of butyric acid and acetate metabolites (Gerritsen et al., 2014).

The functional analysis using the KEGG database demonstrated significant upregulation of carbohydrate metabolism, energy metabolism, and lipid metabolism pathways, indicating that the FBP diet could effectively support the nutritional needs of finishing pigs. Moreover, the enhanced biosynthesis and biodegradation of secondary metabolites, bacterial secretion system, and nitrogen metabolism pathways indicated that the FBP diet had a substantial impact on the biological function of the intestinal microbiota. Additionally, the significant upregulation of mineral absorption and metabolism showed that the FBP diet could contribute to mineral deposition.

Spearman's rank correlation and linear correlation analyses were performed to reveal the relationship between differentially altered intestinal bacterial species and minerals in finishing pigs. We found that *Christensenellaceae*, *Unclassified_k_norank_d_Bacteria*, *Clostridium_sensu_stricto_1*, *Terrisporobacter*, *Turicibacter*, *Cellulosilyticum*, *norank_f_Ruminococcaceae*, and several others, were positively correlated with such elements as Cu, Fe, P, Zn, and Se in the muscle and liver. These results suggest that dietary supplementation with FBP may increase the deposition of certain minerals in specific tissues. In this study, *Lactobacillus* was negatively correlated with the concentration of Fe in the liver. Previously, Fe deficiency was shown to increase the number of *Lactobacillus* and *Bacteroides* in the faeces, and oral Fe administration resulted in a significant decrease in *Lactobacillus* counts (Paganini and Zimmermann, 2017). Adminis-

tration of iron-containing FBP resulted in a significant decrease in the abundance of intestinal *Lactobacilli*, as reflected by the negative correlation between *Lactobacillus* and iron concentration in the liver. It should be noted that *Unclassified_k_norank_d_Bacteria*, *unclassified_c_Clostridia*, *Cellulosilyticum*, *Turicibacter*, *norank_f_Erysipelotrichaceae*, *Terrisporobacter*, and *Clostridium_sensu_stricto_1* were all negatively correlated with Mn and Mg in the faeces. We also found that faecal ion levels showed a significant negative relationship with *Unclassified_k_norank_d_Bacteria* involved in the conversion of nitrate nitrogen to nitrogen and compost fermentation (Wu et al., 2019). *Unclassified_k_norank_d_Bacteria* may competitively inhibit the binding of nitrate ions and metals, increasing the likelihood of mineral absorption and transport through the intestine. In the present study, *Unclassified_k_norank_d_Bacteria* were positively correlated with minerals in the liver and muscle, and negatively correlated with minerals in faeces. This trend was also recorded for *Unclassified_c_Clostridia*, *norank_f_Erysipelotrichaceae*, *Cellulosilyticum*, *Clostridium_sensu_stricto_1*, *Terrisporobacter*, and *Turicibacter*. It is worth noting that all identified bacteria except *Unclassified_k_norank_d_Bacteria* belonged to the phylum Firmicutes. The FBP diet analysed here significantly altered the colonic microbiota, which was mainly associated with colonisation and proliferation of the dominant phylum Firmicutes, increased mineral deposition in tissues, and reduced mineral content in faeces. An earlier study has found an association between mineral elements and intestinal microbiota (Pajarillo et al., 2021). In this research, we verified correlations between intestinal microbiota composition and mineral elements. However the precise mechanism underlying the mutual effects between mineral deposition and gut microbes remains unknown.

Conclusions

As shown in Figure 6, the fermented *Broussonetia papyrifera* (FBP) diet increased microbial diversity and maintained intestinal homeostasis in finishing pigs. In addition, the FBP diet increased mineral absorption and deposition, while reducing mineral excretion and serum mineral concentrations. Dietary FBP has also improved the function of bacterial metabolic pathways, including energy metabolism and mineral absorption, suggesting its positive impact on intestinal microbiota. This indicates that FBP supplementation could effectively

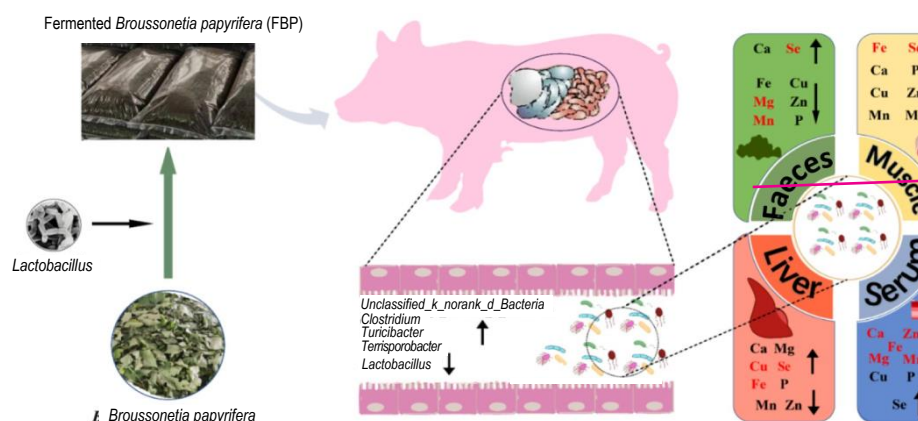


Figure 6. Overall presentation of the fermented *Broussonetia papyrifera* diet on mineral deposition and microbiome in finishing pigs

“↑” indicates an increase, “↓” indicates a decrease

maximise the deposition of minerals in tissues. Considering its beneficial effects on mineral deposition and gut microbiota, the FBP diet could be applied as a promising functional ingredient in pig feeds.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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