# Antioxidant Potential of Pediococcus Pentosaceus Strains From the Sow Milk Bacterial Collection in Weaned Piglets

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**Research**

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# Abstract

**Background:** In modern animal husbandry, the relationship between sow and piglets is closely linked as breeders pay more attention to improving sow nutrition during pregnancy and lactation to favor the growing of neonates. Sow milk is the main food for piglets during their first three weeks of life, which is not only a rich repository of essential nutrients and a broad range of bioactive compounds, but also an indispensable source of commensal bacteria. Maternal milk microorganisms are important sources of commensal bacteria for neonatal gut. Bacteria from hosts' maternal milk may serve as an additive to confer a health benefit on the composition of the indigenous microbiota of piglets.

**Methods:** We first obtained isolates from the sow milk microbiota by the culturomics methods of continuous culture and interval sampling. Then, identified and grouped them based on a nonredundant set of 16S rDNA gene sequences. After assessing their antimicrobial activity against enteropathogens *in vitro*, we selected several strains to further conduct assay in *Drosophila* to evaluate their resistance against oxidative injury. We finally screened out *Pediococcus pentosaceus* (*P. pentosaceus*) SMM914 as candidate strain to verify its antioxidant effect in weaned piglets and study its probiotic function by 16S rDNA sequencing, metabolomics, western blotting and enzyme activity analysis.

**Results:** The 1240 isolates were screened out from the sow milk microbiota and grouped into 271 bacterial taxa. We evaluated 80 *Pediococcus* isolates about their inhibition ability against enteropathogens *in vitro* and then chose top 10 isolates to further test them in *Drosophila*. In 80 *Pediococcus* isolates, *P. pentosaceus* SMM914 showed best performance by inhibition ability against enteropathogens *in vitro* andparaquat challenge in *Drosophila* model. Pretreatment of piglets with SMM914 induces the Nrf2/Keap1 antioxidant signaling pathway and altered the pathways of amino acid metabolism and lipid metabolism in plasma. In colon, *Lactobacillus* was significantly increased in the high dose of SMM914 group compared with the control group.

**Conclusion**: Our study provided useful resources for deeply understanding the relationships between the maternal microbiota and the offspring and supported the theory of Sow and Piglet Integration.


# Introduction

A unique microbiome is established in every individual in their first few years of life. In particular, the first year of life is the crucial stage of the development of the microbiome. Breastfeeding is the main factor affecting the development of the microbiome in this period[1]. Breastfed infants have a decreased risk of acute and chronic diseases, including gastroenteritis and sudden infant death syndrome, because breast milk provides essential nutrients and a broad range of bioactive compounds fordeveloping neonates[2]. In addition, commensal bacteria from breast milk act as pioneer bacteria during the critical stage of initial neonatal gut colonization[3]. Several studies have specifically demonstrated at the strain level that there are some bacteria shared between human breast milk and infant feces by isolating and identifying

bacteria[4]. The breast milk-associated microbiota contribute to the “initial” intestinal microbiota establishment in infants and may help to modulate both short- and long-term infant health outcomes[5].

Due to the high degree of similarity in anatomy, physiology and immunology between humans and pigs, piglets have been extensively used as an ideal model to study factors influencing infants’ gastrointestinal system and health[5]. Additionally, pork is the most widely consumed meat in the world[6]. The normal development of piglets plays a foundational role in influencing the profitability of pork production. Early weaning is an abrupt, stressful event in piglets that often leads to severe oxidative stress and restricts the development of commercial swine husbandry. Breast milk is the main food for piglets during the first three weeks of their lives. The vertical transfer concept that the breast milk microbiota can be transmitted from mother to neonate during breast feeding became accepted gradually[7]. Accumulating evidence has revealed that the breast microbiota has great impacts on health[8]. Although abundant probiotics have been isolated from human milk and bovine milk[9–11], strategies for the preservation, isolation and study of the natural bacteria of sow milk are currently limited. Culture-independent methods have allowed an understanding of the composition and diversity of sow milk microbiota[6], but culture-dependent methods are still critical for the functional identification and utilization of the breast milk microbiota, which can be indirectly or directly beneficial for precise care of early-weaning piglets. However, research systematically investigating the sow milk microbiota using culture-dependent methods has not yet been performed.

In the present work, a comprehensive sow milk microbiota collection was first established by the culturomics of continuous culture and interval sampling, and a total of 1,240 bacteria were isolated. Then, we depended on antagonistic experiments against pathogens *in vitro* and antioxidant tests in *Drosophila* model to select the candidate probiotic species *Pediococcus pentosaceus* (*P. pentosaceus*). *P. pentosaceus* belongs to the gram-positive, catalase-negative, homofermentative bacteria that has long been used as biopreservatives and healthy commercial starters in fermented food[12–14]. Interestingly, the antioxidant activity of *P. pentosaceus* SMM914 in piglets was verified by elevated antioxidant metabolites in plasma and the activation of the Nrf2/Keap1 pathway in livers. It is suggested that SMM914 could represent a promising candidate for maintaining redox balance and conferring health benefits to piglets. These results regarding the sow milk bacterium and its antioxidant function in piglets showed the importance of Sow and Piglet Integration (SPI). Our studies could support future research on the probiotic properties of sow milk microbiota, presenting great potential for promoting healthy livestock breeding.

## Results

# A comprehensive collection of lactic acid bacteria from sow milk

Breast milk is one of the common sources of probiotic strains[15]. We hypothesized that these lactic acid bacteria (LAB) from sow milk could facilitate adaptive, functional changes for the optimal weaning transition of piglets, which are vulnerable to various stressors. However, the diversity and function of

probiotics in porcine milk remain relatively understudied[16]. To establish a sow milk bacterial collection (smBC), the large-scale cultivation and identification of the sow milk microbiota (SMM) were performed by three procedures (Fig. 1, **steps 1–3**).

After the first three steps, we obtained 1240 isolates derived from the sow milk microbiota by the culturomics of continuous culture and interval sampling. These isolates were grouped into 271 bacterial taxa in the CD-HIT analysis based on a nonredundant set of sequences of the V1-V5 region of the 16S rDNA gene with a cutoff value of 99% identity for classification. The Silva version 132 16S rRNA database, NCBI nucleotide collection (nr/nt) database and DAIRYdb reference database were used to classify all isolated prokaryotes into two categories: suspected new species and previously identified prokaryote species (including known species from dairy products). A phylogenetic tree was built based on the calculated distances between pairs of sequences (**Table S1,2**). The results revealed that 151 taxa were assigned to previously described species **(black, Fig. S1**), while the other 120 taxa could not be assigned to any known species (**blue, Fig. S1**), which were defined as suspected new species. The alignment against the DAIRYdb reference database revealed that 107 out of the 271 taxa were assigned to species in dairy products (**red dots, Fig. S1**).

Specifically, 23 species were assigned to previously described species found in both the DAIRYdb reference database and Silva version 132 16S rRNA sequences database or the NCBI nucleotide collection (nr/nt) database, including *A. lwoffii*, *Acinetobacter sp., C. perfringens*, *Pelomonas, Enterococcus sp.*, *E. durans*, *L. amylovorus*, *L. taiwanensis*, *L. garvieae*, *L. mesenteroides*, *Enterobacteriaceae bacterium* DHL-32, *Lactobacillales bacterium*, *Lactobacillaceae bacterium*, *Streptococcaceae bacterium*, *S. enterica* and *Salmonella sp*. Nine species were suspected new species and could not be assigned to any known species in the DAIRYdb reference database. In addition, five species were assigned to a previously described species in the Silva version 132 16S rRNA database or NCBI nucleotide collection (nr/nt) database, but not in the DAIRYdb database, including *Acidovorax sp.* SEPRH9, *S. hyovaginalis, S. mitis*, *Streptococcus sp.* S2 and *S. thoraltensis* (Fig. 2 **and Fig. S1**).

A total of 922 out of 1240 isolates belong to *Lactobacillales* (**Suppl. Data 2**), and this group was dominated by *Lactococcus lactis*, which was roughly consistent with the microbiota composition of sow milk during lactation according to a recent report [16]. The genera *Staphylococci* and *Streptococci* represented 5.81% and 4.03% of the total bacterial isolates, respectively. These results supported the view that commensal *Staphylococci* and *Streptococci* commonly occur in breast milk[17, 18], which may originate from the maternal skin[19]. The collection of 1240 isolates obtained through these efforts provides insight into the diversity of sow milk microbiota and enables more studies on their function associated with mammalian health and diseases.

**Screening of candidate probiotic** P. pentosaceus **strains**

To explore health-promoting bacterial genera, which are not limited to traditional probiotic of *Lactobacillus* and *Bifidobacterium*, we focused on *Pediococcus*. Antagonistic activity against pathogens to control their spread is a prerequisite for a potential probiotic[20]. Piglets commonly encounter

pathogens on farms at increasing frequencies[21], including *Salmonella typhimurium* (*S. typhimurium*) [22], *enterohemorrhagic Escherichia coli* (EHEC)[23], *enterotoxigenic Escherichia coli* (ETEC)[24], *Klebsiella pneumoniae* (*K. pneumoniae*)[25], *Aeromonas punctate* (*A. punctate*)[26], *Staphylococcus aureus* (*S. aureus*)[27], *Listeria monocytogenes* (*L. monocytogenes*)[2] and *Clostridium perfringens* (*C. perfringens*) [28]. The fluctuating size of the inhibition zone against pathogens indicates the inhibitory activities of different *P. pentosaceus* against both gram-negative and gram-positive pathogenic bacteria (Fig. 3a), revealing strain-specific antimicrobial activity against different bacteria[29]. The morphology of the top ten strains was observed (**Fig. S2**), and they were then used in subsequent tests.

For these *P. pentosaceus* strains with strong antimicrobial activity, we next used a simple animal model to rapidly screen bacteria that show potent antioxidant activity *in vivo* (Fig. 3b). Considering the similarities of the intestinal development with mammals and the cost of the mouse model[30, 31], *Drosophila* could be an appropriate model to evaluate the ability of our bacteria in protection of the host from reactive oxygen species (ROS), whose accumulation causes damage to the health of both *Drosophila* and mammals[30, 32]. Resistance to paraquat can be used as a measure of free radical scavenging activity in the *Drosophila* system[32, 33]. After paraquat treatment for 45 hours, the flies colonized with *P. pentosaceus* SMM914 showed a significantly elevated survival rate in response to paraquat challenge ($p < 0.05$, log-rank test) (Fig. 3c).

SMM914 showed a strong ability to inhibit the proliferation of *S. aureus*, while almost one-third of *P. pentosaceus* strains exhibited no antimicrobial ability toward this species. Through the sodium hydroxide neutralization reaction, the antibacterial effect of SMM914 against *S. aureus* and ETEC was abolished, which proved that its bacteriostatic effect is mainly due to the presence of organic acids in either anaerobic or aerobic conditions (**Fig. S3**). We measured the time curves of growth and pH for SMM914. The results revealed that the strain entered the stationary phase after 12 h of fermentation, while the pH was stabilized at approximately 3.9 after 36 h of fermentation under either anaerobic or aerobic conditions (**Fig. S4 a, b**). Additionally, after sequencing verification of the 16S rDNA, L-lactate dehydrogenase 1 and L-lactate dehydrogenase 2 genes, it was revealed that SMM914 is closest to *P. pentosaceus* SRCM100194 (**Table S1**).

# Growth performance and serum biochemical parameters in the pig-feeding trial

Because SMM914 showed bacteriostatic activity and the strongest resistance to paraquat-induced stress in *Drosophila*, it was selected to treat the piglets in the low-dose (LD) group or high-dose (HD) groups prior to early weaning (Fig. 4a). There were no significant differences among the three groups regarding growth performance (**Table S2**). However, the HD group showed a tendency toward an increased length of the small intestine compared to the control ($p = 0.07$, $n = 7$). The consumption of SMM914 in the HD group increased the final body weight by 7.23% ($p > 0.05$, $n = 18$). Regarding visceral indices, a higher heart coefficient was observed in piglets treated with SMM914 ($p < 0.05$, $n = 7$) (**Table S2**). The heart coefficient has been reported to be negatively associated with oxidative stress via changes in angiotensin

II-aldosterone-brain natriuretic peptide[34]. We speculated that the increased heart coefficient observed in this study could be an indicator of alleviated stress.

Blood biochemical parameters demonstrated that treatment with SMM914 reduced ALT, LDH, TP and ALB levels, which are closely related to protein metabolism and reflect liver damage (**Table S3**). Other serum biochemical traits were not influenced by oral gavage. Weaning is frequently associated with liver injury and alters blood chemistry related to liver function[35]. The elevation of ALT and LDH activities in serum can be used as a biomarker of hepatic disorders and the loss of functional integrity under oxidative stress[36–38]. The shift observed in our study was in accordance with another report that the administration of *P. pentosaceus* LI05 significantly prevented increases in TP and ALT in the context of acute liver failure[39]. Thus, compared with the control in our study, liver injuries in the treated groups were ameliorated.

# Pretreatment of piglets with SMM914 induces the Nrf2/Keap1 antioxidant signaling pathway

The liver is a target organ of stress in vertebrates and is commonly accepted to be involved in the secretion of bile salts, the phagocytosis of residual materials and the metabolism of proteins as well as detoxification[40]. When constantly challenged by various endogenous or exogenous free radicals, the liver is susceptible to damages [22, 41]. To provide a theoretical basis for the clinical use of SMM914, this study investigated the alteration of the Nrf2/Keap1 signaling pathway in the liver by western blotting analysis and enzyme activity assays. The Nrf2/Keap1 signaling pathway is conserved across metazoans[42, 43]. Keap1 is a specific repressor of Nrf2 via tight binding. Antioxidant metabolites can cause the dissociation of Keap1 and Nrf2 complex, promoting Nrf2 movement into the nucleus[44]. Nrf2 transfers from the cytosol to the nucleus, resulting in the coordinated transcriptional upregulation of a battery of antioxidant enzymes and detoxifying proteins[45]. CAT and SOD1 are widely recognized as important endogenous antioxidant enzymes that scavenge hydroxyl and superoxide anion radicals[46, 47].

As expected, in the western blotting analysis of this study, the protein level of Keap1 was remarkably suppressed in piglets receiving SMM914 at both high and low doses. We found that SMM914 not only markedly increased the intranuclear protein expression level of Nrf2 but also led to elevated protein levels of NQO-1, HO-1, CAT and SOD1 in a concentration-dependent manner (Fig. 4b-e).

In the enzyme activity assays, the HD group simultaneously increased glutathione peroxidase (GSH-Px) activity, CAT activity and SOD activity ($p < 0.05$) in the livers of the high-dose group (Fig. 4f). Additionally, the HD group showed a significant decrease in MDA, a lipid peroxidation end product, in the liver compared with the control group (Fig. 4f). The western blotting data combined with enzyme activity tests suggested that SMM914 functions as a promising probiotic conferring antioxidant capacity by activating the Nrf2/Keap1 antioxidant signaling pathway in weaning piglets.

# The altered pathways of amino acid metabolism and lipid metabolism in plasma

To provide a better understanding of the antioxidant mechanism of SMM914, we further examined the metabolic profiles of blood plasma from the three groups ($n = 7$). PLS-DA plots showed separated clusters with an optimal goodness of fit ($R^2 = 0.996$, Q2 = 0.681 (Fig. 5a); $R^2 = 0.994$, Q2 = 0.479 (Fig. 5b), indicating that the models were suitable and reliable for prediction. The KEGG enrichment of differential metabolites between the HD group and the control group revealed that the pathways of amino acid metabolism and lipid metabolism were the main perturbed metabolic pathways (Fig. 5c).

In regard to amino acid metabolism, several critical antioxidant metabolites (cysteine-S-sulfate, DL-methionine sulfoxide, L-methionine) (Fig. 5d) closely related to cysteine and methionine metabolism were significantly increased by 1.41-2.03-fold in the HD group compared with the control group ($p < 0.05$). D-proline, L-proline and L-glutamate, (Fig. 5e) which are involved in arginine and proline metabolism, were increased in the LD group compared with the control group by 1.43-fold ($p < 0.05$), 1.30-fold ($p < 0.05$) and 1.34-fold ($p = 0.056$), respectively. In the glycine, serine and threonine pathway, choline was also significantly increased in the HD group (Fig. 5f).

However, the intensities of cholic acid, taurochenodeoxycholate and glycochenodeoxycholate (Fig. 5g), which are involved in the biosynthesis pathway of primary bile acid, were decreased in the LD group to 0.43-fold ($p < 0.05$), 0.58-fold ($p = 0.08$) and 0.47-fold ($p < 0.05$), respectively, compared to the levels in the control. Decreased levels of corticosterone and cortisol (Fig. 5h) were also observed in plasma, which are related to steroid hormone biosynthesis.

The possible protective effect of SMM914 on the weaned piglets is depicted in Fig. 5c. L-methionine is a limiting amino acid in early lactation[48] associated with various key physiologic events, and the increased availability of L-methionine in early lactation could have positive effects on plasma lipid metabolism and overall antioxidant status[49]. Methionine sulphoxide is also biologically available as a methionine source. High methionine bioavailability is likely to increase the entry of L-methionine into the one-carbon metabolism cycle, where S-Adenosyl-L-methionine is then used to generate S-Methyl-5'-thioadenosine and 1-Aminocycloproane-1-carboxylic acid. Through the transsulfuration and transmethylation pathway for the synthesis of the amino acid L-cysteine, L-methionine also serves as a substrate for glutathione, an endogenous sulfur-containing antioxidant[50, 51]. Glutathione is required for regulating the cell redox state and detoxification in all cell types through a direct reaction with free radicals.

Cysteine and methionine metabolism is overlapped with choline metabolism tightly because choline can serve as the substrate for L-methionine synthesis. Choline is an essential vitamin for humans and other mammals to regulate amino acid metabolism[52], particularly when L-methionine levels are not sufficient around parturition[53]. It has been established that choline deficiency induces the generation of ROS[54] and oxidative damage in rats[55], ruminants[56] and fishes[57]. Moreover, dietary supplementation with

choline enhances the antioxidative capacity in IUGR pigs[58]. New evidence has shown that choline deficiency-induced oxidative damage is associated with changes in the transcription of antioxidant enzymes and Nrf2 signaling in the liver and intestine[57]. Furthermore, in mammals, L-glutamate is an abundant amino acid in milk that is required for the synthesis of glutathione and alleviates oxidative stress by increasing antioxidant enzyme activities[59, 60]. Glutathione is decomposed into L-gamma-glutamyl amino acid, and L-gamma-glutamyl amino acid is further converted to pyroglutamic acid. A high level of pyroglutamic acid also contributes to glutathione deficiency and could be an indicator of the oxidative state[61]. Similarly, in our study, the concentration of pyroglutamic acid was significantly downregulated. Collectively, the alteration of these metabolites' intensities is conducive to the accumulation of glutathione, which is consistent with our previous enzyme activity assays showing that a high dose of SMM914 markedly increased GSH-Px activity ($p < 0.05$) in the liver. (Fig. 5f)

On the other hand, several metabolites in lipid metabolism are involved in oxidative injury[62, 63]. Cholic acid increases both the hepatic and systemic expression of oxidative stress[63]. Simultaneously, metabolic syndrome, which is both a cause and effect of oxidative stress, has been reported to be associated with elevated deoxycholic acid levels[64]. Deoxycholic acid can combine with taurine or glycine to form taurochenodeoxycholate or glycochenodeoxycholate, respectively, which are considered as hydrophobic bile acids and induce the phosphorylation of NADPH oxidase and the formation of ROS[62]. In addition, excessive stress can cause the development of neurological disorders[60]. Classic stress hormones including corticosterone and cortisol were also found to be decreased. Interestingly, these metabolites in lipid metabolism are closely related to serum ALT, which reflects damage in the liver[65]. Under psychological and emotional stress conditions, corticosterone and cortisol induce the oxidative load in the brain, with a significant increase in pro-oxidant markers in constantly changing environments[66]. In human infants, after maternal separation at weaning, separation anxiety is an inevitable phenomenon that may raise cortisol levels and even alter the gut microbiota composition through the gut-brain axis[67, 68].

# The reshaped colon microbiota in piglets by SMM914

The changeover from milk to solid feed strongly influences the development of the gut microbiota[69, 70]. Disorders in the composition of the microbiota can induce oxidative stress and chronic metabolic diseases through the liver-gut axis[71, 72]. In this study, the colonic microbiota was further investigated by using 16S rDNA gene amplicon sequencing. All samples from weaned piglets approached the saturation plateau based on Shannon-Wiener rarefaction curves (**Fig. S5**), suggesting that the sampling was sufficient for nearly all bacterial species. The shared and specific OTUs are shown in a Venn diagram (Fig. 6a). The bacterial community of the three groups shared 687 OTUs. There were 12 unique OTUs in the LD group and 35 in the HD group. Twenty-five OTUs were detected in the LD and HD groups but not in the control group. No differences were observed between the control and HD groups in terms of α-diversity (**Fig. S6**).

To intuitively measure the extent of the similarity of the overall microbiota, the results of PCA based on distance revealed a separate clustering of samples between the HD group and the control group, but the

colonic specimens of the LD group were not separated from those of the control group. These results indicated that the high dose of SMM914 reshaped the microbiota structure of the colon but not remodeled the LD group (Fig. 6b).

At the family level, the relative abundance of *Lactobacillaceae*, *Lachnospiraceae*, *Christensenellaceae* and *Ruminococcaceae* in the LD group and HD group were increased by 43.54%, 11.14%, 44.71%, and 32.37% and by 219.79%, 17.17%, 418.68%, and 57.91%, respectively, compared with the control group (**Fig. S7**). Specifically, the results revealed that the genus *Lactobacillus* was significantly increased in the HD group compared with the control group ($p < 0.05$). SMM914 also promoted the growth of the genus *Lachnospiraceae* AC2044 ($p < 0.001$) and the genus *Lachnospiraceae_uncultured* ($p < 0.05$) in the HD group (Fig. 6c).

Previous compelling investigations have demonstrated that during the suckling period, *Lactobacillus* plays a protective role against oxidative damage by upregulating the expression of glutathione reductase and glutathione S-transferase[73, 74]. The *Lachnospiraceae* family participates in the breakdown of carbohydrates and potentially contributes to antioxidative properties[75]. For example, methionine attenuates oxidative stress in rats, which was achieved through higher abundances of *Lactobacillus* and *Lachnospiraceae*[76]. Furthermore, dietary provision of sodium butyrate in broilers is reported to significantly depress the MDA concentration in the jejunal mucosa, which is associated with the microbial community, including a striking increase in *Lachnospiraceae*[77]. The *Ruminococcaceae* family is always negatively related to disease severity[78]. The *Christensenellaceae_R_7 group* plays a positive role in intestinal immunomodulation[79]. In the present work, the genus *Christensenellaceae_R_7* ($p < 0.01$), the genus *Ruminococcaceae* UCG-005 ($p < 0.01$) and the genus *Ruminococcaceae* UCG-014 ($p < 0.05$) showed enrichment in the HD group (Fig. 6c, d).

Conversely, at the family level, the relative abundances of *Bacteroidaceae* and *Prevotellaceae* in the HD group were decreased by 81.57% and 65.56%, respectively (**Fig. S7**). Specifically, the genus *Bacteroides* was observed to decrease in the HD group compared with the control group ($p < 0.05$). SMM914 also inhibited the relative abundance of *Prevotella* ($p < 0.05$) and *Prevotella 2* ($p < 0.05$) at the genus level. These decreased bacteria have been reported to be associated with oxidative stress. For example, chitosan oligosaccharides increase the antioxidant capacity by inhibiting the abundance of harmful bacteria, including *Bacteroides* and *Prevotella*[74]. Improving the cellular antioxidant potential is a promising approach for inflammatory bowel disease (IBD) prevention. The dysbiotic microbiota of IBD is mostly characterized by an increase in *Prevotellaceae* and a decrease in *Ruminococcaceae* and *Lachnospiraceae*[80]. Hence, it appeared that SMM914 administration selectively promoted the transition of a microbial community to adapt to oxidative stress by assisting those more favorable genera but simultaneously inhibiting undesirable ones.

# Discussion

In recent years, breeders have attached great importance to improving sow nutrition during pregnancy and lactation from the maternal source, rather than merely purchasing expensive creep feed for piglets at a later stage to effectively increase production and economic benefits. Here, we put forth the concept of SPI, which is an integral nutritional regulation scheme based on the physiological stages of sows and piglets on pig farms and the interrelationship of the microbiota between the two generations. In intensive animal husbandry, although the early weaning technique is beneficial for sow productivity, this strategy leads to severe stress in piglets[81]. At this life stage, piglets experience a series of stressors, including separation from the mother, transport, the mixing of litters, diet transition and frequent exposure to potential pathogens[82]. To face the challenge from pathogens, antibiotics have been routinely used in farm animal production, but their abuse threatens the health of animals and humans. One promising alternative to antibiotics in animal feed is LAB as a probiotic[83].

During the initial development of mammalian neonates, breast milk is considered a nutritious food and a natural source of commensal bacteria[84, 85] that have adapted to satisfy neonatal needs in evolution[86]. In the present work, we focused on bacteria from sow milk by using the culturomics of continuous culture and interval sampling and established a set of methods for studying the function of *Pediococcus spp*. The results of high-throughput sequencing and untargeted metabolomics underscored the importance of certain maternal microorganisms for improving the antioxidant capacity of the offspring. Conceivably, this culture and identification work is an important step that can reveal the LAB repertoire of sow milk to analyze the relationships between the maternal microbiota and that of the offspring and to deeply understand the concept of SPI. The supplementation of these probiotics could be a novel way to protect piglets from early-weaned stress syndrome. In addition, pigs are not only considered one of the most important livestock species worldwide but also ideal models for simulating conditions in humans because of the striking similarity of their intestinal physiopathology to that of human infants. There are abundant microorganisms to be explored in human milk and sow milk[6, 86]. Metagenomic approaches have generated countless sequences that have not been assigned to living purified bacteria, and a culturomic strategy has been reported to double the species number of microorganisms isolated from the human gut[87]. Although culturomic are time and labor consuming, it is a more challenging task to evaluate the functional properties of such enormous isolates. To measure antioxidant capacities *in vivo*, we used a rapid *Drosophila* model combined with a subsequent piglet experiment to screen out next-generation probiotics in weaning piglets.

# Conclusion

In this study, SMM914 shows great potential to function as a promising alternative to antibiotics to relieve oxidative stress in early-weaned piglets. However, standards for safety and stability are not clear enough at present, owing to the complexity of intestinal microorganisms and the personalized treatment background of hosts. During the selection of potential probiotic strains, we should not only pay attention to their function but also consider other selection criteria carefully, including rigorous safety and stability assessments. For example, once a widely used probiotic contained many antibiotic resistance genes, it would accelerate the acquisition of resistance among bacteria via horizontal transfer. In the future, it will

be necessary to further strengthen the research on the safety and stability of SMM914 to pave the way for the application of maternal probiotics, especially regarding its survivability during granulation and storage conditions.

# Methods

### Culture media and bacterial strain isolation.

Healthy second-parity sows with similar breeding dates raised on a pig breeding farm (Changsha, China) were employed in this study. The sows received no antibiotics within the 4 weeks prior to breast milk sampling. The areolar skin around the teats was successively swabbed with alcohol tampons and warm saline-lubricated sterile swabs. Using sterile tubes, fresh ordinary milk was collected from six sows during lactation (**Fig. S1, step 1**).

Considering that bacterial populations can survive through cell death and the recycling of dead cells[88], the continuous culture and intermittent sampling was performed for 30 days in a diverse and dynamic environment in an anaerobic incubator ($N_2$ = 90%, $CO_2$ = 5%, and $H_2$ = 5%) at 37 °C (**Fig. S1, step 2**). Using sow milk as an inoculum, MRS (Oxoid, Code# CM0359, UK)[89], M17 (Oxoid, Code# CM0817, UK)[89], TPY (Hopebio, Code# HB0397, China)[90] and GYP (Hopebio, Code# HB8539, China)[91] media were utilized to cover as much LAB diversity as possible. The bacterial cells were harvested every other day by centrifugation at 4000 g for 10 min, and the cell pellets were resuspended in sterile normal saline. Then, the cells were spread on agar plates and anaerobically cultured in a DG250 Anaerobic workstation (DWS, UK) at 37 °C for 24 h-72 h. The identification of the isolates was first carried out by using morphological and phenotypic methods. Colonies were restreaked on agar plates, and all the smBC isolates were stocked in 25% (v/v) glycerol broth at -80℃ at the College of Life Science, Hunan Normal University, China (**Fig. s1, step 3**). SMM914 was deposited at the China General Microbiological Culture Collection Center (CGMCC20160).

# Characterization and classification of the isolated bacteria

After isolation and purification, DNA was extracted from pure cultures. The V1-V5 region of 16S rDNA genes was amplified using Takara PrimerSTAR Max DNA Polymerase with a pair of LAB-specific primers, 15f (5'- GCTCAGGAYGAACGCYGG – 3') and 687r (5'- CACCGCTACACATGRADTTC-3') for the identification of the isolates[92]. The PCR-amplified products were identified by Sanger sequencing (Sangon Biotech Ltd., China). The sequencing error-prone areas (50 bp) at both ends were removed. The partial 16S rDNA sequences were searched against the NCBI nucleotide collection (nr/nt) database using BLASTN. The best match for each sequence was selected, and the taxonomy was assigned.

The nonredundant set of 16S rDNA datasets was clustered by using CD-HIT with sequence identity of 0.99[93]. The phylogenetic relationship between isolates was determined by aligning the nonredundant set of 16S rDNA gene sequences to construct a maximum-likelihood tree by using FastTree.

We also performed 16S rDNA sequence alignment of 1240 isolates against the Silva version 132 16S rRNA database, the NCBI nucleotide collection (nr/nt) database and the DAIRYdb reference database using BLASTN with a threshold of 1e-05 e-value, 99% coverage and99% cutoff. A suspected new species was defined below these values against the Silva version 132 16S rRNA database and NCBI nucleotide collection (nr/nt) database[93]. The sequencing read data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (Suppl. data s2).

# Antibacterial assay

The following indicator strains were used: *S. typhimurium* ATCC 14028, EHEC ATCC 43894, ETEC O149: K88, *K. pneumoniae* ATCC 13883, *A. punctata subsp. Caviae* ATCC 15468, *S. aureus* ATCC 25923, *L. monocytogenes* ATCC 19115 and *C. perfringens* ATCC 13124.

Agar well diffusion assays[94] were utilized to evaluate antimicrobial activity against these indicator pathogenic strains *in vitro*. Briefly, pathogens were grown in Luria-Bertani (LB) broth at 37 °C for 8 h and then diluted at a volume ratio of 20 μL to 4 mL LB and mixed well. Fifty microliters of the diluted liquid was spread evenly on each soft LB agar plate containing 0.8% agar. The residual liquid was evaporated on a ventilated clean bench. Next, holes were punched in each agar plate via sterile iron pipettes, with a depth of 6 mm and a diameter of 5 mm. *P. pentosaceus* strains were grown in MRS broth at 37 °C for 18 h. Then, the supernatants of *P. pentosaceus* fermentation broth were precisely added to the holes with a 30 μL volume per well. The central well of each plate was filled with 30 μL MRS broth as the negative control. After 48 h of incubation at 37 °C, antibacterial activity was observed as a halo of inhibition in the bacterial lawn formed around the sample, and the diameter of the zones of inhibition was measured. The evaluation of each sample was repeated in triplicate.

**Paraquat resistance assays in** Drosophila

The *Drosophila* experiments were conducted under a 12 h light:12 h dark cycle at 25 °C on cornmeal-molasses medium. Six-day-old mated female *Drosophila* $w^{1118}$ were collected under $CO_2$ anesthesia and starved for 2 h. Each group consisted of 3 vials, and each vial contained 20 female flies. In the bacterial association assays, the colony-forming units (CFUs) of *P. pentosaceus* were enumerated using MRS agar plates following standard microbiological procedures. Groups of adult female flies were colonized with pure cultures ($1 \times 10^{10}$ CFUs) of the *P. pentosaceus* strains for 3 days, including SMM847, SMM853, SMM862, SMM867, SMM881, SMM906, SMM907, SMM908, SMM914 and SMM918. Distilled water without *P. pentosaceus* was used as a negative control. Then, these flies were transferred to vials containing 2 pieces of Whatman paper soaked with 200 μL 5% (w/v) sucrose containing 12 mmol/mL paraquat (methyl viologen dichloride, Cat# 856177, Sigma-Aldrich, USA). Each group was supplied with fresh paraquat vials every day. The 12 mmol/mL concentration was chosen because it was empirically shown that this concentration was lethal to more than 80% of female $w^{1118}$ flies within 2 days.

# Piglet feeding trial and tissue sampling

The Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, reviewed and approved the experimental procedures involving piglets. As described in Jun Hu et al[95], a total of 54 newborn suckling piglets (Landrace × Yorkshire) were chosen from nine second-parity sows and randomly assigned to three treatments ($n$ = 18) among each litter, including a control group (physiological saline, 2.0 mL each time, Control), a low-dose SMM914 solution group ($10^8$ CFU/ml, 2.0 mL each time, LD group) and a high-dose SMM914 solution group ($10^9$ CFU/ml, 2.0 mL each time, HD group). The solution of saline or bacterial cells was infused into each piglet's mouth with a syringe without a needle. All suckling piglets were subjected to oral gavage every other day from the age of 10 to 18 days and were weaned at 21 days.

# Sample collection

Piglets per group from each of the 7 litters were selected to be euthanized for sampling at 28 days. Ten milliliters of blood was collected into heparin sodium anticoagulant tubes via direct cardiac puncture immediately after death and subjected to untargeted metabolism analyses. Another 10 mL of blood was collected in vacuum tubes and centrifuged at 3000 rpm at 4 °C for 10 min. The serum samples were kept at -80 °C until analysis. After the opening of the abdomen, tissues including the liver, spleen, kidney, and heart were weighed, dissected, and then snap-frozen in liquid nitrogen. Colon contents were stored at -80 °C until the extraction of bacterial DNA.

Serum concentrations of parameters reflecting lipid (cholesterol, triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, lipase), protein (total protein, TP; albumin, ALB), carbohydrate (glucose, alpha-amylase, lactic dehydrogenase) metabolism, liver functionality including total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), the activity of aspartate transaminase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptadase (GGT), seroglobulin (GLO), alkaline phosphatase (ALP), lactic dehydrogenase (LDH) and cholinesterase (CHE), as well as kidney functionality including creatinine (CREA), blood urea nitrogen (BUN) and uric acid (UA) were determined using commercial kits according to the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China) and were identified with a TBA-120FR Automatic Chemistry Biochemistry Hiiometer (Hitachi Co., Tokyo, Japan).

# Western blotting analysis

Western blotting was performed as previously described with some modification[73]. Liver samples ($n$ = 6) were powdered under liquid nitrogen and lysed in radioimmunoprecipitation assay buffer with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology). The supernatant was obtained by centrifugation at 12,000 × g for 10 min at 4 °C. The denatured proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes at 200 mA for 1 h. The membranes were blocked with 5% nonfat milk in Tris-buffered saline mixed with 0.5% Tween-20 (TBST) at room temperature for 2 h and then incubated with antibodies against Kelch-like ECH-associated protein 1 (Keap1) (SC-19917, Proteintech, USA), nuclear factor-E2-related factor 2 (Nrf2) (SC-98974, Proteintech, USA), NADPH quinineoxidoreductase-1 (NQO-1) (11451-1-

AP, Proteintech, USA), hemeoxygenase-1 (HO-1) (27282-1-AP, Proteintech, USA), catalase (CAT) (66765-1-Ig, Proteintech, USA), Cu/Zn-superoxide dismutase (SOD1) (10269-1-AP, Proteintech, USA), PCNA (60097-1-Ig, Proteintech, USA) or β-actin (SC-47778, Proteintech, USA), which were diluted with 5% nonfat milk in TBST. The membranes were washed 3 times in TBST and then incubated with a secondary antibody. Finally, the membranes were washed with TBST solution and visualized with a chemiluminescence instrument.

# Enzyme activity analysis

Liver tissue samples were homogenized in saline, followed by centrifugation (2500 × g, 4 °C, 10 min) to obtain the supernatant ($n = 7$). Malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in the liver were determined with commercially available colorimetric diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. The procedures were carried out in duplicate with three parallel samples.

# 16S rDNA gene amplicon sequencing

Intestinal digesta samples were collected after sacrifice. Bacterial DNA was extracted using a QIAamp DNA Stool Kit (Qiagen, Gaithersburg, MD, USA) according to the provided protocols ($n = 7$). The V3-V4 region of the bacterial 16S rDNA gene was amplified by PCR in triplicate in a 20 μL mixture (2 min at 95℃, followed by 25 cycles of 30 s at 95℃, 30 s at 55℃, 30 s at 72℃ and 5 min at 72℃). Using the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, USA), the PCR products were purified and then quantified by QuantiFluor™-ST (Promega, Madison, WI, USA). Library quality was assessed, and the amplicon library was sequenced on the Illumina MiSeq platform. The analysis pipeline followed a recently published review[96]. QIIME (version 1.17) was used to split raw FASTQ files[97], remove primers and barcodes, and perform quality control. High-quality reads were selected and clustered into operational units (OTUs) based on a 97% similarity cutoff using UPARSE v7.1[98] of USEARCH 10.0. Chimeric sequences were identified and removed using UCHIME[99]. The taxonomy of each 16S rDNA sequence was analyzed by the RDP Classifier (http://rdp.cme.msu.edu/) against the bacteria database of SILVA[100]. QIIME was also used for the analysis of alpha diversity and beta diversity.

# Untargeted metabolomics

The plasma samples (100 μL) were thawed at 4 °C and homogenized in 400 μL of precooled methanol/acetonitrile (1:1, v/v) for 60 s. The untargeted metabolic profiling analysis was conducted by using an ultra-performance liquid chromatography (UPLC) system (1290 Infinity LC, Agilent Technologies, Santa Clara, California, USA) coupled to a quadrupole time-of-flight (TOF) mass spectrometer (Triple TOF 5600, AB SCIEX) with electrospray ionization (ESI) in positive and negative ionization modes. For chromatographic separation, 2 μL of the extracted sample was injected by an autosampler system at 4 °C at a delivery flow rate of 300 μL/min into a liquid chromatography column with a column temperature of 25 °C. The mobile phase consisted of A (water + 25 mM ammonium acetate + 25 mM ammonia hydroxide) and B (acetonitrile). The gradient was 95% B and 5% A for 1 min, with a linear reduction to 65% B and 35% A over 13 min, a reduction to 40% B and 60% A over 2 min, maintenance for 2 min and an

increase to 95% B and 5% A over 0.1 min, with a 5 min re-equilibration period. Before injection, quality control samples were used to monitor the stability and repeatability of the data produced by the instrument. The screening criteria for differential metabolites were based on a variable importance projection score > 1 and $p < 0.05$ (Student's t-test). The metabolites were analyzed by comparing the molecular ions with compounds in the available biochemical databases, and the pathway analysis of the identified compounds was conducted using the KEGG website (http://www. genome. jp/kegg).

# Statistical analysis and visualization

SPSS software (version 19.0; IBM Corp., Chicago, IL, USA) was used to evaluate the experimental results with Duncan's multiple comparison test to determine the statistical significance of the differences among treatment groups. The results were expressed as the means with their standard errors. Probability values below 0.05 were considered statistically significant, whereas $0.05 < p < 0.10$ was used to indicate a tendency toward significance. Different letters in the same graph indicate significant statistical differences ($p < 0.05$). Cultivated bacteria were compared with OTUs in corresponding root microbiota members with greater than 97% 16S rRNA gene similarity to examine culture-dependent coverage. Cladograms of the Sow Milk Bacterial Collection were visualized with GraPhlAn v.0.9.7[101], and the scripts were reused from a previously published paper[102].

# Accession codes

The numbers and GenBank IDs of 16S rRNA genes of all smBC isolates are listed in Suppl. Data s2.

# Abbreviations

SPI
Sow and Piglet Integration; Nrf2:nuclear factor-E2-related factor 2; Keep1:Kelch-like ECH-associated protein 1; NQO-1:NADPH quinineoxidoreductase-1; HO-1:hemeoxygenase-1; CAT:catalase; MDA:Malondialdehyde; GSH-Px:glutathione peroxidase; SOD:superoxide dismutase;LAB□lactic acid bacteria□ SMM□sow milk microbiota.

# Declarations


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## Authors' contributions

JY and YLY contributed in conceiving and designing the experiments. LLW, QHL and DZ designed and performed most experiments. XLZ, YX collected the milk samples. YNQ, YZ and YWC conducted antimicrobial activity experiment. QYW, QYH, JLH and YMY performed paraquat resistance assays. CML, YCD, LW, PL, THC and TYY contributed to piglet-feeding trial. ZZZ, YXL, GHZ, JZL, PH and HSY performed the data analysis. ZZZ, JZL, HSY, and QYW revised the work. JY, LLW and XLZ drafted the work. All authors read and approved the manuscript.

## Availability of data and materials

The raw sequence data reported in this paper have been deposited (PRJCA003003) in the Genome Sequence Archive in the BIG Data Center, Chinese Academy of Sciences, under accession code CRA002995 for bacterial 16S rRNA gene sequencing data, which are publicly accessible at http://bigd.big.ac.cn/gsa.

## Ethics approval and consent to participate

Not applicable

## Consent for publication

Not applicable

## Competing interests

No conflict of interest exists in the submission of this manuscript, and the manuscript has been approved by all authors for publication. The authors declare that they have no competing interests.

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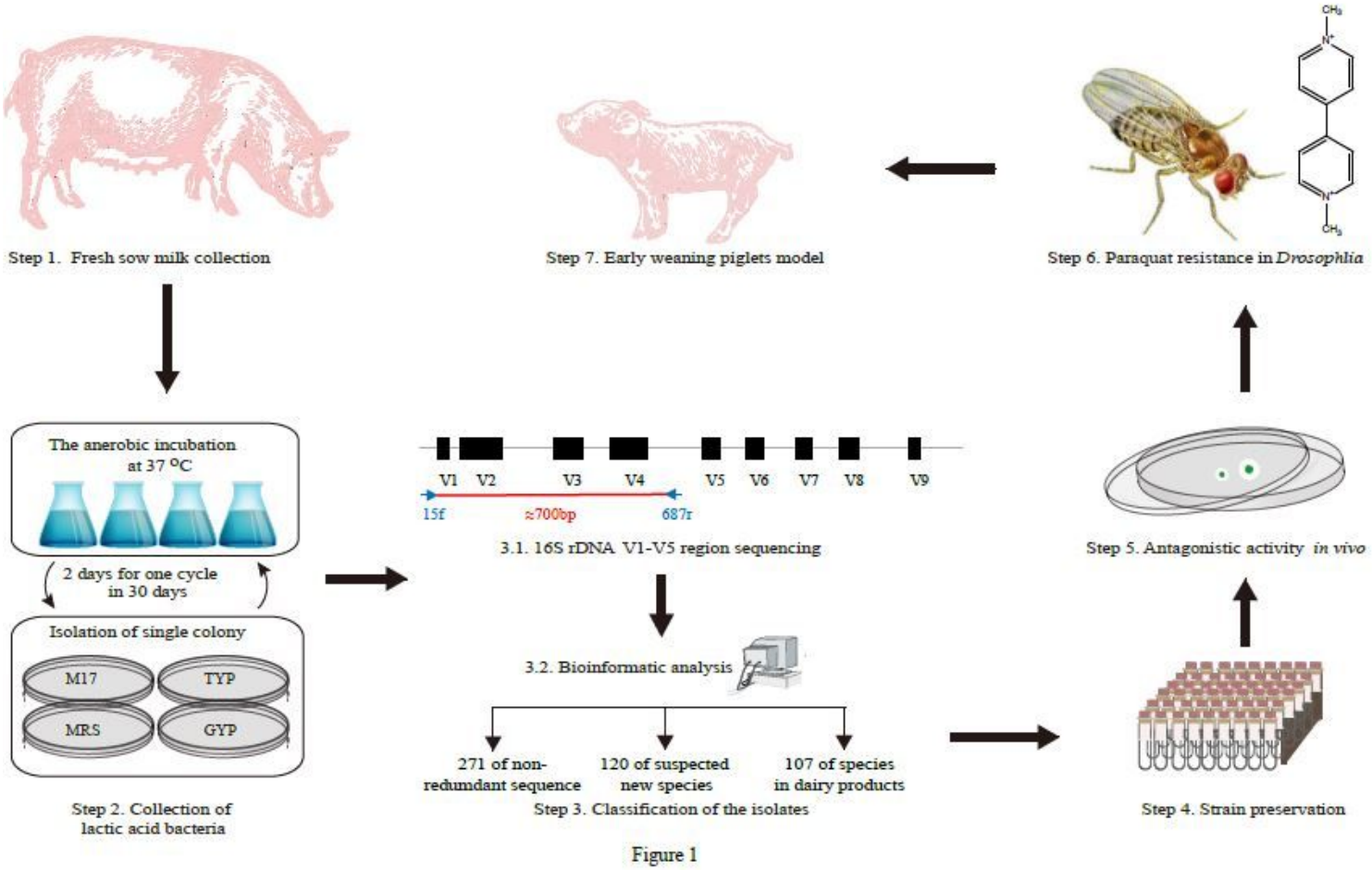
# Figures




## Figure 1

The workflow for large-scale bacterial cultivation from sow milk and the characterization of probiotic functions.

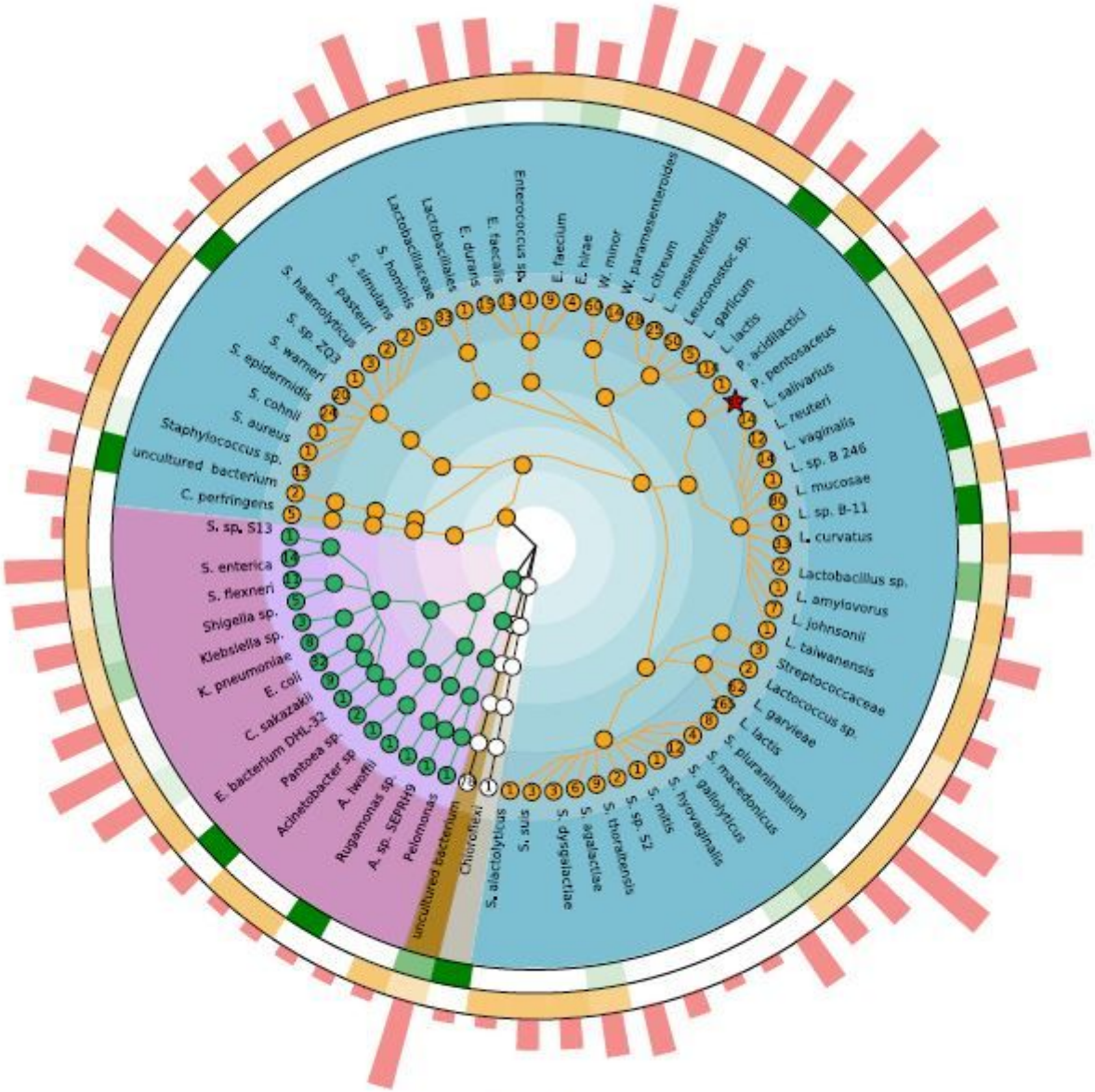



Figure 2

## Figure 2

The Sow Milk Bacterial Collection. The inner circles depict taxonomic assignments of bacteria for the indicated phyla, classes, orders, families, genera, and species that were identified. The species names are labeled, and the numbers of different taxa within each species are provided at the nodes. P. pentosaceus is indicated in red and used in subsequent research. The probability (%) of suspected new species and known species identified from the dairy products is shown in the outer ring with the green or yellow heat map, respectively. The outermost bar plots represent the numbers of isolates (log2 transformed) of each species.

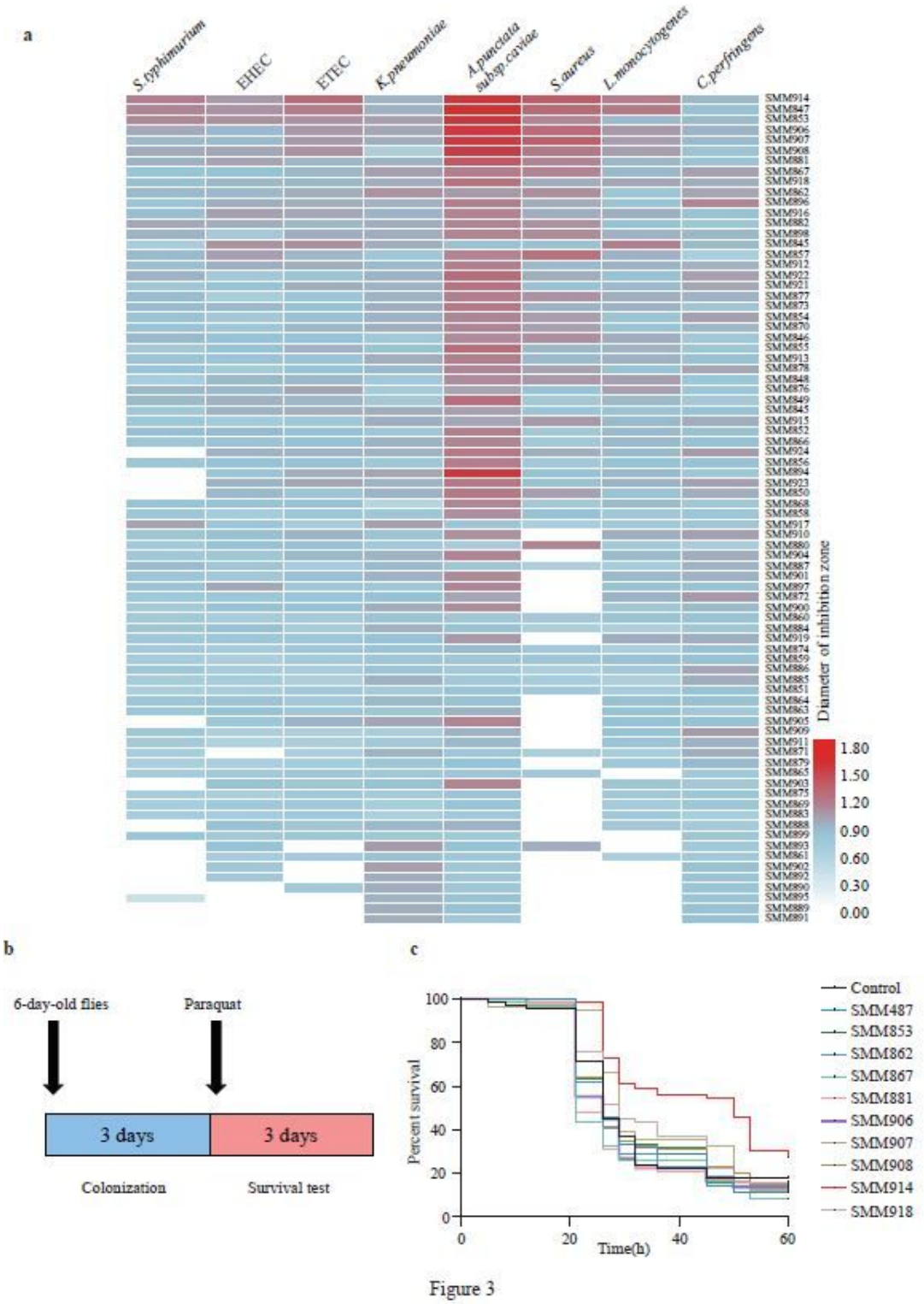



Figure 3

## Figure 3

Screening of potential probiotics prior to a piglet feeding experiment. (a) Antagonistic activity against pathogens in vitro. (b) Graphical representation of experiments conducted to assess the survival rate. (c) P. pentosaceus confers protective effects upon Drosophila. Statistical differences were calculated by the log-rank test.

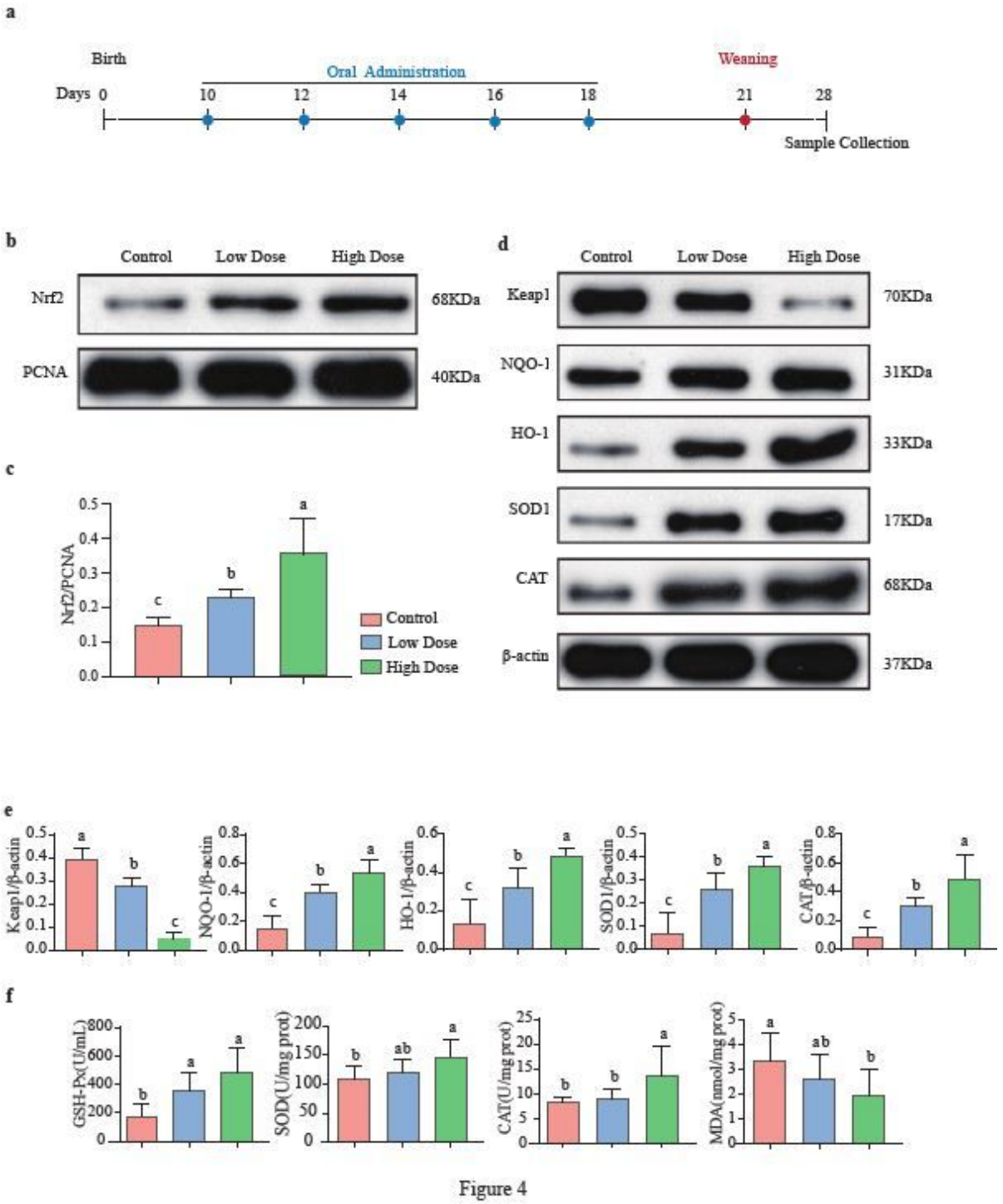



Figure 4

## Figure 4

Effects of the oral administration of SMM914 on the antioxidation capacity and activation of the cytoprotective Nrf2 pathway in piglets. (a) Experimental outline in piglets (n = 18). Piglets were kept for 10 days after birth for adaptation. Oral administration of SMM914 was performed on days 10, 12, 14, 16 and 18 at different doses. At day 7 postweaning, seven piglets per treatment were randomly selected for slaughter for sample collection. (b-c) Bar graph of western blotting showing Nrf2 protein expression

levels in the liver normalized to PCNA expression in the nucleus. (d-e) Western blotting analysis of antioxidant protein (Keap1, NQO-1, HO-1, SOD1 and CAT) levels in the liver and bar graph of data showing their protein expression normalized to β-actin expression in hepatic cells (n = 6). (f) The enzyme activities of GSH-Px, SOD, CAT and MDA were measured in liver lysates. Data are the mean ± s.e.m, n = 7.

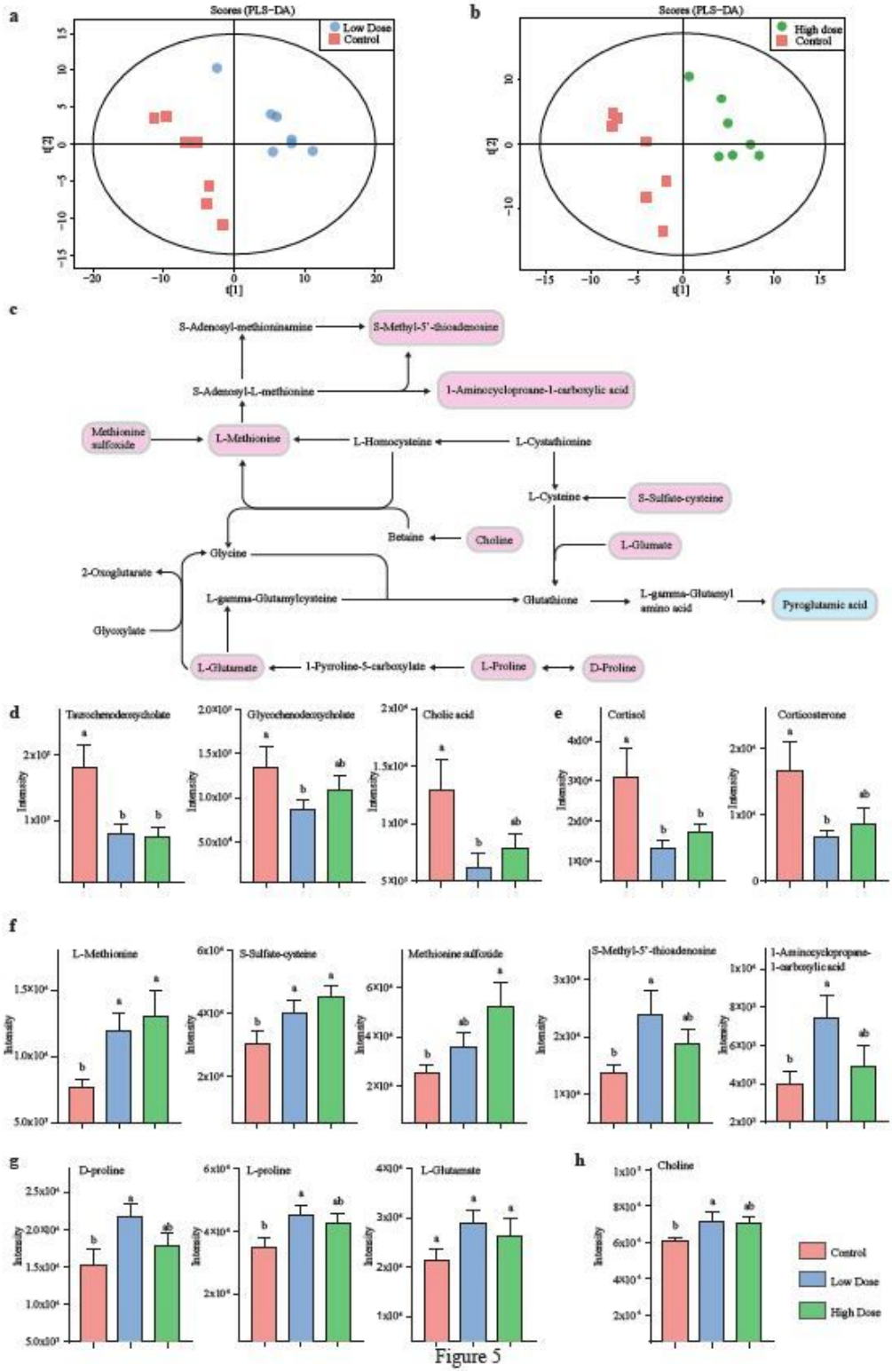



Figure 5

## Figure 5

The metabolic profile of plasma. (a-b) Score plots of the PLS-DA analysis for the plasma metabolome, (a) showing the scatter between the control and low-dose groups. (b) Scatter between the control and high-dose groups. (c) The integrative metabolism pathway according to the KEGG pathway database. Compared with the control group, the blue metabolites represent the intensities of metabolites that were downregulated, while the red metabolites represent the intensities of metabolites that were upregulated. (d-h) The perturbed metabolism pathways and metabolites in response to SMM914 treatment mainly include glutathione metabolism; cysteine and methionine metabolism; glycine, serine and threonine metabolism; and arginine and proline metabolism. Data are the mean ± s.e.m, n = 7.

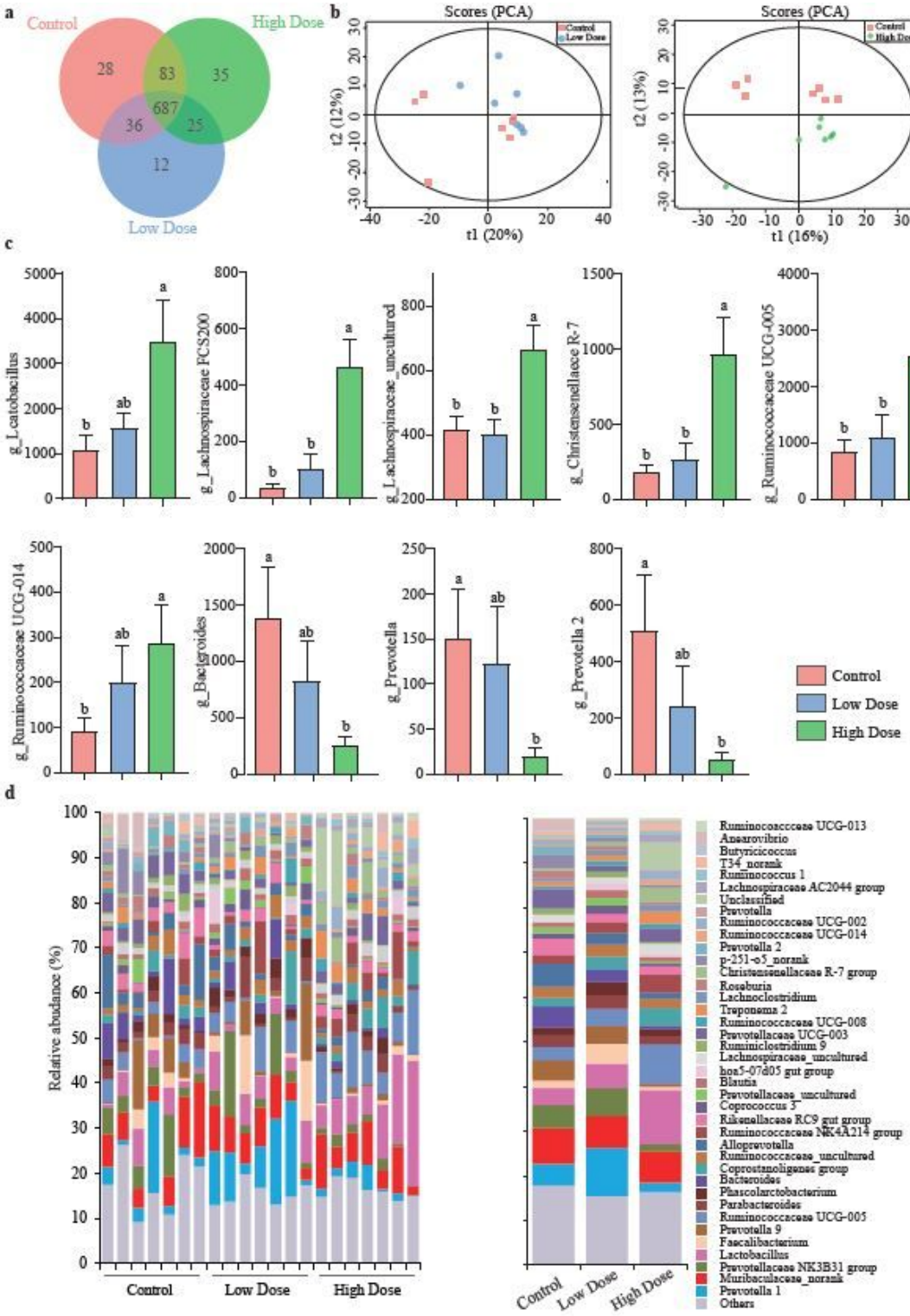



Figure 6

## Figure 6

Microbial composition analysis in the colon. (a) The Venn diagram for operational taxonomic units among the control group, low-dose group and high-dose group. (b) Scatterplot of principal component analysis in bacterial communities. Principal components (PCs) 1 and 2 explain 10.21% and 8.17% of the variance, respectively. (c) The alterations of probiotics and harmful genera in bacterial communities. (d)

Individual (left) and averaged (right) taxon summary of bacterial genera in colon contents. Data are the mean ± s.e.m, n = 7.

## Supplementary Files

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