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Small Intestinal Function, Transcriptomic Profiles, and Gut Microbiota in Ningxiang Pigs, Berkshire Pigs, and Their Crossbred Offspring

Xiaohong Hou
Chinese Academy of Sciences
Ping Wang
Chinese Academy of Sciences
Qian Wang
Chinese Academy of Sciences
Zihan Guo
Chinese Academy of Sciences
Zhao Jin
Chinese Academy of Sciences
Shishu Yin
Chinese Academy of Sciences
Hu Gao
Chinese Academy of Sciences
Jingchun Sun
Chinese Academy of Sciences
Ruimin Ren
Hunan Agricultural University
Fengming Chen
Changsha Medical University
Yulong Yin
Chinese Academy of Sciences
Kang Xu
xukang2020@163.com

Chinese Academy of Sciences

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Abstract

Differences in intestinal digestive and absorptive capacity among pigs with different genetic backgrounds may contribute to variation in nutrient utilization. This study compared small intestinal morphology, brush border enzyme activities, transcriptomic profiles, and ileal microbiota among Ningxiang (NX) pigs, Berkshire (BKS) pigs, and their crossbred offspring (BN). NX pigs exhibited higher villus-to-crypt (V/C) ratio in the duodenum and jejunum and higher duodenal activities of alkaline phosphatase, maltase, and aminopeptidase N than BKS pigs ($P < 0.05$). BN pigs exhibited greater duodenal villus height and a higher ileal V/C ratio than both parental groups ($P < 0.05$), indicating segment-specific differences in intestinal morphology. Transcriptomic analysis revealed that differentially expressed genes (DEGs) between NX and BKS pigs were enriched in pathways related to nutrient metabolism and transport, including starch and sucrose metabolism, fat digestion and absorption, bile secretion, transmembrane transport, and insulin secretion. Comparisons between BN and its parental groups further identified segment-specific enrichment of pathways related to energy metabolism, nutrient processing, and cellular signaling. Correlation analyses highlighted several candidate genes, including sucrase-isomaltase (*SI*), glutathione peroxidase 3 (*GPX3*), pentraxin 3 (*PTX3*), phospholipase C beta 4 (*PLCB4*), angiotensinogen (*AGT*), and insulin-like growth factor 1 receptor (*IGF1R*), whose expression levels were associated with intestinal morphological traits and brush border enzyme activities. Ileal microbiota analysis revealed differences in microbial diversity and community composition among groups, with *Lactobacillus* enriched in NX pigs and distinct abundance patterns of *Escherichia-Shigella* and *Romboutsia* across groups. Correlation analyses further linked selected microbial genera with host gene expression and intestinal phenotypes. Collectively, these findings demonstrate genetic background-associated differences in small intestinal digestive and absorptive characteristics, with variation across intestinal segments, and identify candidate genes and microbial taxa for further investigation of the factors potentially underlying differences in nutrient utilization among pigs.

Introduction

The small intestine is the primary site of nutrient digestion and absorption in monogastric animals and plays a critical role in feed nutrient utilization and overall production performance [1–3]. Nutrient digestion and absorption involve various enzymes associated with the small intestinal brush border, including aminopeptidase N (APN), alkaline phosphatase (AKP), and disaccharidases such as maltase and sucrase [4, 5]. These enzymes contribute to nutrient digestion and intestinal physiological homeostasis, and their activities may serve as indicators of intestinal functional status and vary with host genetic background [6, 7]. In addition, the gut microbiota contributes to host nutrient metabolism, and its composition and potential functional capacity may be associated with host genetic background and intestinal physiological characteristics [8].

Differences in genetic background may contribute to variation in small intestinal function among pig populations. Through long-term adaptation and selective breeding, indigenous pigs such as Ningxiang

(NX) pigs have developed distinctive biological and production characteristics, whereas introduced breeds such as Berkshire (BKS) pigs have been selected for desirable production traits [9, 10]. Crossbreeding between indigenous and introduced pig populations is an important strategy for combining desirable traits, including meat quality and production performance. However, the intestinal digestive and absorptive characteristics of crossbred offspring and the mechanisms underlying their differences from the parental populations remain insufficiently understood.

Previous studies have reported differences between NX pigs and the crossbred offspring of NX and BKS pigs in the digestion of basal corn nutrients, crude fiber utilization, and amino acid digestion and absorption patterns [11, 12]. However, the relationships between these differences and intestinal morphology, brush border enzyme activities, host gene expression, and gut microbiota remain unclear, and the underlying molecular regulatory mechanisms require further investigation.

Therefore, this study compared intestinal morphology and brush border enzyme activities in the duodenum, jejunum, and ileum of NX pigs, BKS pigs, and their crossbred offspring (BN). Transcriptomic and ileal microbiome analyses were conducted to characterize gene expression profiles, identify enriched functional pathways and differentially abundant microbial taxa, and investigate their associations with intestinal digestive and absorptive characteristics. We further integrated gene expression, intestinal phenotypic, and microbiota data to explore associations among host gene expression, microbial community composition, and intestinal digestive and absorptive traits. This study provides insights into the molecular and microbial factors potentially associated with variation in intestinal digestive and absorptive characteristics among pigs and may inform future efforts to improve nutrient utilization through genetic selection.

Materials and methods

Animal feeding and sample collection

A total of 42 barrows were used in this study and assigned to three groups according to genetic background: Ningxiang (NX), Berkshire (BKS), and crossbred (BN), with 14 pigs per group. All pigs were obtained from Hunan Chuweixiang Animal Husbandry Co., Ltd. (Ningxiang, Hunan, China). They were fed a corn-soybean meal-based diet and had ad libitum access to feed and water. At 260 ± 5 days of age, the pigs were electrically stunned and immediately slaughtered by exsanguination at a commercial abattoir in accordance with standard operating procedures. Immediately after slaughter, approximately 1-cm-long segments of the duodenum, jejunum, and ileum were collected, immediately fixed in 4% paraformaldehyde, and stored at 4°C until histological analysis. Ileal contents were collected simultaneously and stored at -80°C for subsequent microbiota analysis. Additional tissue samples from the duodenum, jejunum, and ileum were collected, rinsed with sterile physiological saline, and stored at -80°C for subsequent analyses.

Intestinal morphology and digestive enzyme assays

Fixed intestinal tissue samples were paraffin-embedded and sectioned at 4 μm, followed by hematoxylin and eosin (H&E) staining. Images were captured and analyzed using CaseViewer to measure villus height (VH) and crypt depth (CD), and the villus height-to-crypt depth (V/C) ratio was subsequently calculated. Frozen intestinal tissue samples were thawed, homogenized, and centrifuged, and the resulting supernatants were collected for analysis. The activities of AKP, maltase, and APN were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China).

Intestinal transcriptomics sequencing

Three pigs per group were randomly selected for transcriptomic analysis. Tissue samples were collected from the duodenum, jejunum, and ileum of each pig, yielding a total of 27 samples. Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific Inc., USA), and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Samples with an RNA integrity number > 8.0 and an A260/A280 ratio > 1.9 were used for library preparation. RNA-seq libraries were constructed using the TruSeq RNA Sample Preparation Kit (Illumina, USA) and sequenced on an Illumina HiSeq 2500 platform. Clean reads were aligned to the Sus scrofa reference genome (Sscrofa11.1) using TopHat2 (v2.0.3.12), and gene-level read counts were generated using HTSeq-count. Differential expression analysis was performed using edgeR, with FDR ≤ 0.05 and $|\log_2 FC| > 1$ used to identify differentially expressed genes (DEGs). Functional and pathway enrichment analyses were performed using R package DAVID and PANTHER 15.0 software.

RT-qPCR validation

To validate the RNA-seq results, real-time quantitative PCR (RT-qPCR) was performed using a LightCycler 480 instrument according to the manufacturer's instructions. The primers used are listed in Table 1. β-actin was used as the reference gene, and relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [13].

Table 1
Primers used in this study

Game	Primer sequence(5'-3')	Primer size(bp)
β-actin	F: CTGCGGCATCCACGAAACT	19
	R: AGGGCCGTGATCTCCTTCTG	20
LOC118025941	F: CTGCAACCTTGAAGAGATTC	20
	R: CCATATACATCCAGTCGCTC	20
ASGR2	F: ACCCTTAACAGTTGCAGCC	19
	R: CCAGATTGGGTCTTCTCTCT	20
SPINK5	F: AACATGGTGAAAGACGGAAA	20
	R: TAGCTTCTCGCTCAAAGATG	20
GABARAP	F: CTTCTGATCTCACAGTTGGTC	21
	R: GGGTGGAATGACATTGTTGA	20
ALOX5	F: CTGAACTACTCAAAAGCGATG	21
	R: AGATTTTCTCAAAGTCGGCA	20
ALOX5AP	F: CTGAATCAAACATGGATCAGGA	22
	R: GACACAGTTCTGGTTGGCAG	20
SLC9A3	F: GAGGAAGTGCCTGGTGA	17
	R: CAGGTGGAAGCCGATCTTG	19
PROM2	F: TCCTCTCTGTGGTGCAG	17
	R: TACCGCACCACCTCATC	17
NEUROG3	F: GGACTGGTGAATGAGTTTTG	20
	R: CACTACAACTTCTCAGACCA	20
LDHB	F: GGTTTCGCTATCTTATGGCT	20
	R: TCACTATCATTGTCCGTTCC	20
PKP1	F: CCGACAATTACAACAACTACG	21
	R: CCCGTTGTAGATCGGATAG	19
FUT2	F: TGAGCATGGCTGGTTCT	17
	R: GTGCTGGAAGGTTCGAGAG	19
CYP26B1	F: TTCAAGACTCACTTGCTGG	19

	R: AGATCTTGGAGAAGACCTTG	20
LOC110258569	F: GAAGAGCTGAGAGGGTACT	19
	R: CTGAACTATTCTGGTGCCTT	20
AGT	F: TCAGACATAAATAGGCCTCG	21
	R: TCACTCTTGCGACCCTTA	18
GCK	F: CACTCACCTGGAAGAAGC	18
	R: GGATCTGCTCCGCCTTTT	18
CREB3L1	F: AATCAAGAACAAGATCTCGGC	22
	R: TTCGTTGTTCTCGGATGTAA	21
CREB3L4	F: TGCTGTTTTCCTTAAGTCGG	20
	R: TCTGAAATCCATGCTTCTAGCC	22
SI	F: TCTTGTCAGACTCAAATGGCAA	22
	R: AACTTTACCTCAACCTCGCT	20
PGM1	F: CGGAAGCAATAACGGATAAGA	21
	R: CCACAATTTCCACTGTGAAG	20
CCL20	F: GCCGTGAATATCCTCAGTCAAA	22
	R: CAGCACAGCGAGGTTCTTTT	20
EFCAB7	F: ATGATCGCGGAGTTGTTATT	20
	R: CTCTGAAAGCGTTGACTTCT	20
AQP5	F: CTCAACAACAACACGACTCC	20
	R: CCGGTGAAGTAGATCCCCAC	20
PLB1	F: CTGTCTGCACTTCTCTGAAC	20
	R: CCACTGGTTCCATCATGT	18

Intestinal 16S sequencing

Twelve pigs were randomly selected from each group, and ileal content samples were collected for microbiota analysis. Microbial DNA was extracted using the TGuide S96 Magnetic Soil/Stool DNA Kit. The V3-V4 region of the bacterial 16S rRNA gene was amplified using the primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT). PCR products were purified by 1.8% agarose gel electrophoresis, and sequencing libraries were constructed and sequenced on the

Illumina NovaSeq 6000 platform using paired-end sequencing. Raw reads were quality-filtered and trimmed using Trimmomatic v0.33 and Cutadapt v1.9.1, respectively, followed by paired-end read merging and length filtering using USEARCH v10. Denoising and chimera removal were performed using DADA2 implemented in QIIME2 v2020.6. Amplicon sequence variants were then used for taxonomic annotation and microbial community analyses, including α-diversity (Chao1, ACE, Shannon, and Simpson indices), β-diversity (principal coordinates analysis, PCoA), LEfSe analysis (LDA score > 4.5), and correlation analysis. Potential microbial functional profiles were predicted using PICRUSt2, with functional annotation based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS version 22.0. Differences among groups were assessed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for post hoc comparisons. Differences were considered statistically significant at $P < 0.05$. Spearman's rank correlation analysis was used to assess associations among multi-omics data, with $P < 0.05$ considered statistically significant.

Results

Small Intestinal Morphology and Brush Border Enzyme Activities Exhibit Segment-Specific Differences among Pig Groups

As shown in Fig. 1A, the morphological characteristics of the small intestine differed among the pig groups in a segment-specific manner. In the duodenum, VH was significantly greater in the BN group than in the NX and BKS groups ($P < 0.01$) (Fig. 1B), whereas CD was significantly greater in the BKS group than in the NX group ($P < 0.05$) (Fig. 1C). Accordingly, the V/C ratio was significantly higher in both the NX and BN groups than in the BKS group ($P < 0.05$) (Fig. 1D). In the jejunum, no significant differences were observed among the three groups in VH or CD; however, the V/C ratio was significantly higher in the NX group than in the BKS group ($P < 0.05$) (Fig. 1D). In the ileum, CD was significantly lower in the BN group than in the BKS group ($P < 0.05$) (Fig. 1C), while the V/C ratio was significantly higher in the BN group than in the NX and BKS groups ($P < 0.05$) (Fig. 1D). Overall, small intestinal morphological differences were segment-specific, with VH and CD differences mainly observed in the duodenum and ileum, and V/C ratio differences also detected in the jejunum.

Analysis of brush border enzyme activities further revealed distinct segment-specific differences among the pig groups (Fig. 1E-G). In the duodenum, the activities of AKP, maltase, and APN were significantly higher in the NX group than in the BKS and BN groups ($P < 0.05$), In addition, AKP activity was significantly higher in the BN group than in the BKS group ($P < 0.01$). In the jejunum, no significant difference was detected in AKP activity among the three groups. However, maltase activity was

significantly higher in the NX group than in the BKS and BN groups ($P < 0.001$ and $P < 0.01$, respectively), whereas APN activity was highest in the BN group. In the ileum, no significant differences were observed among the three groups in AKP, maltase, or APN activity. Overall, significant differences in brush border enzyme activities among the pig groups were observed primarily in the duodenum and jejunum, whereas no significant differences were detected in the ileum.

Molecular Mechanisms Underlying Differences in Small Intestinal Digestive and Absorptive Functions between NX and BKS pigs

To investigate the molecular basis of differences in small intestinal digestive and absorptive functions between the NX and BKS groups, RNA sequencing was performed, followed by differential gene expression and Gene Ontology (GO) enrichment analyses. The DEGs were enriched in diverse functional categories, including enzymatic activity, transmembrane transport, and receptor-mediated signaling, some of which may be associated with intestinal digestive and absorptive functions. Notably, the enrichment profiles differed among intestinal segments. In the duodenum, the DEGs were predominantly enriched in polypeptide N-acetylgalactosaminyltransferase activity, nucleoside:sodium symporter activity, ATPase activity, coupled to transmembrane movement of substances, alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase activity and CCR chemokine receptor binding (Fig. 2A). In the jejunum, the DEGs were mainly enriched in ATPase activity, coupled to transmembrane movement of substances, GTPase activity, alditol: $NADP^+$ 1-oxidoreductase activity, galactosylceramidase activity, opioid receptor binding (Fig. 2B). In the ileum, the DEGs were primarily enriched in ATPase activity, D-aspartate oxidase activity, glycine C-acetyltransferase activity, opioid receptor binding, type 2 angiotensin receptor binding (Fig. 2C).

KEGG pathway enrichment analysis further revealed segment-specific enrichment patterns among the DEGs between the NX and BKS groups. In the duodenum, the DEGs were mainly enriched in insulin secretion and starch and sucrose metabolism (Fig. 2D). In the jejunum, the enriched pathways included ABC transporters and fat digestion and absorption (Fig. 2E). In the ileum, the DEGs were mainly enriched in bile secretion and the cAMP signaling pathway (Fig. 2F). Collectively, these findings indicate that the functional enrichment profiles of small intestinal DEGs differed among intestinal segments, potentially involving carbohydrate metabolism, nutrient transport, lipid digestion and absorption, and bile secretion. These segment-specific molecular patterns may be associated with differences in small intestinal digestive and absorptive functions between the NX and BKS groups.

Transcriptomic Characterization of Small Intestinal Differences Between BN Crossbred Offspring and Their Parental Lines

To further characterize the molecular differences between the crossbred offspring (BN) and their parental lines, NX and BKS, GO enrichment analysis was performed. In the NX vs. BN comparison, duodenal DEGs were mainly enriched in functional terms related to ATPase activity and cholesterol

binding, among others, whereas jejunal DEGs were mainly enriched in arachidonate 5-lipoxygenase activity and other related functional terms. Ileal DEGs were primarily enriched in terms associated with 11-cis retinal binding, vitamin D 24-hydroxylase activity, and other related functions (Fig. 3A). In the BKS vs. BN comparison, duodenal DEGs were mainly enriched in GO terms related to epinephrine secretion and chronic inflammatory response. Jejunal DEGs were primarily enriched in terms associated with hydrolase activity and bioactive lipid receptor activity, whereas ileal DEGs were mainly enriched in vitamin catabolic process, iron coordination entity transport, and cell-cell adhesion (Fig. 3B).

KEGG pathway enrichment analysis further revealed segment-specific enrichment patterns in the comparisons between BN group and its two parental lines. In the NX vs. BN comparison, duodenal DEGs were primarily enriched in pathways such as insulin secretion and PI3K-Akt signaling, whereas jejunal DEGs were mainly enriched in arachidonic acid metabolism and glycolysis/gluconeogenesis. In the ileum, DEGs were primarily enriched in cortisol synthesis and secretion and the AGE- RAGE signaling pathway (Fig. 3C-E). In the BKS vs. BN comparison, duodenal DEGs were mainly enriched in selenocompound metabolism and starch and sucrose metabolism (Fig. 3F). Jejunal DEGs were primarily enriched in the glucagon signaling pathway and glycine, serine, and threonine metabolism (Fig. 3G), whereas ileal DEGs were mainly enriched in vitamin digestion and absorption and glycolysis/gluconeogenesis (Fig. 3H).

Notably, in NX vs BN, genes such as phospholipase C beta 4 (*PLCB4*), acid phosphatase 3 (*ACP3*), *IGF1R*, phosphoenolpyruvate carboxykinase 1 (*PCK1*), angiotensinogen (*AGT*), and angiotensin II receptor 1 (*AGTR1*) were enriched across multiple pathways; In BKS vs BN, phosphoglycerate mutase 2 (*PGAM2*), α-1,2-fucosyltransferase 2 (*FUT2*), vasoactive intestinal peptide (*VIP*), EF-hand calcium-binding domain protein 7 (*EFCAB7*), Toll-like receptor 2 (*TLR2*), and lactate dehydrogenase B (*LDHB*) were associated with multiple enriched pathways. To validate the RNA-seq results, 25 DEGs were randomly selected for RT-qPCR analysis. The expression patterns observed by RT-qPCR were consistent with those obtained by RNA-seq (Fig. 3I).

Distinct Ileal Microbiota Profiles Among Pig Breeds and Their Hybrid Offspring

The V/C ratio in the ileum was higher in the BN group than in the parental groups. Given the important role of the ileum in microbial colonization and activity within the small intestine, subsequent analyses focused on characterizing ileal microbiota differences among the two parental breeds and their crossbred offspring. The Shannon, ACE, and Simpson indices were significantly higher in the BKS group than in the BN group, while all three indices were also significantly higher in both the BKS and BN groups than in the NX group (Fig. 4A-C). PCoA based on β-diversity revealed significant differences in ileal microbial community composition among the three groups (Fig. 4D). Together, these findings indicate that ileal microbial diversity and community composition differed among the three groups with distinct genetic backgrounds.

At the phylum level, the dominant phyla in the ileal contents were *Firmicutes* and *Proteobacteria* (Fig. 4E). Among the 10 most abundant phyla, *Firmicutes* exhibited the highest relative abundance in the NX group, followed by the BKS group, and lowest in the BN group. In contrast, the BKS group exhibited higher relative abundances of *Fusobacteriota* and *Actinobacteriota* than both the NX and BN groups. The BN group, however, showed higher relative abundances of *Proteobacteria* and *Bacteroidetes* than both the NX and BKS groups. At the genus level, the 10 most abundant genera included *Escherichia-Shigella, Lactobacillus, Clostridium_sensu_stricto_1, Romboutsia, Terrisporobacter, Streptococcus, unclassified_Bacilli, Klebsiella, Actinobacillus*, and *unclassified_Muribaculaceae* (Fig. 4F). Differential abundance analysis showed that the relative abundances of *Escherichia-Shigella, Clostridium_sensu_stricto_1, Romboutsia*, and *Terrisporobacter* were higher in both the BKS and BN groups than in the NX group. *Lactobacillus* exhibited the highest relative abundance in the NX group. The relative abundance of *Streptococcus* was higher in the BKS group than in both the NX and BN groups, whereas *Klebsiella* and *Actinobacillus* were more abundant in the BN group than in both parental groups.

LEfSe analysis identified differentially abundant microbial taxa as potential biomarkers among the three pig breeds, using a LDA score threshold of > 4.5 (Fig. 4G). Eight potential biomarkers were identified in the NX group, including Firmicutes, Bacillus, and Lactobacillus. The BKS group had four potential biomarkers, including *Clostridia, Peptostreptococcales_Tissierellales, Peptostreptococcaceae*, and *Romboutsia*. The BN group exhibited 11 potential biomarkers including *Bacteroidetes, Clostridium_sensu_stricto_1, Proteobacteria*, and *Gammaproteobacteria*. These group-specific taxa further highlighted differences in ileal microbial composition among the three groups.

To further investigate potential functional differences in the ileal microbiota, PICRUSt2 was used to predict microbial functional profiles, followed by KEGG pathway analysis. The results showed that compared with the NX group, the microbial community in the BN group was mainly enriched in pathways including metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, two-component system, and homologous recombination (Fig. 4H). Similarly, compared with the NX group, the BKS group showed enrichment of predicted microbial functions related to metabolic pathways, quorum sensing, pyruvate metabolism, two-component system, and homologous recombination (Fig. 4I). These findings suggest that the ileal microbiota of the BN and BKS groups differed from that of the NX group in their predicted functional profiles.

Integrative Analysis Reveals Associations Among Genes, Intestinal Phenotypes, and Ileal Microbiota

To investigate the molecular basis of differences in small intestinal digestive and absorptive capacity, we performed integrative correlation analyses involving DEGs, intestinal phenotypic traits, and dominant ileal microbial taxa. In the NX vs. BKS comparison, functional enrichment analysis identified terms and pathways related to carbohydrate binding, positive regulation of insulin secretion, and bile secretion. Among these genes, *SI*, *GPX3*, and *PTX3* were more highly expressed in the NX group and were significantly positively correlated with maltase and AKP activities. In contrast, apoptotic protease

activating factor-1(*APAF1*), *PLCB4*, solute carrier family 6 member 14 (*SLC6A14*), and solute carrier family 28 member 3 (*SLC28A3*) were more highly expressed in the BKS group and showed significant negative correlations with maltase and AKP activities. Specifically, *APAF1* was negatively correlated with VH, whereas phospholipase C beta 4 (*PLCB4*) and *SLC6A14* were negatively correlated with APN activity. In addition, *SLC6A14* was negatively correlated with both VH and maltase activity. Notably, *GPX3* and *PTX3* were significantly positively correlated with V/C ratio and negatively correlated with CD, while *SI* and *GPX3* were positively correlated with APN activity (Fig. 5A).

In the NX vs BN comparison, functional enrichment analysis highlighted pathways related to glycolysis/gluconeogenesis and sphingolipid metabolism, including sphingolipid biosynthesis. Among the DEGs, *ACP3* and *AGTR1* were highly expressed in the NX group and significantly positively correlated with APN and AKP activities. *AGT* and *AGTR1* were positively correlated with maltase activity, whereas *AGTR1* was negatively correlated with VH. Conversely, *PLCB4* and *IGF1R* were highly expressed in the BN group. *PLCB4* was significantly negatively correlated with maltase activity but positively correlated with VH and CD. In contrast, while *IGF1R* was negatively correlated with AKP, APN and maltase activities and positively correlated with V/C ratio and VH (Fig. 5B).

In the BKS vs. BN comparison, functional enrichment analysis identified pathways related to glycine, serine, and threonine metabolism, glycolysis/gluconeogenesis, and starch and sucrose metabolism. Among the associated DEGs, *PGAM2, LDHB*, and *FUT2* were more highly expressed in the BN group, whereas *EFCAB7* was more highly expressed in the BKS group. *PGAM2* and *LDHB* were significantly positively correlated with VH and V/C ratio and negatively correlated with CD, whereas *FUT2* was negatively correlated with APN activity. In contrast, *EFCAB7* was significantly positively correlated with maltase and APN activities. Furthermore, *TLR2* was positively correlated with maltase activity, while *VIP* was negatively correlated with VH and V/C ratio but positively correlated with APN and maltase activities (Fig. 5C).

We next integrated the dominant ileal microbiota with DEGs and intestinal phenotypic traits to further investigate associations between microbial composition and host intestinal characteristics. In the NX vs. BKS comparison, *Lactobacillus* was significantly positively correlated with *GPX3* and *PTX3* but negatively correlated with *APAF1* and *PLCB4*. Conversely, *Escherichia-Shigella* was significantly positively correlated with *APAF1* and *PLCB4* but negatively correlated with *GPX3*.

Both *Clostridium_sensu_stricto_1* and *Romboutsia* were significantly positively correlated with *APAF1* (Fig. 5D). In the NX vs. BN comparison, *Lactobacillus* was significantly positively correlated with *AGT* and *AGTR1* but negatively correlated with *PLCB4, IGF1R*, and *PCK1*. *Escherichia-Shigella* was significantly positively correlated with *PLCB4* and *PCK1* but negatively correlated with CD, *AGT*, and *AGTR1*. Both *Clostridium_sensu_stricto_1* and *Romboutsia* were significantly positively correlated with V/C ratio and *PCK1* but negatively correlated with CD. In addition, *Romboutsia* was negatively correlated with *AGTR1* (Fig. 5E). In the BKS vs. BN comparison, *Lactobacillus* was significantly negatively correlated with VH, whereas *Romboutsia* was significantly positively correlated with *EFCAB7* (Fig. 5F).

Discussion

The small intestine is the principal site of nutrient digestion and absorption in animals [14]. Along the proximal-to-distal axis, the duodenum, jejunum, and ileum exhibit distinct structural and functional characteristics [15]. The intestinal mucosal architecture, digestive enzyme activities, and gut microbial colonization patterns collectively contribute to nutrient utilization and intestinal homeostasis [16, 17]. In the present study, the V/C ratio in the duodenum and jejunum was significantly higher in NX group than in BKS group. This finding is consistent with previous reports showing more favorable intestinal mucosal morphology in Taoyuan pigs than in Duroc pigs, suggesting that differences in intestinal morphology may be associated with genetic background and breed-specific characteristics in pigs [18, 19]. The V/C ratio is an important morphological indicator of intestinal mucosal development and is commonly used to assess intestinal structural integrity and potential absorptive capacity. A higher V/C ratio generally indicates a more favorable balance between villus development and crypt depth and may be associated with improved nutrient absorption and intestinal barrier function [20, 21]. These morphological differences may be related to the long-term adaptation of indigenous pig populations to their production environments and dietary conditions. Chen et al. reported that a low-energy diet was more favorable for the overall regulation of growth performance and meat quality in Ningxiang pigs, further suggesting their specific nutritional adaptation [22]. Regarding digestive enzyme activities, the NX group exhibited significantly higher duodenal activities of AKP, maltase, and APN, as well as higher jejunal maltase activity, than the BKS group. These results are consistent with previous findings that Taoyuan black pigs and their crossbred offspring exhibit higher digestive enzyme activities than exotic lean-type pig breeds [23]. The higher activities of enzymes involved in carbohydrate and protein digestion may enhance the hydrolysis of dietary nutrients and thereby contribute to intestinal digestive capacity. However, whether these differences translate into improved overall nutrient utilization or reflect adaptations to specific dietary conditions requires further investigation.

Host genetic background may contribute to the functional regionalization of the small intestine through segment-specific differences in gene expression. Transcriptomic analysis revealed limited overlap among DEGs between the NX and BKS groups across th the duodenum, jejunum, and ileum, suggesting that the effects of host genetic background on intestinal gene expression vary among intestinal segments rather than following a uniform regulatory pattern throughout the small intestine. In the duodenum, a major site of initial nutrient digestion, DEGs were predominantly enriched in starch and sucrose metabolism and ATPase activity coupled to transmembrane movement of substances. *SI* is a brush-border enzyme complex that contributes to the hydrolysis of dietary carbohydrates, including sucrose and starch-derived oligosaccharides, and its maltase activity may therefore contribute to the observed differences in carbohydrate digestion [24]. In the jejunum, a major site of nutrient absorption, DEGs were enriched in pathways associated with ABC transporters and fat digestion and absorption. Differential expression of solute carrier genes, including SLC28A3 and SLC6A14, may reflect differences in intestinal nutrient transport, given their respective roles in nucleoside and amino acid transport [25, 26]. These findings provide molecular evidence suggesting that genetic differences between NX and BKS group may contribute to the functional characteristics of jejunal nutrient absorption. In the ileum, DEGs

were primarily enriched in peptidase activator activity, vitamin digestion and absorption, and bile secretion. These enrichment patterns may reflect differences in processes related to peptide digestion, vitamin handling, and bile acid metabolism, further highlighting the distinct molecular characteristics of the ileum [27, 28].

The gut microbiota is an important component of the intestinal mucosal microenvironment and contributes to the regulation of host intestinal function, nutrient metabolism, and immune homeostasis [29]. In the present study, the ileal microbiota of the NX group was characterized by a relatively high abundance of taxa such as *Firmicutes* and *Lactobacillus*, whereas the BKS group exhibited higher abundances of potentially detrimental genera, including *Escherichia_Shigella* and *Clostridium_sensu_stricto_1*. Notably, *Lactobacillus* was identified as a microbial marker distinguishing the NX group from the BKS group. Its higher relative abundance was associated with differences in the abundance of other microbial taxa, including potentially detrimental bacteria. A similar microbial feature was also observed in the BN group, although the extent to which the hybrid offspring shared the microbial characteristics of the NX group should be interpreted in light of the group-specific results. Previous studies have reported that *Lactobacillus* supplementation can increase VH, expand the effective absorptive surface area of the intestinal mucosa, and improve average daily gain and feed conversion efficiency in piglets [30]. In addition, porcine-derived *Lactobacillus salivarius* has been reported to adhere preferentially to ileal mucosal epithelial cells and inhibit the mucosal colonization of pathogens such as *Escherichia coli* and *Salmonella*, potentially supporting intestinal health and nutrient utilization [31]. Certain strains, including *Lactobacillus delbrueckii*, have also been reported to modulate TLR2 and TLR4-associated signaling pathways, alleviate intestinal oxidative stress and mucosal injury, and help maintain intestinal barrier integrity [32]. Collectively, these findings suggest that the relatively high abundance of Lactobacillus in the NX group may be associated with its intestinal characteristics. However, further studies are needed to determine whether this association reflects a functional contribution of the microbiota to intestinal morphology and digestive capacity.

Correlation analyses revealed that *Lactobacillus* abundance was positively correlated with the expression of *GPX3* and *PTX3*, which are involved in antioxidant defense and immune regulation [33, 34], as well as with the intestinal V/C ratio. In contrast, *Lactobacillus* abundance was negatively correlated with the expression of *APAF1* and *PLCB4*, which are involved in apoptotic signaling and intracellular signal transduction, respectively [35]. These associations suggest that *Lactobacillus* abundance may be linked to intestinal mucosal characteristics and host defense responses, although the underlying mechanisms remain to be elucidated. In the BKS group, the relative abundance of *Escherichia_Shigella* was positively correlated with *APAF1* and *PLCB4* expression but negatively correlated with *GPX3* and *PTX3* expression. This correlation pattern may reflect an association between the ileal microbial community and host gene expression; however, it does not establish impaired host defense, altered epithelial cell turnover, or a direct contribution to differences in intestinal morphology and digestive function. Overall, the intestinal characteristics observed in the NX group may be associated with interactions between host genetic background and the gut microbiota, potentially contributing to differences in mucosal architecture and digestive enzyme activities. Although these characteristics may be consistent with the proposed

adaptation of indigenous pig populations to particular dietary conditions, their relationship with adaptation to low-nutrient-density, high-fiber diets requires further investigation [10, 36].

Notably, the BN group exhibited significantly more favorable ileal mucosal morphological traits than both parental groups, suggesting a potential heterotic phenotype in intestinal morphology. This finding highlights the possibility that crossbreeding may generate intestinal phenotypes that exceed those observed in either parental group, rather than simply reproducing the parental phenotypic characteristics [37]. Notably, the BN group exhibited significantly more favorable ileal mucosal morphological traits than both parental groups, suggesting a potential heterotic phenotype in intestinal morphology. This finding highlights the possibility that crossbreeding may generate intestinal phenotypes that exceed those observed in either parental group, rather than simply reproducing the parental phenotypic characteristics [38]. However, pathway enrichment alone cannot establish the presence of non-additive genetic effects, which require further validation.

Interactions between the gut microbiota and host genetic background may be associated with differences in intestinal function among pig groups and may contribute to the potential intestinal functional heterosis observed in the hybrid offspring. Previous studies have demonstrated that host genetic background significantly influences gut microbiota composition in pigs [39]. In the present study, the BKS group exhibited the highest microbial richness, whereas the microbial diversity of the BN group was intermediate between that of the two parental groups. These findings suggest that host genetic background may be associated with differences in ileal microbial community structure among the three groups. Functional enrichment analysis further revealed associations between microbial pathways related to secondary metabolite biosynthesis and metabolism in the BN group and host functional pathways involved in vitamin metabolism and glycolysis. These associations may reflect potential links between microbial metabolic functions and host gene expression in the hybrid offspring. However, further studies are needed to clarify whether these relationships contribute to differences in intestinal nutrient metabolism and function. Given the distinct microbial profiles and host functional characteristics observed among the groups, host–microbiota interactions may provide a useful framework for investigating the potential mechanisms underlying intestinal functional differences and heterosis in pigs [40]. In addition, LEfSe analysis identified group-associated microbial biomarkers, including *Lactobacillus*, *Roseburia*, and *Clostridium*, which may serve as potential indicators for pig population discrimination and targets for breeding-assisted selection and precise modulation of intestinal microbiota. These findings may have practical implications for pig breeding and intestinal health management. Overall, intestinal functional differences among pig groups may be associated with variations in intestinal morphology and microbial community composition.

Conclusions

The NX group exhibited a higher V/C ratio and greater activities of several intestinal digestive enzymes than the BKS group, indicating differences in intestinal morphology and digestive capacity between the two genetic backgrounds. The BN group exhibited more favorable intestinal morphological

characteristics than both parental groups in the ileum, suggesting potential heterosis in intestinal morphology and related digestive and absorptive functions. Transcriptomic analysis revealed segment-specific molecular differences among the three groups, with *SI*, *PLCB4*, *AGT*, and *PGAM2* identified as candidate genes for further investigation of intestinal digestion and absorption. In addition, distinct gut microbial profiles were observed among the three groups, with *Lactobacillus*, *Roseburia*, and *Clostridium* identified as group-associated microbial biomarkers for the NX, BKS, and BN groups, respectively. These findings provide insights into the relationships among host genetics, intestinal function, and gut microbiota, and may inform further research on pig breeding and intestinal health.

Abbreviations

ACP3	**acid phosphatase 3**
AGT	angiotensinogen
AGTR1	angiotensin II receptor 1
AKP	alkaline phosphatase
APAF1	apoptotic protease activating factor-1
APN	aminopeptidase N
CD	crypt depth
DEGs	differentially expressed genes
EFCAB7	EF-hand calcium-binding domain protein 7
FUT2	α-1,2-fucosyltransferase 2
GO	Gene Ontology
GPX3	glutathione peroxidase 3
H&E	hematoxylin and eosin
IGF1R	insulin-like growth factor 1 receptor
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDHB	lactate dehydrogenase B
PCK1	phosphoenolpyruvate carboxykinase 1
PCoA	principal coordinates analysis
PGAM2	phosphoglycerate mutase 2
PLCB4	phospholipase C beta 4
PTX3	pentraxin 3
RT-qPCR	real-time quantitative PCR
SEM	standard error of the mean
SI	sucrase-isomaltase
SLC28A3	solute carrier family 28 member 3
SLC6A14	solute carrier family 6 member 14
TLR2	toll-like receptor 2
V/C	villus-to-crypt
VH	villus height

ACP3	**acid phosphatase 3**
VIP	vasoactive intestinal peptide

Declarations

Ethics approval and consent to participate

This animal study was conducted in strict accordance with international ethical guidelines and was approved by the Animal Ethics Committee/Institutional Animal Care and Use Committee (IACUC) of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (No. ISA-2023-0020, approval date:20 February 2023).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

Hou X.H. participated in writing the original draft, conducting investigations, data visualization and data curation. Wang P. and Wang Q. participated in original draft writing, investigation and data curation. Jin Z. and Guo Z.H. participated in investigation and data curation. Yin S.S. participated in manuscript review and editing as well as methodology establishment. Gao H. participated in project administration and methodology. Sun J.X. and Ren R.M. participated in manuscript review, editing and methodology development. Yin Y.L. was involved in manuscript review and editing, funding acquisition and conceptualization. Xu K. participated in manuscript revision and editing, project administration,

methodology construction, funding acquisition and conceptualization of the research. All authors have read and approved the final manuscript.

Data Availability

Raw sequencing data have been deposited in the NCBI BioProject database under accession numbers PRJNA1364994 and PRJNA1479259.

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Figures

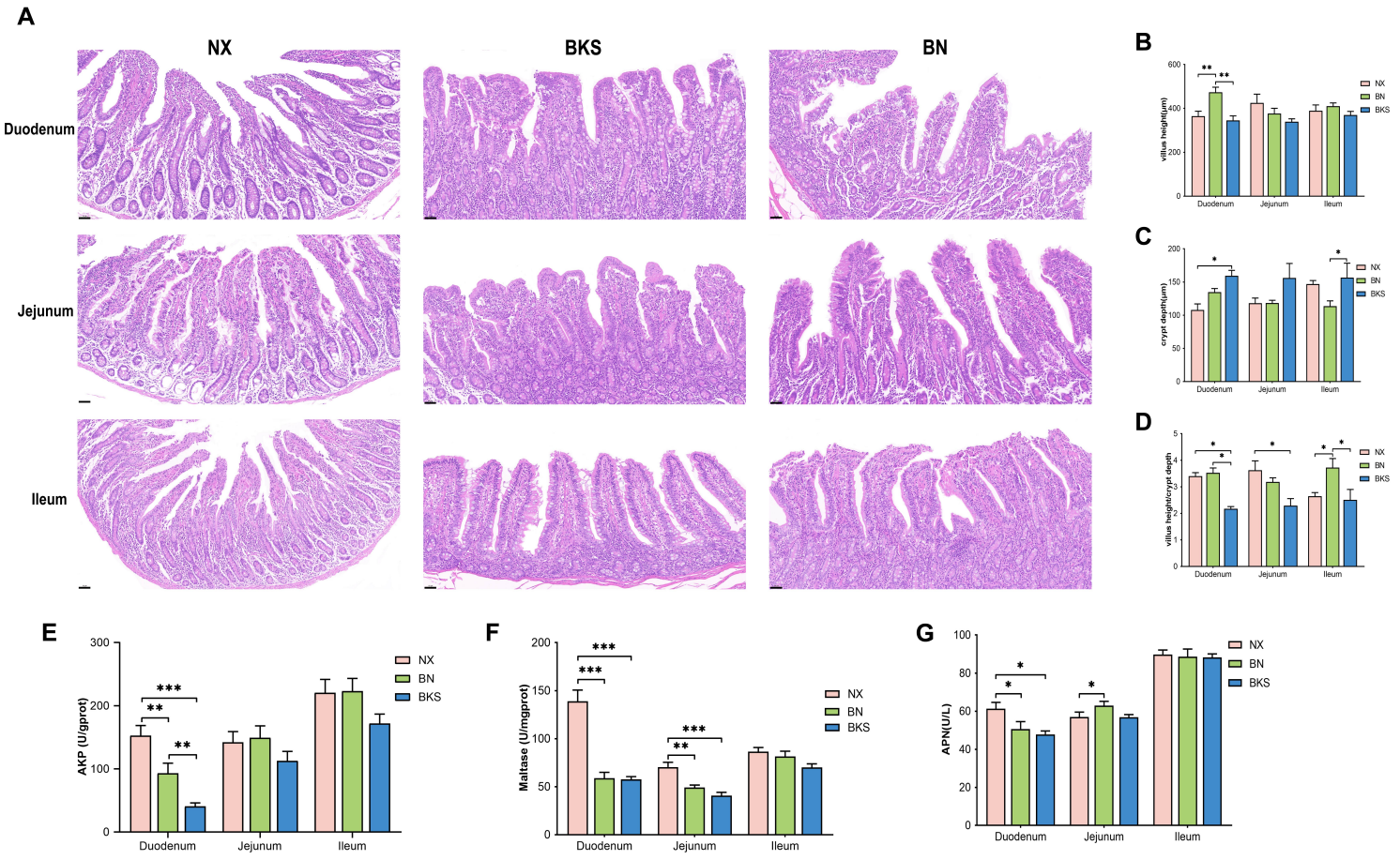


Figure 1

Comparison of small intestinal morphology and brush border enzyme activities among pig groups. (A) Representative H&E-stained sections of the duodenum, jejunum, and ileum. Scale bar = 50 μm. (B-D) VH, CD, and V/C ratio in the duodenum, jejunum, and ileum, respectively. (E-G) Activities of brush border enzymes, including AKP, maltase, and APN, in the duodenum, jejunum, and ileum, respectively. Data are

presented as mean ± SEM. Asterisks indicate statistically significant correlations: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

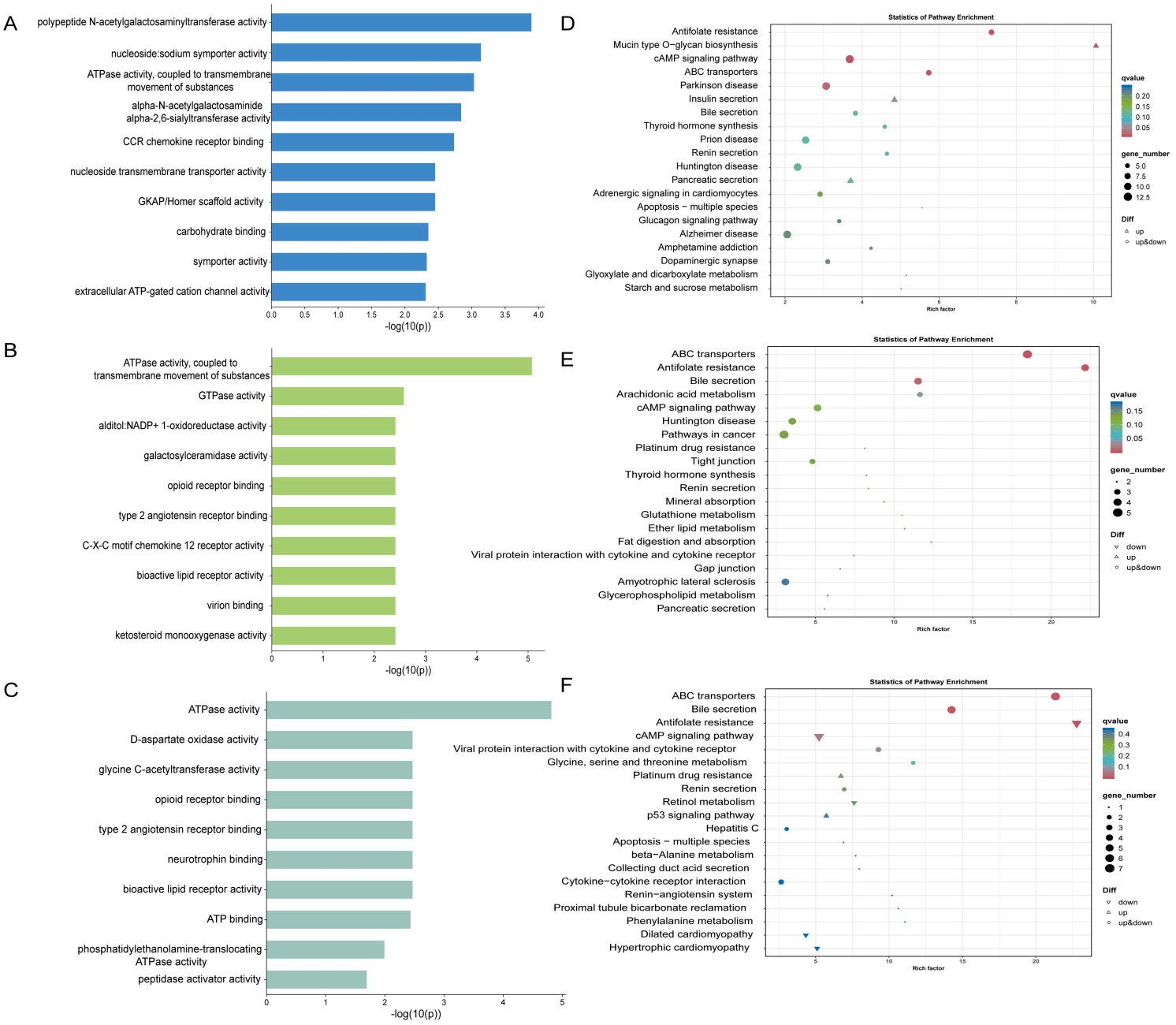


Figure 2

GO and KEGG enrichment analyses of DEGs between the NX and BKS groups across small intestinal segments. (A-C): GO functional enrichment analysis of DEGs in the duodenum (A), jejunum (B), and ileum (C). (D-F): KEGG pathway enrichment analysis of DEGs in the duodenum (D), jejunum (E), and ileum (F).

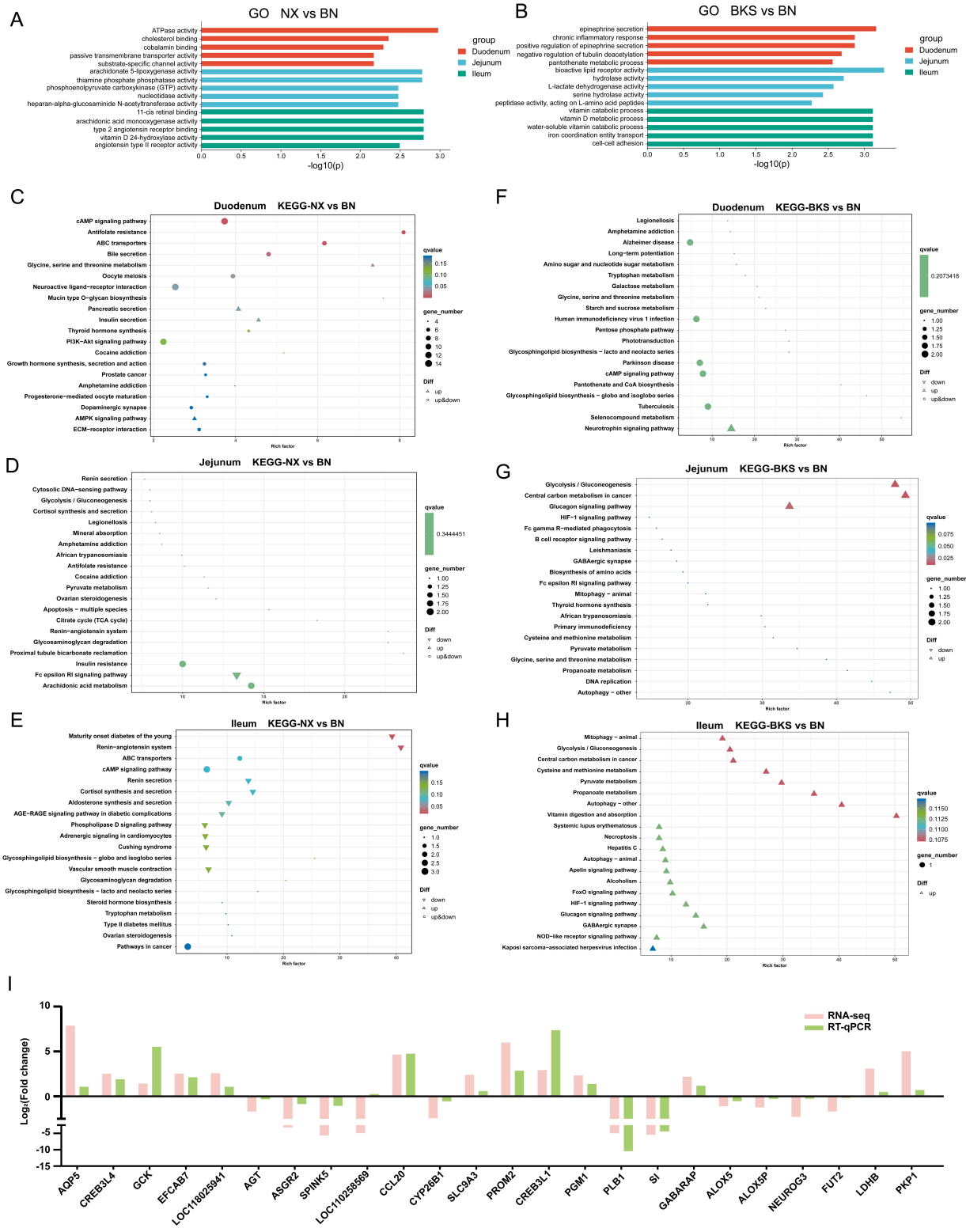


Figure 3

GO and KEGG enrichment analyses of DEGs in the small intestine of BN crossbred offspring compared with their parental lines, and RT-qPCR validation. (A) GO enrichment analysis of DEGs in the NX vs. BN. (B) GO enrichment analysis of DEGs in the BKS vs. BN comparison. (C-E) KEGG pathway enrichment analyses of DEGs in the duodenum, jejunum, and ileum, respectively, in the NX vs. BN comparison. (F-H)

KEGG pathway enrichment analyses of DEGs in the duodenum, jejunum, and ileum, respectively, in the BKS vs. BN comparison. (I) RT-qPCR validation of 25 randomly selected DEGs.

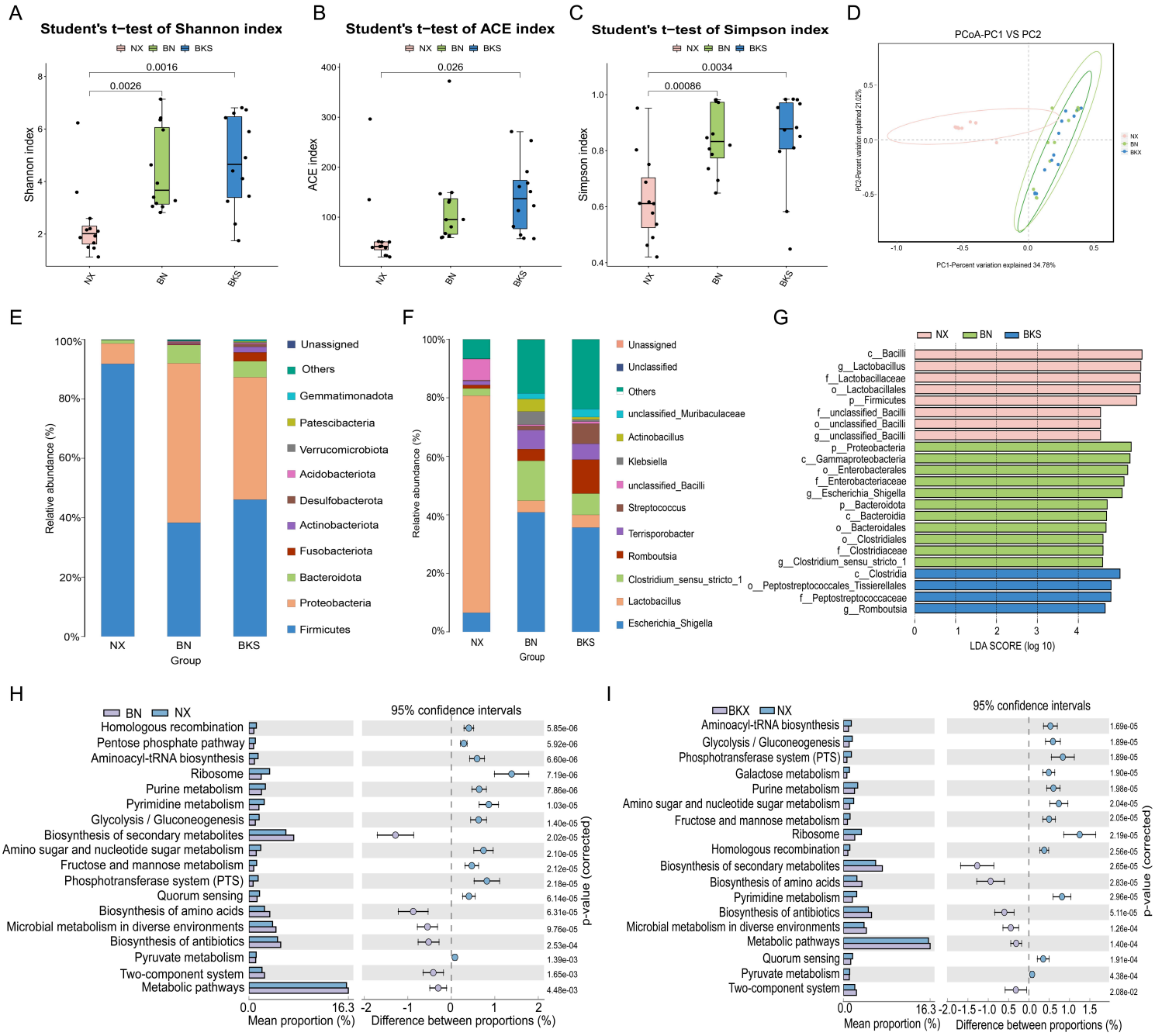


Figure 4

Diversity and taxonomic composition of the ileal microbiota in pig breeds and their hybrid offspring. (A-C) α-Diversity of the ileal microbiota based on the Shannon, ACE, and Simpson indices, respectively. (D) PCoA based on β-diversity, showing differences in ileal microbial community structure among the NX, BKS, and BN groups. (E) Relative abundance of the top 10 bacterial phyla in ileal contents. (F) Relative abundance of the top 10 bacterial genera in ileal contents. (G) LEfSe analysis identifying differentially abundant microbial taxa among the three groups, with an LDA score threshold of > 4.5 for identifying

potential biomarkers. (H-I) KEGG pathway analyses of predicted microbial functional profiles of the ileal microbiota in the BN and BKS groups compared with the NX group, respectively.

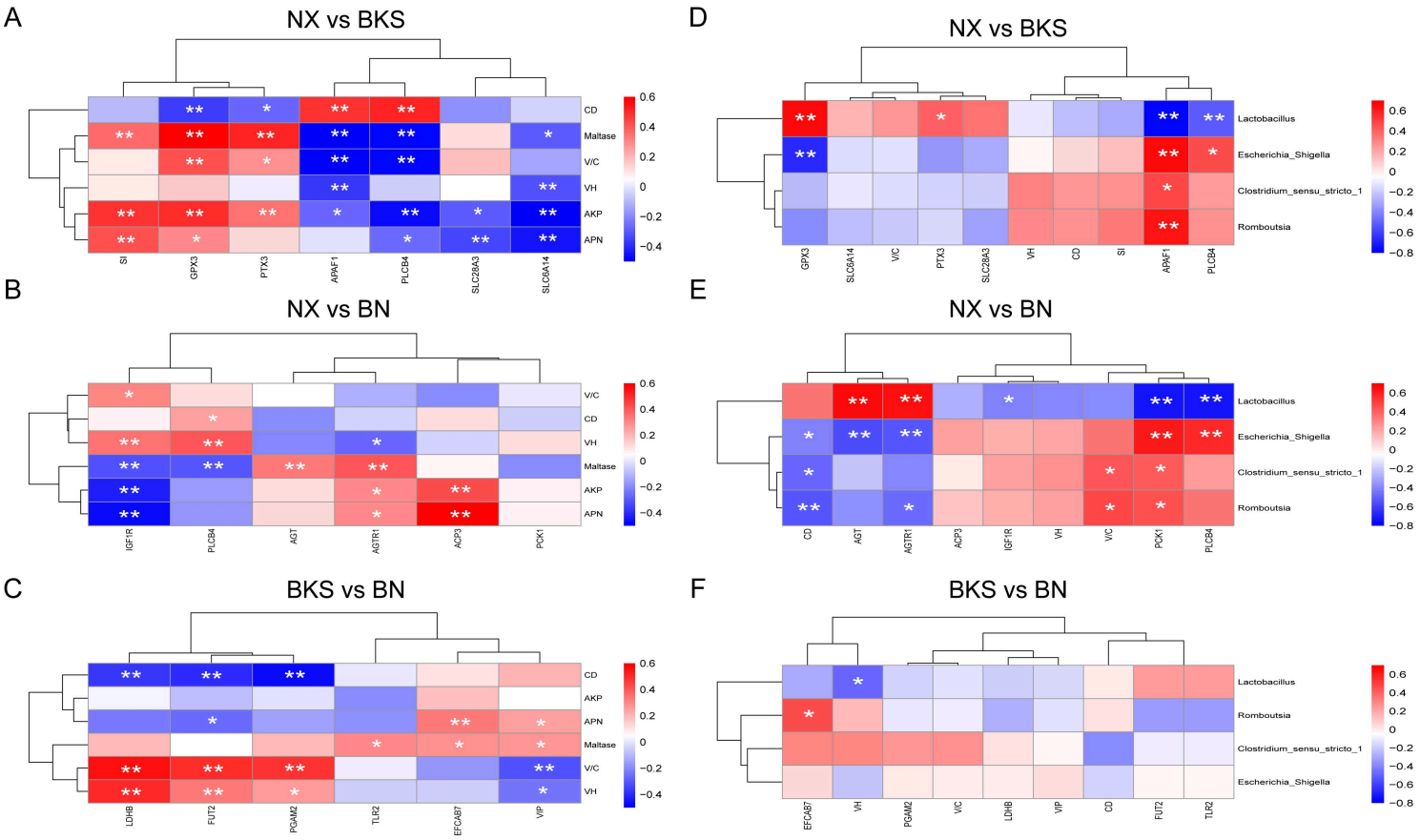


Figure 5

Correlation analysis integrating differentially expressed genes, intestinal phenotypes, and ileal microbiota among the NX, BKS, and BN groups. (A-C) Correlations between selected DEGs and intestinal phenotypic traits in the NX vs. BKS, NX vs. BN, and BKS vs. BN comparisons, respectively. (D-F) Correlations between dominant ileal microbial taxa and selected DEGs and intestinal phenotypic traits in the NX vs. BKS, NX vs. BN, and BKS vs. BN comparisons, respectively. Red and blue indicate positive and negative correlations, respectively, with color intensity representing the strength of the correlation. Asterisks indicate statistically significant correlations: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.